Utilities of reverse genetics system as a platform for the development of Porcine Reproductive and Respiratory Syndrome (PRRS) virus vaccine

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Utilities of reverse genetics system as a platform for the development of Porcine Reproductive and Respiratory Syndrome (PRRS) virus vaccine

by

Dong Sun

A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Veterinary Microbiology

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Ames, Iowa
2013
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DEDICATION

To

my parents

for their understanding, encouragement and support.
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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) causes reproductive disorder in breeding pigs and respiratory symptoms in pigs of all ages. The virus continues to bring significant economic losses to swine industry, and is considered to be one of the most important swine pathogens. Vaccination has been utilized to aid or facilitate PRRS control. Among various types of vaccines used, attenuated live virus vaccines appear to be more efficacious than killed virus vaccines. However, the effect of vaccination with attenuated live virus vaccines is crippled by several safety and efficacy issues. First, the vaccine development process is time-consuming. Second, the vaccine has the potential to revert to virulent. Third, the vaccine tends to provide full protection only to homologous strains. Three independent studies were conducted to address these issues and provide a concept for future vaccine development.

The first study was based on a previous in vitro work in our laboratory which demonstrated that chimeric viruses containing mixed structural genes from two genetically and antigenically distinct strains (VR2332 and JA142) of PRRSV in an organized manner were susceptible to antisera generated against both of the donor strains. In the present study, three chimeric viruses (JAP5, JAP56 and JAP2-6) were constructed by replacing ORF5, ORFs 5 and 6, and ORFs 2-6 of VR2332 with the corresponding gene(s) of JA142 respectively and were evaluated in vivo for their ability to confer pigs the cross protection against the VR2332 and JA142 strains. A total of 114 pigs were divided into 6 groups and each group was intramuscularly inoculated with one of the three chimeric viruses (n=16 per group), VR2332 (n=24), JA142 (n=24), or sham inoculums (n=18). At 44 days post
inoculation (dpi), these pigs were divided further into 15 groups (n= 6 or 8 pigs per group) and challenged intranasally with VR2332, JA142, or sham inoculum. Although no pigs demonstrated severe clinical signs or lesions after the 2nd inoculation, all pigs inoculated with chimeric viruses prior to challenge had significantly \((p<0.001)\) lower levels of viremia when compared with the challenge control group. These results suggest that chimeric viruses which have mixed ORFs 5-6 with other structural genes of two distinctive PRRSV strains can confer pigs cross protection against both of the donor viruses.

The second study utilized the same concept of the first study but was expanded to assess if use of chimeric viruses can be universally applicable to obtaining broader cross protection among PRRS viruses. To evaluate the strategy of chimeric virus, this study selected four distinct wild-type 1648-02, 17198-6, MN184 and SDSU73 strains of PRRSV whose nucleotide identities range from 86.7% to 92.2% and generated 12 chimeric viruses by mixing their ORFs 3-4 or ORFs 5-6. Their susceptibilities to the neutralizing activity of hyperimmune serum generated against each one of the four wild-type strains were assessed \textit{in vitro} by fluorescent foci neutralization (FFN) assay. No or minimum level of cross neutralization existed among the four wild-type strains, and not all chimeric viruses obtained broader cross neutralization. Broader cross neutralization could be obtained when chimeric viruses were constructed in an organized manner depending on the immunobiological importance of ORFs 3-4 and ORFs 5-6 products of each strain. The neutralization of MN184 and SDSU73 were mainly mediated by ORFs 5-6 products, while ORFs 3-4 products played very limited roles in virus neutralization. Products from ORFs 5-6 and ORFs 3-4 of 1648-02 were all implicated in virus neutralization, but ORFs 5-6 products were more important \((p<0.001)\). For 17198-6, ORF5-6 and ORF3-4 products were equally involved in virus
neutralization. Chimeric viruses 17198-MN184, 17198-SDSU73 and 17198-1648 were highly susceptible to both anti-17198 and anti-MN184 hyperimmune sera, suggesting that the chimeric virus strategy can be universally applicable to obtain broader cross protection among PRRSVs independent on strains.

The third study focused on assessing whether non-structural proteins play a role in the cross neutralization between viruses. In this study, eight different patterns of structural gene mixing between JA142 and VR2332 were constructed into three cDNA infectious clone backbones. Chimeric viruses were then tested for their susceptibilities to JA142 and VR2332 antisera. No matter which backbone was used to construct the chimeric viruses, chimeric viruses carrying the same structural genes showed almost the same degree of susceptibility to the JA142 and VR2332 antisera, suggesting non-structural gene of the backbone has limited impact on the specificity of viral immunity. On the other hand, chimeric viruses with different gene mixing patterns displayed significant diversity in their susceptibilities to the JA142 and VR2332 antisera. Cross neutralization testing demonstrated that determinants for VR2332 neutralization were located in ORF3 or ORF4 whereas determinants for JA142 neutralization were located in ORF6. ORFs 2 and 7 products of JA142 displayed conflicting roles in virus neutralization. Their impacts remain to be further studied.

In conclusion, chimeric virus strategy may be a way to induce broader cross-neutralizing antibody leading to better cross-protective humoral immunity against heterologous PRRSVs in pigs. As the nonstructural genes of backbone did not affect the specificity and sensitivity of virus neutralization, chimeric viruses containing avirulent non-structural genes of the backbone (i.e., infectious clone) and well-organized structural genes
of PRRSV may be used to obtain the improved efficacy and safety of vaccination when a live virus based vaccine is used.
CHAPTER 1. GENERAL INTRODUCTION

Dissertation organization

This dissertation is composed of an abstract, a following general introduction, three research papers, a general conclusion and an acknowledgement. Chapter 1 is the general introduction, which provides a brief overview of the current knowledge on the functional genomics of PPRSV. Chapter 2, 3 and 4 are research papers which have been prepared to be submitted to the Journal of Virology. References, tables and figures of each research paper are following the discussion section of their corresponding paper. The last chapter contains the general conclusions of the studies.
LITERATURE REVIEW: Functional genomics of PRRS virus

1. Introduction

Porcine Reproductive and Respiratory Syndrome (PRRS) was first recognized in the United States in 1987 and in Europe in 1990[91, 139]. The disease is characterized by: a) reproductive failure in sows which is manifested by abortion, stillborn, mummification, premature farrowing, reduction in conception rate, and estrus disruption [24, 96, 97, 185] and in boars manifested by hypospermatogenesis, decreased semen quality, and loss of libido [25, 75, 149, 158, 183, 184]; and b) respiratory disorder in all ages of pigs with a non-specific lymphomononuclear interstitial pneumonitis [70, 153]. Since its first recognition, the disease has swept over most of swine-producing countries throughout the world. Immune suppression caused by PRRS virus infection can usually lead to secondary infection of other pathogens and further worsen the disease situation of pig farms [66, 152]. Beyond direct production losses, costs for biosecurity, diagnosis, and pharmaceutics can be increased significantly. PRRS is considered to be one of the major health and economic issues for swine operations, and most updated estimates have been made. In The Netherland, the loss during an 18-week outbreak was €59 to €379 to one sow; the costs after the outbreak varied between €3 and €160 to each sow. And the outbreak caused €126 to each sow on average [130]. In the United States alone, it has been estimated that PRRS brings approximately 660 million US dollars losses to the swine industry annually [79]. The worldwide economic impact of PRRS would be even greater although actual assessment has not been made.

PRRS continues to be a significant problem in swine industry, and occasionally introduces new issues. For example, in 1996, the abortion storm broke out in Iowa and many
other swine producing states in the US. Sows and gilts had fevers of 40-41°C, mortality of 5% to 10%. And 10%-50% mid- or late-term abortions were observed in herds [69]. In 2006, the outbreak of highly pathogenic PRRS in China characterized by high fever (40-42°C) and high morbidity up to 100% with 90% mortality [105, 187]. The unexpected emergences of virulent PRRSV strains continuously drew a strong attention to this pathogen.

PRRS virus (PRRSV), the etiological agent of PRRS, was first isolated in Lelystad, the Netherlands, in 1991 by using porcine alveolar macrophage (PAM), and was therefore named Lelystad virus [212]. Later in the United States, PRRSV was first isolated in 1992 by using a continuous cell line CL2621, and was designated as ATCC VR-2332 [26].

As observed under an electronic microscope (EM), the PRRSV particle is an spherical enveloped virion of 45-72 nm in diameter containing a double-layer nucleocapsid core that averages 39 nm in diameter and is separated from the envelope by 2-3 nm [16, 34, 114, 170]. As such, PRRSV is sensitive to lipid solvents. The virus is also sensitive to environmental factors. Temperature has a significant effect on the half-life of PRRSV. In aerosols, PRRSV is more stable at low temperatures and low relative humidity [78]. In cultured media at pH 7.5, the virus is stable at both -70°C and -20°C; the half life of the virus is 140 h at 4°C and 3 h at 37°C, respectively. At pH 6.25 and 4°C, the PRRSV half life is 50 h; while at pH 6.0 and 37°C, its half life is 6.5 h [15]. In manure, the half life at 4°C and 40°C is 120.5 h and 1.7 h respectively [107].

All viruses could be divided into seven groups (six groups initially) according to their types of genome and methods of replication [6]. In that classification system, PRRSV, which has a positive sense, single-stranded RNA genome, belongs to virus group IV. The PRRSV genome, which is 15kb in size, encodes at least 10 open reading frames (ORFs), namely
ORF1a, ORF1b, ORF2, ORF2b, ORF3, ORF4, ORF5, ORF5a, ORF6, and ORF7. According to the International Committee on Taxonomy of Viruses (ICTV), PRRSV is grouped along with three other members: equine arteritis virus (EAV, genome size around 12kb), lactate dehydrogenase-elevating virus (LDV, genome size around 14kb), and simian hemorrhagic fever virus (SFHV, genome size around 15kb) into the Arteriviridae family within the Nidovirales order (also includes families Coronaviridae, Mesoniviridae, and Roniviridae) due to their significant similarity in morphology, genome organization and transcription strategy [21, 164].

Like other arteriviruses, in the process of PRRSV replication, a set of 7 subgenomic mRNAs (sgmRNAs) sharing the same 5’ leader sequence and 3’ terminal sequence are generated [27, 119]. For each sgmRNA, only the first ORF is translated. It has been proposed that sgRNAs are transcribed from the genomic RNA in the negative strand synthesis step, and subsequently sgmRNAs are produced using these negative-sense sgRNAs as template [137, 157]. ORF1a and ORF1b, which together take up 2/3 of the genome from the 5’ end, encode nonstructural proteins including RNA-dependent RNA polymerase that are indispensable for virus replication. ORF2 through ORF5 encode glycoproteins GP2, GP3, GP4 and GP5 (or E protein in some early studies) [164]. ORF2b is completely embedded in ORF2 and encodes a non-glycosylated small protein named E or 2b. The recently discovered protein GP5a is expressed from the same sgmRNA as GP5 but in an unappreciated manner. And the size is only 51 aa, which is much smaller than the size of GP5 (200 aa) [56, 85]. ORF6 and ORF7 encode two non-glycosylated proteins matrix protein (M) and nucleocapsid protein (N), respectively [164].
PRRS viruses have been divided into two genotypes: type 1 (European type, Lelystad virus as the prototype) and type 2 (North American type, VR-2332 as the prototype). These 2 genotypes share only about 70% of their full-length nucleotide identities [52, 129, 151]. Antigenically, these two types are distinct as well. Antiserum generated against viral proteins or virion of each genotype demonstrated significantly different reactivity against each other [33, 90, 198, 211]. Moreover, within each type, a great degree of genetic and antigenic variations have also been reported by numerous investigators [48, 59, 142, 230, 234]. Remarkable geographical variability in sequence has been reported among type 1 PRRS viruses, which, has further divided them into three subtypes based on ORF7 sequence. Subtype 1 has spread into most regions throughout Europe and subtypes 2 and 3 exist in Eastern European countries [59, 172]. Degree of serological cross reactivity among the three subtypes is low [89, 172]. Type 2 PRRS viruses have been divided into 9 clusters based on ORF5 sequence. An average genetic diversity between clusters is 12.5%, with a largest pairwise distance being as high as 27.8% [160].

The origin of PRRSV remains unclear. Intensive studies have been performed after the recognition of PRRSV to trace the evidence of PRRSV existence before PRRS outbreaks in the late 1980s. In Canada, a serological evidence for PRRSV antibody was demonstrated in serum samples from 2 of 51 herds collected in 1979 and from 8 of 51 herds collected in 1980 [19]. In the United States, PRRSV-specific antibody-positive serum samples have been identified since 1985 [244]. In Europe, antibody against PRRSV was detected in samples collected in 1987 in former East Germany [133]. Findings from these retrospective studies suggest that PRRSV had been hibernating in pig populations before clinical symptoms became evident. Furthermore, phylogenetic analyses of both type 1 and type 2 PRRS viruses
suggest that a common ancestor might have been introduced to domestic pigs around 1979 [58, 160]. Among nidoviruses, LDV, whose genome shares approximately 53% nucleotide identity with PRRSV, is most genetically close to PRRSV; whereas EAV and SHFV, which share approximately 42% and 45% genomic nucleotide identities with PRRSV respectively. It has been hypothesized that PRRSV might be originated from LDV [144], which infected wild pigs initially in the Central Europe. The virus was then brought to the United States with infected wild boars imported from Europe, and the virus evolved separately on both continents until transmitted to domestic swine resulting in disease. However, a serological survey of feral swine did not substantiate this hypothesis since anti-PRRSV antibody was detected in domestic swine earlier than feral swine (Jeff Zimmerman, personal communication). More clues are required to draw a solid conclusion on the origin of PRRSV.

PRRS has devastated and remains to be a major disease problem to the swine industry worldwide. To control the disease effectively, good farm management practice including biosecurity is always critical and should be emphasized. For example, one should use disposable boots or de-contaminate boots with bleach bath before and after getting into herd/barn [36]. Use of an air filtration system would minimize potential aerosol transmission of PRRSV into farm or between buildings on site [35, 37], although air transmission of PRRSV remains unclear. All-in/all-out pig flow combined with thorough cleaning and disinfection of facilities between batches of pigs is highly recommended to prevent transmission from old pigs to young pigs [65]. For prevention of PRRS at the population level, vaccination is an effective tool. Several licensed commercial vaccines (e.g. Fostera™ PRRS, Porcilis® PRRS, Ingelvac® PRRS MLV) are available to swine practitioners and producers. However, significant genetic and antigenic diversity among PRRS viruses in the
field has limited the protection efficacy provided by a specific vaccine. Suboptimal cross protection by monovalent vaccines has been a major impediment to preventing or controlling PRRSV by vaccination. PRRSV has a higher evolitional rate (on the order of $10^{-2}$/site/year) compared to those (on the orders of $10^{-3}$ to $10^{-5}$/site/year) of standard RNA viruses [77]. The virus could evade the host immunity established by vaccination through mutation or recombination. To minimize or overcome these obstacles in achieving an effective and protective immunity against PRRSV, much deeper and thorough understanding of PRRSV, especially characteristics and immunobiological functions of the genome and viral proteins, is necessary. Thus, this review will focus on the PRRSV genome and each viral protein to depict their structure, function and role in immunity.

2. Un-translated Region (UTR)

Positive sense single-stranded RNA viruses utilize genomic RNA as both a messenger RNA and templates for genomic RNA synthesis, encapsidation and subgenomic mRNA transcription [136, 164]. The regulation and coordination of different stages of virus infection has been a hotspot for RNA virology. It is believed that UTR of the RNA viral genome is the key domain in balancing these processes, via different sequence motifs, alternative high-order structures where the RNA-dependent RNA polymerase (RdRp) complex starts the synthesis of plus or minus strand and interacts with other viral or host factors [18, 64, 194, 245]. The RNA genome of PRRSV ranges in size between about 15,000 nucleotides (nt) and 15,500 nt. As in all other arteriviruses, the 5' and 3' end of PRRSV genome is capped and polyadenylated, respectively [164]. The 5' UTR and 3' UTRs are critical for virus replication, and microRNA targeting either 5' or 3' UTR could result in a 90%
reduction of the viral genome copy number and a six log TCID$_{50}$ decrease in virus titer during virus replication [225].

Like other members of Nidovirales, PRRSV produces a set of 3’ co-terminal nested sgRNAs for protein expression. The sgRNAs share the same genomic 5’ leader sequence, which is the 5’ UTR of PRRSV. The formation is mediated by transcription regulating sequences (TRSs) located at the 3’ end of the leader sequence (leader TRS) and upstream of each ORF start codon (body TRS). In equine arteritis virus and simian hemorrhagic fever virus the leader TRS sequences were identified as UCAAC [42] and TTAACC [238] respectively. The TRS sequences of two types of PRRSV are not the same; UCAACC has been identified as type 1 PRRSV TRS [121], whereas U/GUA/G/CACC has been identified as type 2 PRRSV TRS [119]. The formation of sgRNA is generated in the negative strand synthesis step. The transcription is initiated at 3’ UTR and jumps to join the 5’ UTR with the regulation of TRS. The 3’ UTR plays an important role in regulation of transcription. Despite the conservation of leader-body junction sequences in both type 1 and type 2 isolates, the distance between the junction sequence and the downstream ORF is quite variable [120].

2.1 5’ UTR

The 5’UTR of type 1 PRRSV is about 220 nt long and that of type 2 PRRSV is about 190 nt long. The 5’UTRs of both types exhibit approximately 50% nucleotide identity [134], but their functional mechanisms may be different from one another. Changing the 5’ UTR of a North American PRRSV with that of a European strain didn’t affect the viability of the virus, while virus replication was abolished in the opposite way [63], suggesting there must be some elements utilized by European PRRSV in addition to those of North American
PRRSV. Although the 5’ UTR is critical for virus replication, some degree of changes in the 5’ UTR can be tolerated. The 5’ proximal side has been shown to be able to tolerate 7 nt deletions [23] and 16 nt sequence alteration with a combination of deletion and substitution [62].

The critical role of the 5’ UTR in many positive-sense RNA viruses have been associated with the secondary structures of the 5’ UTRs. The poliovirus cloverleaf and hairpin motif in the 5’ UTR help to maintain the stability of viral RNA [8, 61]. A stem-loop structure of dengue virus’ 5’ end has been demonstrated to promote specific RNA synthesis, internal ribosome entry site (IRES), complicated structures formed by the 5’ UTR sequence, and initiate translation [82, 109, 141]. Stem-loop structures have been identified in equine arterivirus and are apparently conserved in other members of the Arterivirus genus [193]. In the case of PRRSV, 5’ UTRs of type 1 and type 2 isolates share only about 50% identities; however, within each type, the sequences are conserved. Secondary structures of both 5’ UTRs were estimated to form 6 major helical stem-loops designated as E-SL1-5 for type 1 PRRSV and N-SL1-5 for type 2 PRRSV respectively. The E-SL1 of type 1 PRRSV could be divided into two minor stem-loops named E-SL1a and E-SL1b, corresponding to the N-SL1 region of type 2 PRRSV. On the other hand, N-SL4 of type 2 PRRSV could be divided into two minor stem-loops designated as N-SL4a and N-SL4b, corresponding to E-SL4 of type 1 PRRSV. Several of these stem-loops have been identified to be related with viability of the virus. The 5’ proximal stem-loop 1 in the highly structured 5’ UTR was invariably required for virus infectivity [62], and the G-C rich stem structure and the stem integrity of N-SL2 have been shown to be crucial for sgmRNA synthesis. A similar stem structure with different sequence of N-SL2 could also be used by the virus for the same purpose [110].


2.2 3’ UTR

The 3’ UTRs of many positive-sense RNA viruses are critical in viral RNA synthesis. Synthesis of both nidovirus’ full-length genomic and subgenomic RNA begins at the 3’ terminus of positive strand of the viral genome and is critical for virus replication. In the case of Coronavirus, stem-loop structures have been discovered to interact with viral or host proteins through pseudoknot interaction between two stem-loop structures for virus replication [80, 108, 237]. This interaction may be mediated by RNA-protein or RNA-protein-protein interactions. The PRRSV 3’ UTR has been discovered capable of binding with many cellular and viral proteins. A 7-base sequence (15009-GAUUGGC-15015 of LV) in the 3’ UTR which is highly conserved among PRRSV isolates, interacts with 7 bases (14664-GUCAAAUC-14671 of LV) of the loop area of hair-pin structure formed by the RNA of ORF7. Mutating 5 of the 7 bases in either of the two strands could abolish the complementary loss of viability of the virus; however, mutating sequences on the both strands while keeping their complementarity restored the virus replication [201].

The type 1 PRRSV 3’ UTR is 114 nt long whereas the type 2 PRRSV 3’ URT is 151 nt in length which is the longest among arteriviruses [117]. Although the 3’ UTR of type 1 PRRSV is about 40 nt shorter than that of Type 2 PRRSV, the extra 40 nt of type 2 PRRSV following ORF7 stop codon seems to be dispensable for virus viability. Swapping type 2 PRRSV 3’ UTR with type 1 PRRSV’s could generate a virus with properties similar to the properties of its parental type 2 PRRSV [182]. Similarly, the 7 nt following the ORF7 stop codon of LV can be removed without affecting the virus viability, while removal of a 32 nt section diminished virus viability [200]. The four nucleotides (15517-AAUU-15520) at the 3’ proximal end of the 3’ UTR are conserved among type 1 and type 2 PRRS viruses. Changing
the 4th U with A or G inactivated the virus, with a C reduced the level of negative strand genomic RNA replication. The AU of the 2nd and 3rd place is critical for virus viability, and no CPE could be observed after five passages. Changing the 1st A to C didn’t change RNA replication much; however, changing it to G caused the negative strand genomic RNA replication level to decrease by 50% as compared to wild type, and changing to U led to lack of transcription. The primary sequence of 15503-AACCA-15507, 14 nt upstream of the 3’ UTR, is also conserved among type 1 and type 2 PRRS viruses. The AACCA and its flanking sequence form a stem-loop structure at the end of the 3’ UTR, but this structure is less important with respect to viral infectivity compared to the primary sequence of these five nucleotides [231].

3. Nonstructural proteins

Nonstructural proteins of PRRSV are expressed by ORF1, which takes 2/3 of the viral genome at the 5’ terminal. ORF1 comprises 2 ORFs: ORF1a and ORF1b which encode two polyproteins pp1a and pp1ab. Normal translation generates pp1a, which is subsequently processed into 10 non-structural proteins (nsp1α, nsp1β, nsp2-6, nsp7α, nsp7β, nsp8). Production of pp1ab relies on ribosomal frame shift (RFS) in the overlapping area between ORF1a and ORF1b [43, 164]. Through the RFS mechanism, the stop codon of nsp8 is abolished. Translation then runs through until the next stop codon in the mRNA1 and leads to the formation of pp1ab that is processed into 13 proteins (nsp1α, nsp1β, nsp2-6, nsp7 α, nsp7β, nsp9, nsp10, nsp11, nsp12). Nsp8 and nsp9 share the same amino acid sequence at the N terminal. The nonstructural protein processing involves the rapid autoproteolytic of nsp1α,
nsp1β, nsp2, and the processing of other nonstructural proteins by nsp4. The structure and function of each nonstructural protein is discussed in details below.

3.1 Nsp1

Arterivirus nsp1 region possesses two papain-like cysteine protease (PCP) domains, named PCPα and PCPβ. The two enzymes are autoproteolytic. Cleavages take place rapidly after translation at the C terminal of each domain and release corresponding fragments from the polyprotein into nsp1α and nsp1β. The PCPα of EAV has lost its protease activity, resulting in only one cleavage taking place by the enzyme corresponding to PCPβ of other arteriviruses at C terminal, releasing nsp1 [41]. The nsp1α and nsp1β cleavage sites of type 2 PRRSV were found between 180M and 181A [22]. The sites of type 1 PRRSV was speculated to be between 180H and 181S. The nsp1β cleavage site is between 383G and 384A [22].

3.1.1 Nsp1α

Nsp1α, composed of 180 amino acids (aa), is 19kDa in size and forms homodimer. Two zinc fingers have been identified. The 1-65 aa, which constitute the first zinc finger domain (ZF1), is followed by the PCPα domain located at 66-166 aa and the C terminal extension (CTE) at 167-180 aa. The second zinc finger (ZF2) is overlapped with the PCPα domain [179]. Upon translation, PCPα cleaves the polyprotein at 180H and releases itself. Inactivation of PCPα was shown to impair sgmRNA synthesis but not to affect genome replication [95]. CTE peptides are inserted into a PCPα substrate binding site of the same molecule, suggesting a cis-acting mechanism. After self-cleavage, the CTE peptide is
stabilized by the PCP domain and doesn’t release itself from the PCP domain. Thus, the self-released nsp1α is no longer proteolytic but more likely to be involved in nonproteolytic activities [179]. ZF is a common motif in transcription factors. The ZF domain of EAV is known not to be necessary for replication but be critical for transcription [189].

PRRSV Nsp1α is able to modulate host immunity by interfering interferon (IFN) and tumor necrosis factor (TNF) production [163, 168, 176]. Mutation of ZF2 and PCPα (C76S, H146Y, C76S/H146Y; C70S, C76S, H146Y and/or M180I) does not change the IFN suppression ability of nsp1α, indicating that ZF1 is the crucial domain for the IFN suppression function [76]. It has been reported that Gly90, Asn91, Arg97, Arg100 and Arg124 of nsp1α are also necessary for TNF-α suppression. CTE is also critical for maintaining the immunosuppressive activity of nsp1α and deletion of CTE abolishes nsp1α’s inhibitory ability [161, 168]. The last four amino acids (i.e. aa 177-180) are not indispensable, i.e., aa 1-176 is totally functional. The Phe 176 is critical for the inhibitory activity of nsp1 α, and mutating it to Ala abolishes such activity [161].

The immunosuppression activity by nsp1α has been reported to be mediated in several steps. Nsp1α could inhibit IκB phosphorylation and block NF-κB translocation to the nucleus [168]. Nsp1α could also block IRF3 association with the CREB binding protein (CBP) in the nucleus without blocking phosphorylation and nuclear translocation but inhibiting IRF3 [93].

3.1.2 Nsp1β

Nsp1β is 23kDa in size and contains 203 aa. It can be divided into four parts, the N terminal domain (NTD, aa 1-48), the linker domain (LKD, aa 49-84), the C terminal papain-
like cysteine protease domain (PCPβ, 85-181), and the C terminal extension (CTE, aa 182-203). PCPβ cleaves nsp1β off from the polyprotein following nsp1α cleavage. The protease active site of PCPβ is formed by Cys90 and His159 (Cys96 and His 165 in LV) [41]. The aa 194-203 fully occupies the putative substrate binding site of PCPβ domain in cis, suggesting that the CTE is the substrate of PCPβ within the same molecule and might prevent further proteolytic activity of PCPβ. Nsp1β exists as homodimer in ethylene glycol disuccinate ester (EGS) crosslinking solution. Such formation relies on residues in NTD and LKD. NTD of nsp1β possess an endonuclease activity utilizing ssRNA and dsDNA as templates rather than ssDNA and dsRNA.

Nsp1β has been identified to be able to suppress type I IFN production [13]. Functions of several cellular factors in the signal transduction pathway are affected. Nsp1β has shown to inhibit expression of ISG15 and ISG56 [138] and block nuclear translocation of STAT1 in the JAK-STAT signaling pathway [22, 138]. Phosphorylation and nuclear translocation of IFN regulatory factor 3 (IRF3) is inhibited, and both IRF3 and NF-κB dependent gene inductions are greatly decreased [13]. The PCPβ domain interacts with cellular transcription co-factor p100 and affects sgmRNA synthesis [190]. Translocation of ISGF3 (composed of STAT1 and STAT2 with IRF9) to the nucleus is blocked. Karyopherin-α1 (KPNA1, also called importin-α5), known to mediate nuclear import of ISGF3, is degraded by nsp1β induced ubiquitination [204]. This ubiquitination function has been suggested to be related to virus virulence since virulent strains VR2332 and VR2385 have this function but attenuated strain Ingelvac MLV vaccine virus does not [204]. The region aa 16-20 of nsp1β has been found to be associated with type I IFN suppression since replacing amino acids within this region with alanine decreased the suppression of type I IFN
transcription. An *in vivo* study showed the mutant with alanine substitution had a reduced growth rate at early stage and then reverted to wild type growth properties [14]. In addition, nsp1 has also been found to suppress the TNF-α promoter activity. Nsp1α and nsp1β have been demonstrated to inhibit the activity of the TFs that bind CRE-κB and Sp1 elements respectively [177].

### 3.2 Nsp2

Nsp2 is the largest product among nonstructural proteins and even among all PRRSV viral proteins. The nsp2 size of VR2332 is about 1196 aa. As the most variant protein between type 1 and type 2 PRRSV, less than 40% amino acid identity was observed [1, 129]. Four domains are identified in the nsp2 protein: a N terminal papain like cysteine protease (PL2), followed by a hypervariable region rich in proline with unspecified function, a hydrophobic transmembrane (TM) region embodying four predicted TM helices, and a conserved C terminal region [74, 243]. The PL2 protease has both *cis* and *trans* acting activities. Cleavage at nsp2/3 joint site (1196G-G1197) by PL2 frees the nsp2. The enzyme catalytic motif is composed of Cys55 and His124, while the Asp89 is essential for PL2 protease activity *in trans* [72]. The PL2 core domain (aa 47-180) and its immediate downstream region (aa 181-323) and transmembrane domain are crucial for virus viability. Deletion of either region is lethal to the virus [71, 72]. In contrast, the N terminal and middle hypervariable region (aa 324-813) are more tolerate to deletions [71]. In addition, rather than only one length of nsp2, several nsp2 isoforms which share the same N terminal but differ in the C terminal have been discovered. The large protein is the nsp2, and smaller species are not essential for virus replication in cell culture [73].
Many RNA viruses replicate in the cytoplasm. To reduce the effects of host cell immune factors and form a regional environment for efficient replication, viruses modify endoplasmic reticulum (ER) with viral proteins to establish separate vesicle ultrastructures [40]. During EAV replication, replication complexes known as double membrane vesicles (DMVs) have been observed [140]. Nsp2 has been identified to be involved in the formation of these vesicles. In cell culture system, silencing of nsp2 reduced the virus titer, while over expression of nsp2 increased the virus titer [203].

In addition, nsp2 has also been discovered to possess the function of modulating innate immunity. Nsp2 has great potential for activating NF-κB. Over expression of nsp2 induced IκB-α degradation and NF-κB translocation and also induced NF-κB dependent IL-6, IL-8, COX-2 and RANTES [51]. Nsp2 inhibit IFN-β production by antagonizing activation of IRF-3 pathway. The cysteine protease domain (PL2) of nsp2 is necessary for IFN antagonism. Antagonizing activation of IRF-3 occurs by inhibiting phosphorylation and nuclear translocation of IRF-3 [103]. Nsp2 is an antagonist of ISG15, which is able to inhibit PRRSV replication. Deletion of N terminal 23aa (aa 402-424 of pp1a) largely abolished the inhibitory effect of nsp2 on ISG15 production, but no virus was rescued, 19aa deletion (aa 402-420 of pp1a) partially relieved the ISG15 antagonist function and rescued live viruses [181]. Nsp2 contains a cysteine protease domain at N terminal that belongs to the ovarian tumor (OTU) protease family. The OTU domain antagonizes type I interferon induction by interfering with the NF-κB signaling pathway, as well as interfering the polyubiquitination process of IκB-α, preventing IκB-α degradation. Mutations abolishing the OTU activity also impaired the ability to inhibit NF-κB and affected virus viability [180].
Another characteristic of nsp2 is that it is highly permissive to deletion and insertions both naturally and artificially. A 36 aa insertion compared to the sequence of VR-2332 has been identified in a vaccine strain [159]. And a 88 aa deletion that is able to differentiate the wild-type PRRSV was produced by continuous passing on cell culture [206]. Two isolates NADC30 and NADC31 containing nsp2 deletions in MN184 were also isolated [17]. By using PRRSV infectious clones, it has been proven that deletions of a 187 aa fragment (aa 480-667 of HuN4-F112) or a 21 aa fragment of BJ-4 could be tolerated by the virus without affecting its viability [150, 229]; constructs with tags inserted in the nsp2 region could also be rescued with inserted tags properly expressed [92, 228].

The PRRSV nsp2 has been intensively studied, and unexpected discoveries continue to come out. More recently, a new protein called nsp2TF (transframe fusion) encoded through -2 PRF at a conserved G_GUU_UUU sequence at an estimated efficiency of around 20% with the N terminal 2/3 of nsp2 was discovered. Nsp2TF overlaps the nsp2 coding region in the +1 frame. The nsp2TF protein seems to be related to virus viability, since mutation abolishing the nsp2TF expression impairs PRRSV replication and produces a smaller plaque compared to that of wild-type [54].

3.3 Nsp3

Nsp3 of PRRSV has not been studied much so far and our knowledge is largely based on studies of the arterivirus model, EAV. The EAV nsp3 is predicted to contain four transmembrane domains, with both the N and C termini residing in the cytoplasm [148]. Both nsp2 and nsp3 can be recovered from the membrane fraction of infected cells. The nsp2 PL2 activity and the cleavage of nsp2/3 are unnecessary for DMV formation. A 50 aa
luminal domain between the first and second transmembrane domain, is conserved among arteriviruses and contains four conserved Cys residues. Mutating these Cys residues impaired the formation of DMVs. In the context of infectious clone, mutating the four Cys is lethal. Nsp3 has a strong interaction with nsp3 even under very stringent conditions [166]. Both nsp2 and nsp3 are necessary and sufficient to induce the formation of double membrane structures that strikingly resemble those found in infected cells [165].

3.4 Nsp 4

Nsp4, a 3C like proteinase, which is 203 aa long, is the main protease responsible for processing [167]. The catalytic triad is composed of Ser118, His39, and Asp64 [227]. After translation, nsp1α, nsp1β and nsp2 are quickly autocleaved. The remaining nonstructural proteins are processed by nsp4. Nsp4 is folded into 3 domains: N terminal β-barrel domain, middle β-barrel domain, and a C terminal domain mixed of α/β structure (aa 157-199). The protease domain is 1-153 aa, and the catalytic site is located between N terminal domain and middle domain [188]. The enzyme is monometric and is activated upon cleavage at the nsp2/3 joint site [243].

Instead of cutting each protein tandemly one by one, nsp4 processes the poly protein using two pathways. In the main pathway, nsp3-8 is cleaved at the nsp4/5 joint site, yielding polyproteins nsp3-4 and nsp5-8. Then nsp5-8 is cleaved at 7/8 only whereas nsp5/6 and nsp6/7 joint sites appear to be inaccessible to the protease. In the alternative proteolytic cascade, which is used at low but significant level in infected cells, the nsp4/5 joint site is uncleaved and the nsp5/6 and nsp6/7 joint sites are processed. Nsp3-8 has to interact with
cleaved nsp2 to allow processing of the nsp4/5 junction. When nsp2 is absent, the nsp4/5 joint site cannot be processed. Nsp3-8 is processed following the alternative pathway [208].

3.5 Nsp5-8

The remaining proteolytic products of pp1a, namely nsp5-8, are not well characterized so far. The hydrophobic domains located in nsp5 postulate the association of nsp5 with membrane [165] and indicates that it may be related to DMV formation.

3.6 Nsp9

The viral genome replication and sgmRNA transcription of arterivirus are carried out in the membrane bound replication/transcription complex. The catalytic core of this complex is composed of nsp9 and nsp10, which possess RdRp and helicase functions respectively [43].

The nsp9 RdRp domain is located in the C terminal part, while the N terminal part has an unclarified function. A study based on *E. coli* expressed recombinant nsp9 protein revealed that the protein is able to initiate the RNA synthesis *de novo* in a template specific manner [12]. A Ser-Asp-Asp (SDD) motif, which is a shared property with coronaviruses and other nidoviruses, is identified in the nsp9 [12] and is similar to the the GDD signature of poliovirus RdRp and other RdRps [131]. This SDD motif is critical for virus viability. Mutation of any of the three residues except for GDD is lethal to the virus. The polymerase function and virus transcription are destroyed by mutations, however, virus replication was not affected [239].

In addition to the important function as replicase, nsp9 has also been identified as playing roles in viral immunogenicity. Two conserved T cell epitopes are identified in
regions aa 119-135 (KEEIALSEAQIACDIR) and aa 151-167 (VRGNPERVKGVLOCKNTRF) of nsp9 in type 2 PRRSV [135].

3.7 **Nsp10**

Nsp10 is a helicase that is part of the replicase complex that unwinds RNA with energy from ATP hydrolysis in the process of replication. The protein is composed of an N terminal zinc finger domain and a C terminal helicase domain with a linker region between them [43]. The zinc finger and the linker region are involved in EAV genome replication and transcription [195, 196]. The helicase domain is grouped into helicase superfamily 1 (SF1), commonly seen in positive strand RNA viruses. Nsp10 has polynucleotide stimulated ATPase and 5’ to 3’ unwinding activities of both RNA and DNA duplex [9, 88]. Nsp10 is also involved in viral immunity. Two T cell epitopes have been identified in the nsp10 protein aa 209-225 (VRILAGGWCPGKNSFLD) and aa 217-233 (CPGKNSFLDEAAYCNHL) induces IFN gamma production by PBMC [135].

3.8 **Nsp11**

Nsp11 is a nidovirus-specific uridylate-specific endonuclease (NendoU). This enzyme is considered to be a marker of Nidoviruses, since no counterparts of this enzyme have ever been discovered in other viruses. The enzyme activity is independent of Mn$^{2+}$. The enzyme has pyrimidine specificity with preference for cleavage at uridylates [128]. The mechanism of RNA hydrolysis of NendoU is similar to that of bovine pancreatic RNase A [53]. Nsp11 cleaves both single-stranded and double-stranded RNA substrates 3’ of pyrimidines with preferred cleavage at single-stranded uridylates. The fact that expression of
enzyme active nsp11 is toxic to cells and the NendoU knockout mutants remain capable of RNA synthesis suggest that the enzyme is targeting cellular RNA substrates.

The IFN inhibitory activity of nsp11 has been reported, and this inhibitory activity is mediated via RIG-I signaling pathway. Nsp11 inhibits IRF3 phosphorylation and blocks the nuclear translocation of IRF3 [232]. Endoribonuclease activity of nsp11 is critical for the inhibition [162]. The aa His3735, His3750, and His3779 of pp1ab are catalytic sites for NendoU activity, and mutating them to Ala aborted the inhibitory activity of nsp11 [232].

3.9 Nsp12

Few studies of arterivirus nsp12 have been performed so far, thus current knowledge of this protein is largely unknown yet.

4. Minor structural proteins

GP2, GP3, and GP4, expressed by sgmRNA2, sgmRNA3, and sgmRNA4 are contained in smaller amounts. The three proteins are incorporated in the virus envelope as multimeric complexes [30, 217]. Another protein expressed by sgmRNA2, ORF2b, or E protein, is also part of these complexes. Lacking of any one of the four proteins, incorporation of other proteins is affected, indicating the interaction of them [217]. It has been suggested that the GP2, GP3, and GP4 complex of EAV is disulfide linked [213, 215], while in the case of PRRSV, this remains to be determined. These proteins are unnecessary for viral particle production, but particles without minor structural proteins are not infectious [217]. The complex is related to cell tropism. PRRSV containing EAV ORF2, ORF3, and ORF4 regions can achieve the broad cell tropism of EAV [186]. Proteins GP2, GP3, and GP4
are N glycosylated. All of these three proteins have glycosylation sites essential for infectious virus production [31, 210].

4.1 GP2

GP2 is a 29-30 kDa large glycoprotein [122] composed of 256/249 residues (in type 2 and type 1 respectively). There are four domains included in the protein: 1) an N terminal signal sequence between residues 1 and 40 (1 to 37 in type 1), 2) a roughly 168 residue ectodomain, 3) a TM helice around 20 residues, and 4) a 20 residue endodomain [216]. Two N-glycosylation sites are identified in both type 1 and type 2 strains, but at different locations. In type 1 viruses, Asn173 and Asn179 are glycosylated, while in type 2 viruses, Asn178 and Asn184 are glycosylated. Both sites are conserved among type 1 or type 2 viruses [31, 121]. The role of N-glycosylation on virus infectivity is inconclusive. Mutation at both sites in LV strain suggested that none of them are critical for virus viability. However, a study based on type 2 PRRSV suggests that the N-glycosylation at 184 is required for infectious virus production [31].

ORF2b, the coding sequence of protein 2b or E, is totally embedded in ORF2. E protein is a non-glycosylated protein with 73 amino acid residues and the size of 10kDa. The start codon of 2b is 6 nt downstream of the ORF2 start codon, and the 2b translation is preferred compare to GP2 [223]. E protein is located in the endoplasmic reticulum and golgi complex in marc145 cells, N terminal 15 residues are the ER localization sequence, and 23-50 and 50-73 are also localization sequences [236]. An immunoprecipitate assay suggested that the 2b protein is incorporated into the PRRSV virion [224]. The E protein is critical for virus infectivity, but not for virion particle assembly. An E deleted mutant by mutating start
codon ATG to GTG is able to enter cells but further steps of replication are interrupted. The E protein consists of a single TM helix and is able to form an homo-oligomeric ion channel similar to the influenza M2 protein. Ion channel blocking agents and lysomotrophic basic compounds both inhibited PRRSV replication during the uncoating process [101]. Two Cys at 49 and 54 are highly conserved among North American isolates. Mutating the two Cys to Ser didn’t affect virus infectivity and replication, indicating that the two Cys are not essential for virus viability [100]. A myristoylation site has been identified at both type 1 and type 2 E protein N terminus [223]. Inhibition of myristoylation by adding chemical 2-hydroxymyristic acid, or removal of the myristoylation motif, decreased virus titer, which means that myristoylation of the E protein is not indispensible for virus viability but could facilitate virus growth [50]. Yu, et. Al., further illustrated that the myristoylation site affects the subcellular localization of the N terminal 15 residues of the E protein. Myristoylation sites and the hydrophilic C terminal are not essential to the membrane association of E protein, but they do orient the N terminus toward the cytoplasm and C terminal toward the ER lumen [236].

4.2 GP3

GP3 is composed of 265 and 254 aa for type 1 and type 2 PRRSV, respectively. GP3 is highly glycosylated, and 6 glycosylation sites in the ectodomain have been predicted. Removal of glycans reduced the molecular weight of GP3 from 42 to 27 kDa [67, 115]. GP3 was detected in the gradient fraction containing purified virion, and was observed by immunogold staining of purified virions from supernatant of infected cells with anti-GP3 antiserum, indicating that it is incorporated in the virion [32]. However, only a minor fraction
of GP3 was found to be secreted in the culture media as soluble membrane free form. Release of GP3 doesn’t generate a putative membrane anchor sequence. Secretory GP3 acquires Golgi-specific modification of its carbohydrate side chains and is folded into a disulfide-linked homodimer form. Translocation from ER to golgi compartment is an obligatory step in cellular secretion of secretory GP3 [115].

The GP3 protein is immunogenic. Adenovirus expressing GP3 is able to induce neutralizing antibodies and cellular immune response, including t cell proliferation responses and cytotoxic T cell responses. Interestingly, truncated GP3 with aa 2-64 deleted could induce significantly higher immune responses than wt GP3 [83]. Two relatively conserved epitopes among North American isolates with minor exceptions have been identified located at aa 67-74 (YEPGRSLW) and aa 74-85 (WCRIGHDRCGED) [240].

4.3 GP4

GP4 is 31 to 35 kDa and is predicted to be a type 1 integral membrane protein with an N terminal signal sequence and a C terminal membrane anchor [121, 214]. GP4 is composed of 183 aa in type 1 PRRSV and 178 aa in type 2 PRRSV [125]. Fragment aa 1-21 is a predicted cleaved signal peptide, and aa 161-181 in type 1 PRRSV and aa 156-177 in type 2 PRRSV is a trans-membrane helix. GP4 is highly glycosylated during transport though the ER-Golgi complex [197]. Four glycosylation sites can be identified as conserved among both type 1 and type 2 PRRSV. Two distinct hydrophobic domains, at the N terminus aa 1-17 and the C terminus aa 165-183, are supposed to be associated with membranes [121]. CD163, a member of the scavenger receptor cysteine-rich (SRCR) superfamily, is a cellular receptor of PRRSV. A recent study indicated that GP4 functions as a glycosyl-phosphatidylinositol
anchor protein colocalize with CD163 in the lipid rafts on cytoplasmic membrane, suggesting the participation of GP4 in virus entry [49].

GP4 is important in virus immunity, and both B cell epitopes and T cell epitopes have been identified. A neutralizing epitope (GVSAQQEKKISFG) has been intensively reported in LV. However, under conditions in which neutralizing antibodies are present against this epitope, mutation could take place to evade neutralizing antibodies both in cell culture and in pigs [28, 29]. Two T cell epitopes of both type 1 and type 2 have been discovered. The first one, CLFAILLAT (aa 175-183) in type 1 and CLLPSLLAI in type 2, is conserved. The second, FLLAGAQL/YI/L (aa 7-15) in type 1 and FLL/VGTKCF/L in type 2, is relatively variable [45]. In addition, type 1 PRRSV has one epitope SAAQQEKKISF (aa 59-67) that doesn’t exist in type 2 PRRSV [124].

5. Major structural proteins

In virion, GP5, matrix protein and nucleocapsid protein are contained abundantly. GP5 and matrix protein, which are the major components of the envelope, form disulfide linked heterodimers [116, 121]. Deletion of either protein could abolish viral particle formation, while in contrast deletion of minor proteins doesn’t affect viral particle production [217]. Nucleocapsid protein is the only protein contained in the PRRSV nucleocapsid that coat the viral genome inside. Co-expression of GP5, matrix and nucleocapsid protein of EAV only is insufficient to generate viral like particles, indicating that other components are needed in viral particle formation [216].
5.1 GP5

GP5 with the molecular weight of 26-30 kDa, is composed of 201 aa for type 1 PRRSV and 200 aa for type 2 PRRSV. Sequence analysis suggests that the protein is composed of four domains: an N terminal putative signal sequence, assumed to be cleaved off [121], followed by an ectodomain of approximately 35 residues with a variable number of possible N-glycosylation sites, a 60-residue hydrophobic region presumed to span the membrane 3 times, and a hydrophilic C terminal region of about 70 residues [127]. GP5 is the most variable protein of PRRSV with only about 55% amino acid identity between the two genotypes [125] and an amino acid identity of about 87%-99% within both genotypes [118, 175]. Four conserved regions (aa 40-57, 67-90, 103-120, and 129-163) have been identified. The N terminus putative signal sequence (aa 3-39) is the most variable region [3]. The GP5 ectodomain is glycosylated. Four N-glycosylation sites at 30, 33 (in some isolates could be 34 or 35), 44, and 51 of VR2332, and two N-glycosylation sites at 46 and 53 of LV have been identified. O-glycosidase had no effect on GP5, suggesting the absence of O-glycosylation [10].

GP5 is involved in virus neutralization and neutralizing antibody induction [68, 143]. Vaccination of DNA vectors containing each ORF of ORF1-7, ORF5 showed the highest titer of neutralizing antibodies, indicating that GP5 is the most important protein in introducing neutralizing antibodies [7]. A neutralizing epitope is located in the middle of the GP5 ectodomain (aa 36-52) of VR2332 [147], while in some type 1 PRRSV, a neutralizing epitope is located at 29 to 35 aa of GP5 [218]. In addition, a proline at aa 24 of LV is critical for the sensitivity of virus to a neutralizing antibody even though it is not included in the epitope [218]. The low level of aa identity (77%) between ectodomain of VR2332 and LV
may be responsible for the lack of cross neutralization between the two genotypes [147]. Using 69 field isolates that were separated into two groups based on their susceptibility to VR2332 VN antibody, a cross neutralization study illustrated that regions of aa 32-34, aa 38-39, and aa 57-59 of GP5 affected virus susceptibility to neutralizing antibodies, and suggested that these sites can be utilized to predict susceptibility of a given strain to another strain in terms of cross neutralization [94]. However, some recent studies cast the role of GP5 in inducing neutralizing antibody into an argument. Recombinant polypeptide of GP5 and M ectodomain of VR2332 has been constructed, and PRRSV infection could induce an ectodomain specific antibody, but this antibody is not associated with neutralizing activity. Animals immunized with polypeptide didn’t neutralize virus infection [104]. Our study also found that the GP5 and M protein of some PRRSV strains doesn’t contribute to virus neutralization (unpublished data). Moreover, antiserum against GP5 aa 37-45 (SHL/FQLIYNL), the epitope previously supposed to be major linear neutralizing epitope region, has been found unable to neutralize classical and HP PRRSV. This peptide cannot inhibit neutralization of pig hyperimmune sera, and the level of its antibody is not related to neutralization [102].

The role of GP5 in cell recognition and virus entry is controversial. Some studies have suggested that GP5 is important in those processes. Murine leukemia virus (MuLV) expressing GP5 was infectious to PAM and MARC145 cells and displayed the same host range with wild type PRRSV [226]. GP5/M complex was ligand for sialoadhesin, an important molecule involved in virus entry whose interaction depends on the presence of sialic acids on GP5 [192]. However, the GP5 ectodomain of EVA replaced with LDV or PRRSV could still infect BHK-21 and RK-13, which are not hosts for LDV and PRRSV,
indicating that GP5 is not the determinant of cell tropism [47]. In addition, the EAV backbone with PRRSV GP5 and M protein only infects the EAV susceptible cell line but not the PRRSV susceptible cell line, suggesting that GP5 and M protein don’t determine the cell tropism of PRRSV [111].

N glycosylation is involved in immune evasion of neutralization. Mutating N44 didn’t generate infectious progeny indicating that the N44 is critical for virus viability. Mutation at sites N34 and N51 are not lethal but did affect virus growth and generated a mutant virus with a lower titer compared to the wild-type virus. Mutants also demonstrated an increased sensitivity to swine antiserum and moreover, pigs inoculated with N glycosylation abolished strains induced significantly higher neutralizing antibodies against the wild-type virus than the wild-type virus itself [4]. Similarly, N 53 of LV, corresponding to N51 of VR2332, is not indispensable for virus viability, while N46 of LV, corresponding to N44 of VR2332, is also critical for virus viability [216]. However, another study has indicated that removal of N-glycosylation of GP5 at 30, 35, 44, or 51 didn’t affect virus growth in vitro but did affect virus growth in pigs. Removal of all N-glycosylation didn’t induce viremia and antibodies. Removal of N44 increased sensitivity of mutant virus to antiserum, but didn’t elicit a high level of neutralizing antibodies to wt PRRSV [209]. Mutating the N glycan of GP5 at 30, 33, 44, and 51 to remove glycan increased the level of neutralizing antibody response compared to that of wild-type GP5 [84].

GP5 contains both B cell epitopes and T cell epitopes. Several B cell epitopes have been identified in the locations of aa 37-45, aa 146-156, aa 164-180, and aa 192-200, where mutations could block antibody recognition [145, 146, 242]. Two relatively conserved T cell
epitopes located at aa 117-131 (LAALICFVIRLAKNC) and aa 149-163 (KGRLYRWRSPVII/VEK) have been discovered in GP5 of type 2 PRRSV [199].

PRRSV could induce apoptosis in infected cells. Signs of apoptosis such as internucleosomal DNA cleavage, degradation of rRNA, and chromatin condensation were clearly observed in both MA104 and PAM cells infected with PRRSV. The same phenomenon was also observed in cells with GP5 protein expression [174]. Adenovirus expressed GP5 has also been identified to be pro-apoptotic [60]. The region of the N terminal 1-119 aa is capable of the induction of apoptosis, and the 90-119 region is fundamental. The C terminal region doesn’t induce cell death by itself [55].

More recently, a new PRRSV protein was discovered almost simultaneously by two different groups using phylogenetic analysis and infectious clone confirmation [57, 85]. ORF5a protein is essential for virus viability. Mutation of ORF5a by optimizing ORF5 caused death of the virus. Inactivation of ORF5a in both type 1 and type 2 PRRSV was lethal for the virus, but the death could be recovered by expressing ORF5a protein in trans [178]. Comparing ORF5a expressing PAM cells and normal PAM cells found 16 proteins associated with cell growth, cytoskeleton networks, and cell communication, metabolism, protein biosynthesis, RNA processing and transportation [132].

5.2 Matrix protein

The molecular weight of M protein was estimated to be about 19 kDa [10, 121]. The M protein has 173 aa for type 1 and 174 residues for type 2 and is more conserved than other PRRSV structural proteins. It is a type III membrane protein with a 16 aa N terminal ectodomain followed by a transmembrane region that is supposed to span the membrane
three times and an 84 aa C terminal endodomain. The N terminal ectodomain of the M gene is required for virus viability. Partial deletion of the ectodomain by removing aa 9-16 failed to produce a viable virus. Viruses were rescued when the M protein ectodomain of LV was replaced with the corresponding region of LDV, EAV, and VR2332; however, virus growth was impaired, and virus titer was much lower. They maintained the original cell tropism but didn’t gain tropism of other viruses, indicating that the M ectodomain is not the determinant of cell tropism [202]. Heraran sulphate is a molecule to which PRRSV attaches to during the early infection stage. Following this interaction, the virus gradually interacts with sialoadhesin. Heparan sulphate is not necessary for sialoadhesin to function as PRRSV internalization receptor, but does enhance the interaction of the virus with sialoadhesin [38]. It has been shown that, in complex with GP5, M could bind to heparin [39].

The M protein ectodomain is immunogenic. Chimeric viruses replacing the M protein ectodomain of LV with the corresponding region of LDV, EAV and VR2332 are immunologically distinguishable from the wild-type LV [202]. The M protein has been suggested as being more involved in cellular immunity since it could induce a higher degree of T cell proliferation compared to other proteins [11]. A T cell epitope ALKVSRGRLLLGLHL located at aa 33-47 of the M protein is universal among type 2 PRRSV. Other epitopes located at aa 9-23, aa 57-71, and aa 93-107 may have 2-4 aa substitutions among the different strains [207].

5.3 Nucleocapsid protein

The PRRSV nucleocapsid (N) protein is 15 kDa in size and encoded by ORF7 from the smallest sgRNA (sgmRNA7). It is a highly basic protein with an isoelectric point at pH
The N protein is the most abundantly expressed viral protein in infected cells and comprises up to 40% of the protein content in a virion [10, 221]. The N protein of type 2 PRRSV is composed of 123 amino acid residues, while type 1 PRRSV’s N protein varies in length from 124aa to 132 aa although it is most commonly128 aa [81, 171, 173]. The N protein is supposed to form disulfide-linked homodimer in assembled viral nucleocapsid [116]. In addition, the N protein is phosphorylated at serine residue immediatley or shortly after the synthesis of the protein. The phosphorylated N protein is incorporated in mature virion and remains phosphorylated throughout its life cycle [221].

The protein is composed of an N terminal RNA binding domain and a C terminal dimerization domain. The N terminal 11 aa seems to be not important for virus growth: replacing them with a 15 aa peptide did not affect virus titer and growth speed [106]. The unstructured N terminal is rich in basic amino acids to facilitate the interaction with the viral genome [5]. The C terminal contains two tandem antiparallel β strands (β1, β2) flanked by two α helices at both ends (α2, α3), and one extra short α helix (α1) lying at the upstream of α2 [46]. Under reducing conditions that disturbs disulphide linkages, the N protein binds less RNA than the non-reducing condition. Mutating the 23 Cys to Ser was shown to produce an identical result indicating that the disulphide-linkage formed by 23 Cys is critical for RNA binding [87]. Changing regions of aa 21-30, 41-50, or 111-123 to sequential Ala resulted in mutant N protein failing to bind RNA [87]. A previous study identified the region 41-47 as the RNA binding region [233]. It could be that the mutation changed the positive charge and reduced the interaction with negatively charged RNA molecules. Deletion of the last 11 amino acids changed the protein conformation, indicating that the conformation may also be required for RNA binding [220]. The N protein dimerizes through noncovalent interactions
after synthesis [222]. The end-to-end contacts are accommodated by interactions between Val112, Ile115 and Arg116 in α3 and Ser 93 and Asp94 in the β loop. Hydrogen bonds between Ser70 in α2 and Glu103 in β2 are also needed [46]. The role of the N terminal seems to be controversial. The N protein with the first 57 aa deleted could form dimers in crystal, suggesting that it is non-essential in dimerization [46]. On the other hand, another study has demonstrated that amino acid residues 30-37 are essential for the N protein interaction, and deletions that included this region failed to form homodimers. In addition, RNA may also participate in dimerization of the N protein [222].

The N protein is the only protein contained in nucleocapsid. By comparing PRRSV with alphaviruses, predicted α helix (α0) in the N terminal domain aa 21-35 is supposed to play a role in assembly [170]. The formation of PRRSV nucleocapsid is not clarified, and two models have been proposed. The first model suggests that N dimers formed through C terminal interaction compose a single layered shell. RNA is coated inside the shell through binding with the N terminal domain [44, 46]. A new model has proposed that two opposite N dimers form a ring structure through the N terminal region α helix. RNA is embraced by this ring and interacts with the N terminal positively charged region, especially aa 35-51. This interaction forms a twisted chain composed of RNA and N protein. The chain bundles into a spherical structure and leaves a hollow interior [170].

The N protein is the most immunogenic viral protein, but anti-N antibody is not neutralizing and not protective [126]. Antigenic variation exists between type 1 and type 2 PRRSV strains, and monoclonal antibodies SDOW17, EP147, and Vo17 could recognize type 2 N protein, while only SDOW17 among those could recognize the N protein of LV [113]. Linear B cell epitopes located in regions of amino acid residues 51-58, 50-66, 79-87 of
type 2 PRRSV and 25-30 of type 1 PRRSV have been identified as being conserved among the two genotypes of PRRSV \([2, 20, 123, 241]\). Linear B cell epitopes at 2-12 and 40-46 of LV are conserved in type 1 but not type 2 PRRSV \([123]\).

The N protein has the function of modulating cytokine production. Strong IL-10 producing cells and Treg was observed in N protein pulsed monocytes derived dendritic cells \([219]\). N protein is able to inhibit IFN-β production induced by dsRNA and interfere with dsRNA induced phosphorylation \([156]\). N protein is also able to interact with cellular transcription factor NF-κB, the inhibitor of the MyoD family-a (I-mfa) domain-containing protein (HIC), and IRF3 \([112, 169]\), implies the possible regulation of host gene expression. In addition to cytokine modulation, N protein interacts with the host cell protein synthesis machinery as well as with the RNA post transcriptional modification machinery, especially at the level of translation initiation \([86]\). The N protein binds poly a binding protein (PABP) and enhances translational efficiency by circularizing mRNA. Silencing PABP could result in reduced levels of RNA synthesis, viral encoded protein, and virus titer \([205]\).

The N protein localizes to the nucleus and primarily nucleolus within the nucleus of infected MARC-145 and PAM cells. Sequence analysis reveals two nuclear localization signals (NLS), namely NLS-1 and NLS-2, located at N terminal aa 10-13 and 41-47, either of which could bring EGFP to the nucleus \([154, 155]\). A nucleolar localization signal (NoLS) sequence is located between residues 41 to 72. Mutation and complete removal of NLS-1 doesn’t change its localization, but mutating the key Lys with uncharged aa in the NLS-2 abolished nuclear/nucleolar localization of the protein \([99, 155]\). Residues KKNKK in the NLS-2 are identified to be the core domain of localization \([155]\). The trafficking of N protein between cytoplasm and nucleus is two way, i.e. the N protein is able to import to and export
from the nucleus [235]. Importin-α, Importin-β, and CRM1 have been suggested as playing roles in the in/out nucleus transport of the N protein [155, 191]. Mutating the 23 Cys to Ser abolished dimerization of N, but N is still localized to the nucleolus, indicating that the N protein nuclear localization is independent of dimerization [98]. The growth of NLS abolished virus was crippled to a titer 100-fold less than wild-type virus. An animal study showed that pigs had a significantly shorter mean duration of viremia than wt, and developed significantly higher titers of neutralizing antibodies, and NSL-abolished viruses restored their nucleus translocation ability [99].

6. Conclusion and Perspectives

PRRSV continues to be a major concern in the swine industry since its recognition. Vaccines have been developed and widely used. However, continuous genetic mutations and antigenic changes of the virus and its ability to modulate and evade the host immune system have limited the efficacy of vaccine, thus impeding effective control of the disease. In the past decades, our knowledge of PRRSV genomics and proteomics has been greatly enhanced through studies on PRRSV and other arteriviruses or nidoviruses. Nevertheless, detailed mechanisms describing the immunobiology, pathogenesis, and immune modulation and evasion remain largely unknown. The biochemical, structural and functional characterizations of key viral proteins need to be further studied to lay an foundation for the development of the next generation of PRRSV vaccine that provide a broad prism of protection and high safety.
References


syndrome virus (PRRSV) inferred from DNA sequences of putative ORF-5 and ORF-7 genes. Virus Res 42:159-65.


CHAPTER 2. CROSS PROTECTION CONFERRED BY CHIMERIC VIRUSES CONTAINING STRUCTURAL GENES OF TWO ANTIGENICALLY DISTINCTIVE STRAINS OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS

Dong Sun, Won-Il Kim, Vickie Cooper, Yong-II Cho, Chong Wang,
Eun-Jin Choi, Kyoung-Jin Yoon

Abstract

Because significant antigenic variation exists among field isolates of porcine reproductive and respiratory syndrome virus (PRRSV), suboptimal cross protection among different virus strains impedes the effective control of PRRS via vaccination. Our previous study showed that chimeric viruses containing mixed structural genes from two distinct strains (VR2332 and JA142) of PRRSV in an organized manner were highly susceptible to the viral neutralizing activity of antisera generated against both strains. In this study, three chimeric viruses (JAP5, JAP56 and JAP2-6) were constructed by replacing ORF5, ORFs 5 and 6, and ORFs 2-6 of VR2332 with the corresponding ORF(s) of JA142, respectively and were evaluated in pigs for their ability to confer cross protection against challenge with the VR2332 and JA142 strains. A total of 114 pigs were divided into 6 groups and each group was intramuscularly inoculated with one of the 3 chimeric viruses (n=16 per group), VR2332 (n=24), JA142 (n=24), or sham inoculums (n=18). At 44 days post inoculation (dpi), these pigs were divided further into 15 groups (n= 6 or 8 pigs per group) and challenged intranasally with VR2332, JA142, or sham inoculum. Although no pigs demonstrated severe clinical signs or lesions, all pigs inoculated with one of the chimeric viruses prior to challenge had lower levels of viremia than the challenge control pigs. Prior inoculation of JAP56 drastically decreased viremia to almost undetectable levels in pigs challenged with
both VR2332 and JA142. These results suggest that chimeric viruses which have mixed structural genes of two distinctive PRRSV strains in an organized manner can induce cross protection against both of the donor viruses.

**Introduction**

Porcine reproductive and respiratory syndrome (PRRS) is characterized by reproductive disorder in breeding animals and respiratory distress in pigs of all ages [4, 18]. Emergence of PRRS was almost simultaneously recognized in the United States and Europe in late 1980’s [25, 47]. Isolation of PRRS virus (PRRSV), the causative agent of PRRS, was first reported in Europe in 1991 [57] and later in North America in 1992 [9]. Since then, the virus has been identified in most of pig-producing regions worldwide and has caused significant economic losses in swine industries [10, 41, 48]. Annual economic loss in the US swine industry due to PRRS has been estimated to be US$ 500-660 millions [21, 43].

PRRSV is a member of the family *Arteriviridae* in the order *Nidovirales* [6] and is an enveloped virion containing a single-stranded positive-sense RNA genome. PRRSV has been classified into two genetically and antigenically distinct groups, European (Type 1) and North American (Type 2) genotype [39, 42]. Remarkable genetic and antigenic variations have been observed between the genotypes and within the same genotype [8, 14, 15, 26, 49, 60, 62]. Such genetic and antigenic variation has hampered effective prevention and control of PRRS through immunity-based interventions.

In general, PRRSV induces weak pro-inflammatory cytokines [35, 55] and anti-inflammatory cytokines [7, 12, 16, 54] and stimulates delayed and weak protective immunity [i.e., virus neutralizing (VN) antibody and interferon (IFN)-γ secreting T cells] which seldom
appears until 3 to 4 weeks after infection [13, 38, 56]. Passively acquired VN antibody alone was proven to prevent viremia and reproductive failure in pigs subsequently challenged with virulent PRRSV strains [46, 61], suggesting the critical role of VN antibody in the control of virus infection. Nonetheless, cell-mediated immunity (CMI) is believed to be essential for the clearance of PRRSV since PRRSV has been detected in lungs and lymph nodes despite the presence of VN antibodies in serum or bronchoalveloar lavage fluid [3, 33, 36, 58]. Anti-viral activity of IFN-γ secreted from activated T-cells and NK cells and the clearance of PRRSV-infected cells by cytotoxic T lymphocytes are known to be crucial in cell-mediated protective immunity against PRRSV [2, 7, 37, 53].

The genome of PRRSV encodes at least 10 open reading frames (ORFs). ORF1a and ORF1b encode nonstructural proteins that involve in virus replication. ORF2a, 2b (or E), 3, 4, 5a, 5, and 6 encode envelope-associated structural proteins and ORF7 produces nucleocapsid (N) protein. The GP5 which is encoded by ORF5 has been considered to be the most important protein in inducing VN antibody [17, 50, 59]. In addition to GP5, GP3, GP4 and M protein expressed from ORFs 3, 4 and 6 respectively, have also been reported to play roles in inducing VN antibody. Yet, GP4 of type 2 PRRSV has not been observed to be related with neutralization [5, 30, 32]. More recently, ORF5a which consists of 153 nucleotides with a 143-nucleotide overlap region with ORF5 was identified. Although ORF5a protein specific antibody was detected at 20 days after infection, the role of ORF5a product in inducing VN antibody is unclear [23]. Besides inducing VN antibody, GP3, GP4, GP5, M and N proteins are also reported to have T-cell epitopes that can stimulate CMI response [22, 32, 34].

VN antibodies play important roles in PRRSV immunity [36]. Since several structural proteins of PRRSV have been reported to be associated with virus neutralization, the role of
those proteins in the induction of VN antibody could vary among different PRRSV strains. Our previous study showed that chimeric viruses with combined structural genes from two antigenically distinct PRRSV strains (i.e. VR2332 and JA142) in an organized manner were susceptible to antisera generated against both strains in vitro [30]. Thus the following study was conducted to explore if such chimeric viruses can confer pigs broader cross protection against challenge with those two heterologous donor PRRS viruses.

**Materials and Methods**

**Viruses and Cells.** Two field isolates of type 2 PRRSV, VR2332 [9] and JA142 [40], were used as reference viruses in the study. These 2 strains share 91% nucleotide identity in ORF5 and 93% nucleotide identity in ORFs 2-7. Genetic and antigenic difference between these viruses has been well documented previously [24, 29]. Three chimeric PRRS viruses designated as JAP5, JAP56 and JAP2-6 were constructed previously based on a VR2332-derived infectious clone [30] and used in an animal study. The chimeric viruses were named based on the ORF(s) of the VR2332 infectious cDNA clone replaced by the corresponding ORF(s) of JA142. All the viruses were propagated in MARC-145 cells, a sub-clone of African green monkey kidney cell line MA104, which is known to be highly permissive to PRRSV [27]. MARC-145 cells were maintained in RPMI-1640 (Sigma-Aldrich Corporation, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Atlas Biologicals, Fort Collins, CO), 100U/ml penicillin, 100ug/ml streptomycin and 250ng/ml amphotericin B (hereafter, RPMI growth medium) at 37 °C in a humidified 5% CO₂ atmosphere.
Animal study. A total of 114 three-week-old pigs were purchased from a commercial operation historically known to be free of PRRSV and housed in the Iowa State University Large Animal Infectious Disease Isolation Facility. After acclimation, the pigs were randomly divided into 6 groups and housed separately. Each group was intramuscularly inoculated with one of JAP5, JAP56, JAP2-6, VR2332, JA142 (2mL of $10^3$ TCID$_{50}$/mL per pig), or sham inoculum RPMI-1640 medium). This was considered as live virus vaccination. At 42 days post-inoculation (dpi), pigs of each group were randomly divided further into 2 or 3 subgroups and housed separately in one of 15 rooms (Table 1). Then each pig was intranasally challenged with JA142, VR2332 (2mL of $10^3$ TCID$_{50}$/mL per pig) or sham inoculum at 44 dpi. Two weeks after challenge (58 dpi from the first inoculation), a half of pigs in each subgroup were euthanized for necropsy. The remaining pigs were euthanized at 4 weeks after challenge (72 dpi from the first inoculation).

All pigs were bled at 0, 7, 14, 21, 28, 35, 42, 48, 51, 55, 58, 65 and 72 dpi. During necropsies, pigs were examined for any gross lesions. Lung samples were collected from each pig for histopathology and PRRSV immunohistochemistry (IHC) [19].

The animal use protocol for the study was reviewed and approved by Institutional Animal Care and Use Committee (ISU-IACUC No. 8-07-6407-S) and the study was conducted by fully observing the appropriate animal care and well-being regulation.

Sample processing. Approximately 10% (w/v) homogenate was made from each lung tissue in Earle’s Balanced Salt Solution (Aldrich Corporation, St. Louis, MO) using Stomacher (Sward Laboratory System Inc., Port Saint Lucie, FL). After centrifugation at 3000 x g for 30 min at 4 °C, the supernatant was collected for testing. Blood samples
collected into Vacutainer® SST™ Plus Blood Collection Tubes (BD, Franklin Lakes, NJ) were centrifuged at 2000 x g for 10 min, and supernatants were transferred into new snap cap tubes. All processed samples (lung tissue homogenates and sera) were kept frozen at -80°C until being tested.

**Determination of viral titers in specimens.** Viral RNA was extracted from sera and lung homogenates by using MagMAX™ 96 Viral RNA Isolation Kit (Ambion, Foster City, CA) with Kingfisher® 96 Magnetic Particle Processor (Thermo scientific, Waltham, MA) as per the user’s manual. A commercial real-time RT-PCR was performed on extracts using TaqMan® NA and EU PRRSV Reagents (Applied Biosystems, Foster City, CA). The cycling conditions were 45°C for 10 min, 95 °C for 10 min and 40 cycles of 97 °C for 2 sec and 60 °C for 40 sec. A set of PRRSV (VR2332) with titer of 10^5, 10^4, 10^3, 10^2, 10^1 or 10^0 TCID₅₀/mL were prepared as standards and included in PCR testing to generate a standard curve and regression equation. Virus titers (TCID₅₀/mL equivalent) in samples were estimated by the equation.

**Sequencing.** Lung samples positive for PRRSV by PCR were sequenced for ORFs 5-7 to determine whether the virus detected in each lung homogenate was due to 1ˢᵗ inoculation (i.e., live virus vaccination) or 2ⁿᵈ inoculation (i.e., challenge). ORFs 5 and 6, or ORF7 of PRRSV, respectively were amplified by SuperScript™ III One-Step RT-PCR System (Invitrogen Corporation, Carlsbad, CA) with primers listed in Table 2. The PCR conditions were 50 °C 30 min, 94 °C 2 min, 40 cycles of 94 °C 15 sec, 60 °C 30 sec and 68 °C 3 min, and a final extension of 68 °C for 5 min. PCR products were purified with QIAquick® PCR
purification kit (Qiagen, Valencia, CA) and submitted to the Iowa State University Nucleic Acid Facility with the PCR primers for sequencing. Sequencing results were analyzed using Lasergene® software (DNASTAR Inc., Madison, WI).

**ELISA.** All serum samples were tested for anti-PRRSV antibody using a commercial ELISA kit (HerdChek® PRRS 2XR Porcine Reproductive and Respiratory Syndrome Virus Antibody Test Kit; IDEXX Laboratories, Westbrook, ME) as per the manufacturer’s instructions to assess IgG antibody responses to vaccination and/or challenge. The kit can detect antibody specific for N protein of PRRSV (both type 1 and type 2).

**Virus neutralization assay.** Fluorescent focus neutralization (FFN) assay was performed to assess the titer of VN antibodies in sera as previously described [28]. The serum samples were first heat-inactivated at 56 °C for 45 min and then 2-fold serially diluted with RPMI-1640 growth medium. One hundred μl of each diluted serum were mixed with an equal amount of PRRSV (VR2332 or JA142) at $10^3$ fluorescent focus forming unit per mL (FFU/mL). The mixtures were incubated at 37 °C for 1 hr in a humidified atmosphere with 5% CO₂ supply and then transferred onto MARC-145 cell monolayers prepared in 96-well plates (Corning Inc., Corning, NY) and incubated for another 1 hr at 37 °C in a humidified atmosphere with 5% CO₂ supply. After the removal of the inoculum, cells were replenished with 200 μl of fresh RPMI-1640 growth medium per well and further incubated at 37 °C for 20 hr. Afterwards, cells were fixed with ice cold 80% acetone aqueous solution for 5 min. The fixative was removed and cells were air dried for 30 min. Then cells were reacted with 1:10000 diluted PRRSV N protein specific monoclonal antibody SDOW-17 (Rural
Technologies, Brookings, SD) and stained with 1:250 diluted fluorescein isothiocyanate (FITC) labeled goat anti-mouse IgG (H+L) (KPL, Gaithersburg, MD). Before observation under an invert fluorescent microscope, plates were washed three times with 0.01 M phosphate-buffered saline (PBS, pH 7.2) and then number of virus-specific fluorescent foci in each well was counted. VN antibody titer was expressed as the reciprocal of the highest dilution in which 90% or higher reduction in the number of FFU (i.e., <10 FFU/well) was observed.

Necropsy, histopathology evaluation and IHC. Lung sections (two sections from the cranial lobe and one from each of middle, accessory, and caudal lung lobes) were fixed in 10% neutral buffered formalin and processed for histopathological examination at Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL; Ames, IA). All tissue section slides with H&E staining or PRRSV IHC staining were examined by a pathologist in a blinded fashion. The gross and microscopic lesions and IHC staining were scored as 0 (no lesion), 1 (mild lesion), 2 (moderate lesion), and 3 (severe lesion) based on the severity of interstitial pneumonia or intensity and distribution of staining [44]. To minimize the variation in scoring, all evaluations were performed by the same pathologist.

Statistical analysis. Data were statistically analyzed by JMP (SAS Institute Inc., Cary, NC). Viremia data were compared by ANOVA with repeated measurement. A contrast was constructed where direct comparisons between the groups were necessary. Lung scores were analyzed by Wilcoxon rank sum test.
Results

Viremia after 1st inoculation (hereafter, vaccination) with wild-type or chimeric viruses. None of pigs was viremic on day 0. Viremia was detected in all of the vaccinated pigs at 7 dpi (Fig. 1A and 1B), when most viruses reached their highest titers. The mean levels of viremia at 7 dpi in the pigs vaccinated with wild-type viruses (i.e., VR2332 or JA142) were $10^{4.6\pm0.1}$ or $10^{5.2\pm0.1}$ TCID$_{50}$/mL respectively and were significantly higher ($p<0.001$) than those in the pigs vaccinated with the three chimeric viruses (ranging from $10^{2.9\pm0.1}$ to $10^{3.3\pm0.1}$ TCID$_{50}$/mL). At 42 dpi, the last bleeding time point before challenge (i.e., 2nd inoculation), viremia was not detected in most (50-87.5%) of vaccinated pigs and the average levels of viremia in all of the groups were lower than $10^{0.7\pm0.1}$ TCID$_{50}$/mL. The sham inoculated group remained negative for PRRSV viremia by 42 dpi.

Viremia levels after challenge with JA142 or VR2332. After challenge (48-72 dpi from the 1st inoculation) with JA142, levels of viremia in the JA142-challenge control group (N/JA142) and heterologous challenge group (VR2332/JA142) were significantly higher than those in other groups ($p<0.001$) as shown in Fig. 1A. On the other hand, all pigs vaccinated with one of the chimeric viruses produced low levels of viremia which were not statistically different ($p>0.1$) from those in the homologous challenge group (i.e., JA142/JA142) and negative control group (N/N). No significant difference in the overall viremia level was observed among groups vaccinated with the chimeric viruses although the JAP5/JA142 group showed significantly ($p<0.05$) higher level of viremia than the homologous challenge group (JA142/JA142) at 51dpi and 58 dpi.
Viremia patterns among groups after challenge (48-72 dpi) with VR2332 are illustrated in Fig. 1B. The VR2332-challenge control group (N/VR2332) produced the highest level of viremia among all of the groups challenged with VR2332 ($p<0.001$), while no significant difference in the overall viremia level was observed among all other groups after challenged with VR2332 even when they were compared to the negative control group (N/N). Group JAP2-6/VR2332 had viremia significantly higher than all other groups except for the VR2332-challenge control group (N/VR2332) at 55 dpi. To our surprise, the heterologous challenge group (JA142/VR2332) generated viremia at the level similar to ($p>0.1$) those found in the homologous challenge group (VR2332/VR2332) and negative control group (N/N).

**Viral loads in lungs.** PRRSV was detected in all of the challenged pigs except for the negative control group (N/N) (Fig. 2). Overall, viral loads were lower in the lungs collected at 72 dpi than those collected at 58 dpi. Furthermore, vaccinated pigs showed lower levels of viral load in the lungs than challenge control groups (N/VR2332 and N/JA142).

**Identification of PRRS viruses detected in lungs after challenge.** Because live viruses were used as vaccines, all PRRS viruses detected in the lungs after challenge were sequenced to determine the source of virus, i.e., vaccination or challenge. As summarized in Fig. 2, both vaccine viruses and challenge viruses were detected in all groups except for group JAP56/VR2332 in which only vaccine virus (i.e., JAP56) was detected. Among the pigs vaccinated with the chimeric viruses, JAP56 persisted in the least number of pigs (7 of 16 pigs) in comparison with JAP5 (8 of 16 pigs) and JAP2-6 (9 of 16 pigs). The challenge
viruses (either VR2332 or JA142) were detected in a less number of pigs when vaccinated with either JAP5 or JAP56 (5 of 16 pigs for both) as compared with pigs vaccinated with JAP2-6 (6 of 16 pigs). The rate of detecting challenge viruses in pigs vaccinated with any of the chimeric viruses (5 of 16 pigs, 31.3% to 6 of 16 pigs, 37.5%) were lower than that in heterologous challenge groups, VR2332/JA142 (5 of 8 pigs, 62.5%) and JA142/VR2332 (3 of 7 pigs, 42.9%).

**ELISA antibody response to vaccination and challenge.** Pigs seroconverted to PRRSV at 2 weeks after vaccination and remained seropositive until the end of the study although S/P ratios were in a decreasing trend until 51 dpi after 42 dpi, i.e., the first week after challenge (Fig. 3.). Significant anamnestic antibody response was not apparent in any of the vaccinated groups after challenge. The negative control group (N/N) remained seronegative during the entire study period.

**VN antibody responses before and after challenge.** Levels of VN antibodies in serum samples were assessed by FFN assay. At 42 dpi (the last bleeding time point before challenge) pigs vaccinated with either VR2332 or JA142 had VN antibody only against the inoculum virus, i.e., no cross-neutralizing activity (Fig. 4). Among the chimeric viruses, JAP5 induced VN antibody (1:4) against VR2332 in one out of 16 pigs whereas JAP56 and JAP2-6 did not induce any VN antibody against VR2332 (Fig. 4A). Similarly, more pigs (5 out of 16 pigs) vaccinated with JAP2-6 produced higher levels (1:2 – 1:4) of VN antibody against JA142 than pigs vaccinated with JAP5 (1 out of 16 pigs; VN antibody titer 1:2) and pigs vaccinated with JAP56 (0 pig) (Fig. 4B).
After challenge with VR2332 at 44 dpi, VN antibody response was not significantly boosted by the VR2332 challenge in any of the subgroups; no group has VN antibody titer higher than 1:2 on average at any time point. (Fig. 5. A-C). On the other hand, VN antibody levels in pigs vaccinated with VR2332 (negative to 1:16 at 72 dpi), JAP5 (1:8 – 1:64 at 72 dpi), JAP56 (1:2 – 1:8 at 72 dpi) or JAP2-6 (1:2 – 1:32 at 72 dpi) were boosted after challenged with JA142 compare to those before challenge (Fig. 5D-F). In contrast, the N/JA142 group did not produce any detectable level of VN antibody specific for JA142 even at 4 weeks after challenge (Fig. 5F).

**Pathological evaluation.** Most of challenged pigs presented mild to moderate gross and microscopic lesions, and distribution and intensity of IHC staining, and only one pig vaccinated with JAP2-6 then challenged with VR2332 at 42 dpi had extensive IHC staining at 58 dpi (Fig. 6). Overall the differences in both microscopic lesion and IHC scores among groups were not significant ($p>0.05$).

**Discussion**

The purpose of the current study is to evaluate if chimeric viruses with mixed structural genes from two distinctive PRRSV strains (VR2332 and JA142) is able to induce cross protective immunity against both donor strains in pigs. Pigs were inoculated first with one of the chimeric viruses or wild-type donor PRRS viruses as ‘live virus’ vaccination and then challenged with VR2332 or JA142 at 44 dpi to assess the degree of cross protection conferred by the vaccination. After the first inoculation (i.e., vaccination), all pigs inoculated
with the chimeric viruses developed significantly \((p<0.001)\) lower levels of viremia than ones inoculated with one of the wild-type donor viruses (Fig. 1A, 1B).

Moreover, pigs vaccinated with chimeric viruses demonstrated a significant level of cross protection against subsequent virus challenge with both VR2332 and JA142. Especially, pigs vaccinated with JAP56 of which ORFs 5 and 6 sequences were introduced from JA142 did not produce significant levels of viremia after challenge with either VR2332 or JA142. Levels of viremia in the JAP56 group were not statistically different \((p>0.1)\) from those observed in the homologous challenge groups (i.e., VR2332/VR2332 or JA142/JA142) and negative control group (N/N), demonstrating that a chimeric virus with mixed genes from two different strains in an organized manner could provide cross protective immunity against both donor viruses.

To define the possible protection mechanisms of the chimeric viruses, VN antibody titers in sera from pigs vaccinated with the chimeric viruses were measured against both JA142 and VR2332. JA142 induced a higher level of VN antibody in more pigs as compared to VR2332. At 42dpi, before virus challenge, 13 of 20 pigs vaccinated with JA142 (ranging between 1:2 and 1:32) and 9 of 24 pigs vaccinated with VR2332 had VN antibody (ranging between 1:2 and 1:8) against only the homologous virus. In the case of pigs vaccinated with chimeric viruses, 5 of 16 pigs vaccinated with JAP2-6 had VN antibody against JA142 (ranging between 1:2 and 1:4), and 1 of 16 pigs vaccinated with JAP5 had VN antibody against both VR2332 and JA142 (VN titer 1:4 and 1:2 respectively). However, no measurable VN antibody was detected in pigs vaccinated with JAP56 even though pigs vaccinated with JAP56 demonstrated cross protection against both challenge viruses based on level of viremia.
VN antibody has been proposed to be an important factor in protecting pigs from PRRSV infection even if it is not the only factor [36, 56]. It was observed that VN antibody of titer 1:8 achieved by passive transfer of concentrated PRRSV antibody (i.e., fractionated IgG) to pigs was able to protect pigs from developing viremia, although virus could still be detected in lungs and lymphoid organs. On the other hand, passive transfer of VN antibody titer as high as 1:32 could confer a half of recipients the sterile immunity against challenge [36], which indicated again that VN antibody alone could not provide the pigs with full protection against PRRS. In the current study, even though no VN antibody was detected in vaccinated pigs before challenge, pigs vaccinated with chimeric viruses were still protected from challenge with two distinct PRRS viruses. Similar observation has also been made in a previous study related to PRRSV [11] and other arterivirus [1], suggesting that other factors such as cell-mediated immunity and cytokine responses rather than VN antibody responses should be explored to define accurate mechanisms for broader protection by inoculation of the chimeric viruses in the future.

After JA142 challenge, the non-vaccinated group developed the highest level of viremia among JA142 challenged groups. The heterologous challenge group (VR2332/JA142) also showed significantly higher viremia level than the other vaccinated groups, indicating that the VR2332 strain does not provide cross protection against JA142. To our surprise, however, no significantly higher level of viremia was detected in pigs vaccinated with JA142 after challenge with a heterologous virus (VR2332) compare to negative control group (N/N, \(p=0.938\)) and homologous challenge group (VR2332/VR2332, \(p=0.506\)) even though the non-vaccinated group developed the highest level of viremia among all of the groups challenged with VR2332. In addition, the group vaccinated with JAP2-6, which had the
largest portion of JA142 structural genes among the three chimeric viruses, also developed low levels of viremia similar to homologous challenge group (VR2332/VR2332). These results indicated that JA142 may be capable of inducing broader cross protection against heterologous PRRSV challenge. In a previous study, the efficacy of Ingelvac PRRS® ATP vaccine, a modified live vaccine originated from JA142, was evaluated against various field isolates (SDSU73, VR2385, and Mn-01-A1) [45]. The ORF5 identity of the three isolates comparing with ATP ranges from 76% to 89%. Though viremia was not evaluated to show the reduction of virus in serum, higher average daily weight gain and milder lesions in vaccinated pigs suggested the better capability of JA142 in protecting pigs from challenge with a wide range of field isolates.

The age of pigs is an important factor to consider regarding the pathogenesis of PRRS virus. When pigs (3-week-old) were first exposed to JA142 and VR2332 at the beginning of the study, average viremia level in each group was $10^{5.2\pm0.1}$ TCID$_{50}$/mL and $10^{4.6\pm0.1}$ TCID$_{50}$/mL, respectively. Six weeks later (i.e., 10 weeks of age), when sham-inoculated pigs were first exposed to JA142 and VR2332, the average viremia level was $10^{4.6\pm0.3}$ TCID$_{50}$/mL and $10^{2.5\pm0.3}$ TCID$_{50}$/mL, respectively. The difference in viremia levels between pigs with different ages was significant ($p<0.001$) for both JA142 and VR2332. Although young pigs at age of less than 1 month old have been commonly used for pathological evaluation for PRRSV [20, 51, 52], 10-week old pigs had to be challenged with VR2332 or JA142 in our study in order to keep the pigs for 6 weeks after vaccination so that the pigs could develop a good level of protective immunity. This might be a reason that only few pigs had severe pathological lesions evaluated by means of gross and microscopic lesions and IHC staining scores. The influence of pig age on clinical response and overall pathogenicity of virus was
also studied by other groups, demonstrating lower viremia levels and clinical scores [31]. Those results further explained our observation in the present study.

In conclusion, the current study proved that chimeric viruses which were constructed by mixing structural genes from two well-known distinct PRRSV strains, VR2332 and JA142, in an organized manner could provide cross protection against both of the donor viruses. This strategy may be applicable to other PRRSV strains and help develop new vaccines, which are able to provide cross protection against various PRRS strains.

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References


Table 1. Design of animal experiment

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<th>Treatment &amp; inoculum</th>
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<td>*<em>Vaccination</em> (0 dpi)</td>
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<td>Sham inoculum</td>
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* A live virus inoculation was considered as vaccination.

⁺ Four pigs from JA142 vaccinated group, two pigs from sham vaccinated group and two pigs from JAP5 vaccinated group were culled for humane reason due to bacterial infection during the study.
Table 2. Primers used in sequencing for ORFs 5 and 6 or ORF7

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Figure 1. Kinetics of PRRS viremia after vaccination (i.e., 1st inoculation) and challenge. Three-week-old pigs were injected with one of the chimeric viruses (JAP2-6, JAP5, and JAP56), wild-type viruses (VR2332, JA142) or virus-free cell culture media (N) at 0 dpi and intranasally challenged with either JA142 (A) or VR2332 (B) at 44 dpi. The level of viremia in each pigs at each time point indicated on the X axis was estimated by a real-time RT-PCR and expressed as TCID\(_{50}\)/ml equivalent (mean± SEM) on the Y axis. Treatment groups are indicated as vaccination/challenge strains.
Figure 2. Viral loads in lungs collected from pigs (n=3-4) necropsied at 2 (58 dpi) and 4 (72 dpi) weeks after challenge with JA142 (A) or VR2332 (B). Viral titers (TCID_{50}/ml equivalent) were estimated by a real-time RT-PCR and were compared between vaccinated groups and non-vaccinated group (*: 0.05<p<0.1; **: p<0.05). PRRS viruses in PCR-positive lungs from pigs vaccinated with JAP5, JAP56, JAP2-6, JA142 and VR2332- were sequenced to differentiate the challenge strains from the vaccine strains. The number of pigs from which either vaccine or challenge viruses were identified was shown in a tabular form under the corresponding bars. a: Viruses from vaccination at 0 dpi, b: Viruses from challenge at 44 dpi, c: Sequencing was not successful as the samples were negative or weak-positive for PRRSV.
Figure 2. continued

<table>
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- - 3 2 1 0 - 1 2 3 0 -
- - 0 0 3 1 - 2 0 1 2 -
- - 1 2 0 2 - 1 2 0 2 -
- - 4 4 4 3 - 4 4 4 4 -
Figure 3. PRRSV-specific serum antibody responses of pigs over time after vaccination or challenge as measured by IDEXX HerdChek® PRRS 2XR ELISA kit. The cut-off S/P ratio value (0.4) recommended by the manufacturer is shown as horizontal dash line. The vertical dotted line indicates the time after the vaccination (44 dpi) with one of the chimeric viruses (JAP2-6, JAP5, and JAP56), wild-type viruses (VR2332, JA142) or virus-free cell culture media (N) when pigs were challenged with either VR2332 or JA142. Treatment groups are indicated with vaccine/challenge strains.
Figure 4. Levels of virus neutralizing (VN) antibody against PRRSV strain VR2332 (A) or JA142 (B) in pig sera collected at 42 days post inoculation (dpi) with one of three chimeric viruses (JAP5, JAP56, and JAP2-6), two wild-type viruses (VR2332 and JA142) or virus-free cell culture medium (N) as determined by a fluorescent focus neutralization assay. Horizontal line indicates the mean VN antibody titer of each group.
Figure 5. Levels of virus neutralizing (VN) antibody in pigs at 14, 21 and 28 days after intranasal challenge with VR2332 (A, B, C) or JA142 (D, E, F). Prior to the challenge at 44 days post inoculation, the pigs were injected with one of the chimeric viruses (JAP2-6, JAP5, and JAP56), wild-type viruses (VR2332, JA142) or virus-free cell culture media (N) as vaccine. The levels of VN antibody in sera were determined by FFN assay. Treatment groups are indicated as vaccination/challenge strains. Mean of each group was indicated as a horizontal bar.
Figure 5. continued

C

72 dpi against VR2332

D

58 dpi against JA142

VN antibody titer (Log2)

VN antibody titer (Log2)
Figure 5. continued

E  
65 dpi against JA142

F  
72 dpi against JA142
Figure 6. Evaluation of lung lesions and IHC scoring. Lungs collected from pigs 2 weeks (58 dpi) or 4 weeks (72 dpi) after challenge with VR2332 (A) or JA142 (B) were evaluated for the severity of gross and microscopic lesions and intensity of immunohistochemical staining and scored on 0 to 3 scale, where 0 is being no lesion or staining, 1 being mild lesion or staining, 2 being moderate lesion/staining and 3 being severe lesion/staining.
CHAPTER 3. CROSS NEUTRALIZATION ANALYSIS OF CHIMERIC PRRS VIRUSES CONTAINING HETEROLOGOUS STRUCTURAL GENES FROM FOUR DISTINCT WILD-TYPE STRAINS

Dong Sun, Won-Il Kim, Yong-Il Cho, Hai Hoang, Kyoung-Jin Yoon

Abstract

The genetic and antigenic variation of porcine reproductive and respiratory syndrome virus (PRRSV) has limited the protection spectrum of vaccines to their homologous strains. Previous studies demonstrated that a chimeric virus containing ORFs 5-6 from JA142 and other structural genes from VR2332 was neutralized by antisera generated against both viruses. The chimeric virus could also protect pigs from challenges by both strains. The same studies demonstrated that ORFs 2 and 7 products were not related with virus neutralization. To evaluate the strategy of providing broad protection against heterologous PRRS viruses by mixing their ORFs3-4 and ORFs5-6, this study used the four distinct wild-type PRRSV strains 1648-02, 17198-6, MN184, and SDSU73 which share 87.4% to 92.2% identity of ORF5 sequence as donor strains to generate 12 chimeric viruses with mixed structural genes from the 4 viruses using a VR2332 infectious cDNA clone as backbone. Susceptibilities of the chimeric PRRS viruses to hyperimmune serum generated against each one of the four donor viruses were tested by fluorescent foci neutralization (FFN) assay. Test results showed that not all of the chimeric viruses obtained broad cross neutralization. Broad cross neutralization could be obtained when viruses were constructed in an organized manner dependent on the immunobiological importance of ORFs 3-4 and ORFs 5-6 of each donor strain. The neutralization of MN184 and SDSU73 appeared to be mainly mediated by ORFs 5-6 products, while ORFs 3-4 products played a limited role. The ORFs 5-6 and ORFs 3-4
products of 1648-02 were all related to virus neutralization, but ORFs 5-6 products were more important. For 17198-6, ORFs 5-6, and ORFs 3-4 products were equally involved in neutralization. Chimeric PRRS viruses 17198-MN184, 17198-SDSU73, and 17198-1648 were highly susceptible to hyperimmune sera generated against both wild-type strains of ORFs 3-4 and ORFs 5-6 origins, suggesting that these may be candidates to provide pigs protection against challenges by distinct heterologous viruses.

**Introduction**

Porcine Productive and Respiratory Syndrome (PRRS) emerged in the late 1980s in both the United States and Europe [17, 34]. Since that time, the disease has swept the swine industry in most pig-producing countries throughout the world and caused significant economic losses [13, 14, 19, 26]. PRRS virus (PRRSV), the etiological agent of PRRS, is a member of the *Arteriviridae* family of *Nidovirales* order [5]. The virus has a positive-sense, single-stranded RNA genome with the size about 15kb. To date, a total of 10 open reading frames (ORFs) have been identified in the genome. ORF1a and ORF1b, which together comprise around 2/3 of the genome at the 5’ terminus, encode non-structural proteins responsible for virus replication. The remaining ORFs at 3’ terminus comprise 1/3 of the genome and encode structural proteins incorporated in virion particles. Viral proteins GP5, M and N, expressed by ORF5, ORF6 and ORF7 respectively, are the main structural components of virion [22, 24]. The N protein is the exclusive protein component of viral nucleocapsid, and is highly immunogenic. GP5 and M proteins, which together occupy more than half of viral proteins, are found in a disulphide-linked heterodimer format in the viral envelope [22]. Deletion of either of these two proteins abolished the production of virions,
suggesting their significant role in PRRSV replication [35]. GP2, E, GP3 and GP4, expressed by ORF2, ORF2b, ORF3 and ORF4 respectively, are contained in the virion at a lower level, and are called minor envelope proteins. These four proteins are incorporated in the envelope as multimeric complexes [8, 35]. Absence of any one or all of these proteins does not affect virion production, but does result in a lack of the other three proteins in virion as well as a lack of infectivity of the virus [35]. The complex has been postulated to be related to cell tropism. PRRSV containing EAV ORF2, ORF3, and ORF4 regions can gain the broad cell tropism of EAV [32].

Both innate and adaptive immunity have been shown to play a role in the defense of PRRSV [21, 27, 29]. Regarding adaptive immunity, passive transfer of PRRSV specific hyperimmune serum could confer pigs sterilizing protection against PRRSV challenges, indicating the important role of neutralizing antibodies in PRRSV immunity [20, 28]. However, even though a neutralizing antibody titer as high as 1:32 is reached, sterilizing immunity could only be obtained in 50% of the pigs [20], suggesting that other factors should also be involved in PRRSV immunity. Intensive studies have shown that cell-mediated immunity is also important in the control of PRRSV. Cytotoxic T lymphocytes are able to target PRRSV infected macrophages to eliminate viruses [7]. Upon activation, T cells and NK cells could secrete anti-viral active IFN-γ to facilitate the clearance of PRRSV [3, 6, 23].

Many studies have illustrated the role of several PRRSV viral proteins in achieving viral immunity. Major structural proteins GP5 and M are known to be the main proteins that induce neutralizing antibodies [2, 11, 15]. Minor structural protein GP3 is also involved in neutralization [4, 16]. GP4 of LV has been demonstrated to contain a neutralizing epitope; this epitope is not, however, conserved among type 1 and type 2 PRRSV strains [25]. In
addition, T-cell epitopes have been discovered in nsp9, nsp10, GP4, GP5, and M proteins. Overall, PRRSV structural proteins GP3, GP4, GP5, and M are highly involved in induction of viral immunity.

Naturally, PRRSV has a much higher rate of mutation compared to other RNA viruses [12]. As a result, PRRS viruses circulating in the field are both genetically and antigenically divergent [9, 30, 36]. The immunity conferred by the vaccination of one virus strain provides limited protection against its distinct strains, and the lack of protection against heterologous PRRSV strains has hampered the control and eradication of PRRS. Thus, vaccines that can confer broad protective immunity against both homologous and heterologous strains are highly desired and could greatly benefit the swine industry in achieving effective prevention and control of PRRS.

One of our previous studies using a chimeric virus of VR2332 origin with ORF5 and ORF6 replaced with those of its heterologous strain JA142 demonstrated that the chimeric virus was susceptible to hyperimmune sera generated against both wild-type strains in vitro [18]. A subsequent in vivo study in our laboratory revealed that such a chimeric virus could provide pigs with protection against challenges of both wild-type viruses, suggesting its ability to induce broad cross protection. The in vitro study also demonstrated that replacing VR2332 ORF2 or ORF7 with those of JA142 didn’t change susceptibilities of chimeric viruses to VR2332 and JA142 antisera, suggesting ORF3-6 portion is critical in virus neutralization. Thus, the current study was designed to test if the strategy of conferring broad cross neutralization by constructing chimeric viruses with mixed ORF3-4 and ORF5-6 from heterologous strains could be widely applied to other strains.
Materials and Methods

Phylogenetic Analysis of PRRSV ORF5 Genes. About 14,000 type 2 PRRSV ORF5 genes collected by Iowa State University Veterinary Diagnostic Lab (ISU-VDL) were aligned initially with reference ORF5 genes. The 14,000 genes were collected from cases submitted to ISU-VDL between 1996 and 2011 from swine operations in most pig raising states in the United States in order to get good representation of type 2 PRRSV strains circulating in North America. The pool of 14,000 genes was reduced for further analysis to approximately 3,000 genes by removing highly identical strains. The 3,000 genes were aligned by MEGA5 software (http://www.megasoftware.net/) with the Clustal W method, and a phylogenetic tree was constructed with the non-rooted Neighbor-Joining method.

Viruses and Cells. Among the 3,000 strains, four representative wild-type strains, MN184, SDSU73, 17198-2, and 1648-06 were selected as donor strains for chimeric virus construction of this study. These four strains are distinct from one another and also distinct from VR2332 and JA142 that were used in our previous study [18]. All viruses were propagated in MARC-145 cells. MARC-145 cells were maintained with RPMI-1640 media (Sigma-Aldrich Corporation, St. Louis, MO) containing 10% (v/v) fetal bovine serum (FBS, Atlas biological, Fort Collins, CO) in a 37 °C incubator with 5% CO2 supplied.

Generation of Hyperimmune Sera. Hyperimmune serum against each of the four selected donor strains was generated in 3-week-old pigs. Twelve pigs were evenly divided into four groups and pigs in each group were intramuscularly inoculated with one donor virus (2mL of 10³ TCID₅₀/mL per pig). Sera were collected periodically and tested for antigen by
PCR and for PRRSV-specific antibodies by ELISA and virus neutralization assay. All pigs were euthanized at 45 days post inoculation for total blood collection. Sera raised against the same virus strain were pooled with the same volume ratio. All sera were then stored in a -20 °C freezer for further use.

**Construction of Chimeric Viruses.** Chimeric viruses combining ORFs 3-4 and ORFs 5-6 from heterologous strains were constructed by using a VR2332 cDNA infectious clone (Fig. 1). Gene recombination was performed by using gene-swapping method as previously described [18]. Basically, each ORF of the four donor strains was amplified by the SuperScript™ III One-Step RT-PCR System (Life Technologies, Grand Island, NY) with primers listed in Table 1, and gel purified with QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA) after agarose gel electrophoresis. A shuttle vector containing VR2332 ORFs 2-7 was used for gene swapping mutagenesis with QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Inc., Santa Clara, CA). ORFs were swapped one by one in the order from ORF 3 to ORF 6. Products were sequenced before putting them back into the infectious clone.

The recombinant structural gene was replaced back into the VR2332 infectious clone by utilizing BsrG I and Hpa I endonuclease sites in the plasmid. Point mutations were performed to induce or remove BsrG I and Hpa I sites in the constructed shuttle plasmid to ensure that the enzyme cutting patterns were the same.

The constructed plasmid was linearized by Acl I and purified by DNAclear Kit (Life Technologies, Grand Island, NY). Linear DNA was transcribed using T7 promoter by
mMESSAGE mMACHINE T7 Kit (Life Technologies, Grand Island, NY). RNA was purified by MEGAclear Kit (Life Technologies, Grand Island, NY).

MARC-145 cells were digested by trypsin and washed twice with DMEM (Sigma-Aldrich Corporation, St. Louis, MO). Washed cells were re-suspended in cooled electroporation buffer (DMEM with 1.25% DMSO) to the concentration of $1.25 \times 10^6$ cells/mL. Subsequently, 400 μL of cell suspension was added to ice cooled 4 mm cuvette and mixed with 5 μg RNA. Cells were shocked by Multiporator system (Eppendorf, Hauppauge, NY) using 1000V 40 μS and incubated on ice for 1 min. The cuvette was then incubated in a 37 °C water bath for 8 min. After the incubation, 1.6 mL of RPMI-1640 containing 10% FBS was mixed with cells in the cuvette and the mixture was transferred to a 6-well plate. When CPE was evident (usually 120 hrs post transfection), viruses were harvested and propagated four more times. All rescued viruses were kept at -80 °C until use. Viruses were named based on their origin of ORFs 3-4 and ORFs 5-6, for example, chimeric virus recombined ORFs3-4 from MN184 and ORFs5-6 from 17198-6 was named MN184-17198.

**Sequencing.** RNAs of the fourth passage rescued viruses were extracted with QIAamp Viral RNA Mini Kit (Qiagen). Structural genes of rescued viruses were amplified by SuperScript™ III One-Step RT-PCR System (Life Technologies) with primer set F: 5’-AAACGGTGAGGACTGGGAGGATT-3’ and R: 5’-GCCATTCACCACACATTTCTCC-3’. After purification, PCR products were submitted to Iowa State University DNA facility for sequencing.
**Virus Neutralization Assay.** Susceptibility of chimeric viruses and the four donor strains to hyperimmune serum against each of the four donor strains was tested *in vitro* by fluorescent-foci neutralization (FFN) assay. Briefly, serum was heat inactivated and was 2-fold serially diluted with cell culture media (RPMI-1640 containing 10% FBS). Then 100 μL diluted serum was mixed with 100 focus forming units (FFU) of virus in 100 μL cell culture media. The mixture was incubated for 1 h at 37 °C and transferred onto monolayered MARC-145 cells in 96-well plates. After 1 h incubation at 37 °C, the mixture was discarded and cells were replenished with 100 μL media per well. Then 20 h later incubating at 37 °C, cells were fixed with ice cold 80% (v/v) acetone-water solution and stained with 1:10000 diluted PRRSV N protein specific monoclonal antibody SDOW17 and subsequently FITC-labeled secondary antibody. After washing with PBS (0.01M, pH 7.2) a few times, the number of fluorescent foci in each well was counted. The VN titer of each serum was expressed as the reciprocal of the highest dilution of the serum in which there was 90% or greater reduction in the number of FFU as compared to that in control wells (i.e., without serum added) was observed. The virus neutralization assay was performed three times separately.

**Data Analysis.** JMP software (SAS Institute Inc., Cary, NC) was used for statistical analysis. To compare the difference in susceptibility of viruses to the neutralizing antibodies, Wilcoxon Rank Sum test was performed.
Results

Analysis of PRRSV ORF5 Genes. Among the ORF5 genes of 3,000 strains, four representative heterologous strains MN184, SDSU73, 17198-2, and 1648-06 were selected for this study. As illustrated in Fig. 2, the four strains are distinct from one another with nucleotide identities ranging from 87.4% to 92.2% and amino acid identities ranging from 84.1% to 92.0%. They are also distinct from VR2332 and JA142 strains that were used in our previous study (Table 2). Basically these viruses represented PRRS viruses circulating in North America.

Susceptibilities of Donor Strains to Hyperimmune Sera. All donor strains were susceptible to their corresponding hyperimmune serum (i.e., homologous neutralization). Compared to the other three viruses that induced levels of neutralizing antibody about 1:32-64, inoculation of MN184 induced a lower level (1:8-16) of neutralizing antibodies (Fig. 3). Levels of cross neutralization of hyperimmune serum against heterologous donor strains were not significant (no higher than 1:2), except for 17198-6 hyperimmune serum, which could neutralize SDSU73 at level of 1:8.

Susceptibilities of Chimeric Strains to Hyperimmune Sera. The susceptibilities of chimeric viruses to hyperimmune sera vary from serum to serum. Susceptibilities of the 12 chimeric viruses to anti-MN184 hyperimmune serum were very clear (Fig. 4 A). Only chimeric viruses containing MN184 ORFs5-6, i.e. 17198-MN184, 1648-MN184, and SDSU73-MN184 were susceptible to the anti-MN184 hyperimmune serum and no other chimeric virus was susceptible to the anti-MN184 hyperimmune serum. Neutralizing
antibody titers against 17198-MN184, 1648-MN184, and SDSU73-MN184 were 1:16, 1:8 and 1:8-16 respectively, which were not significantly different with that of the donor virus \( (p > 0.05) \).

Similar to sensitivities of chimeric viruses to anti-MN184 hyperimmune serum, chimeric viruses containing SDSU73 ORFs5-6, i.e. 17198-SDSU73, 1648-SDSU73, and MN184-SDSU73 could be neutralized by 1:64 diluted anti-SDSU73 hyperimmune serum as the donor virus SDSU73 (Fig. 4 B). Chimeric viruses containing SDSU73 ORFs3-4 could also be neutralized, but the titers (1:2) were significantly lower than neutralization titer of anti-SDSU73 hyperimmune serum to donor SDSU73 virus \( (p < 0.05) \). Other chimeric viruses without genes from SDSU73, were not susceptible to the SDSU73 hyperimmune serum.

Chimeric viruses containing 1648-02 ORFs5-6 were almost as susceptible to anti-1648-02 hyperimmune serum (1:32, 1:16-32, 1:8-16) as donor strain 1648-02 (1:32) (Fig. 4 C). On the other hand, chimeric viruses containing ORFs3-4 of 1648-02 could be neutralized by anti-1648-02 hyperimmune serum, but the titers (1648-17198, 1:4-8; 1648-MN184, 1:4-8, 1648-SDSU73, 1:4) were significantly lower \( (p < 0.05) \). Chimeric viruses containing ORFs3-4 of SDSU73 also showed significantly lower \( (p < 0.05) \) susceptibility (1:2).

All chimeric viruses containing either ORFs5-6 or ORFs3-4 of 17198-6 were similarly susceptible to the anti-17198-6 hyperimmune serum as donor strain 17198-6 (Fig. 4 D). Chimeric viruses 1648-MN184, 1648-SDSU73, MN184-SDSU73, and SDSU73-MN184 were also susceptible to 17198-6 hyperimmune serum, but the titers were significantly lower \( (p < 0.05) \).
Discussion

Numerous studies have indicated that GP3, GP5, and M of PRRSV are involved in virus neutralization [11, 15, 16]. Our previous work discovered that, while a low degree of cross neutralization was observed between heterologous strains VR2332 and JA142, a chimeric virus replacing the ORF5 and ORF6 of VR2332 with those of JA142 was highly susceptible to both antisera generated against VR2332 and JA142 [18]. A subsequent study using this chimeric virus as a live virus vaccine protected pigs against challenges of both viruses, represented by a significant decrease in the level of post challenge viremia. Results implied broadened cross protection against heterologous PRRSV strains originally lacking cross protection could be achieved by inoculating with such chimeric viruses. Thus this study was conducted to evaluate the feasibility of the strategy combining heterologous ORFs3-4 and ORFs5-6 to enhance virus cross neutralization.

Though GP3, GP5, and M have been identified as being involved in virus neutralization, our results suggested that their roles vary from strain to strain. Neutralization of MN184 appears to be solely mediated by the GP5 and M protein, since only chimeric viruses containing MN184 ORFs5-6 were susceptible to the anti-MN184 hyperimmune serum. Both GP5 and M proteins of SDSU73 are believed to be the most important proteins in virus neutralization, since susceptibility of chimeric viruses containing SDSU73 ORFs5-6 to anti-SDSU73 hyperimmune serum were the same as donor strain SDSU73. Yet, GP3 and GP4 also appear to have minor roles in the neutralization of SDSU73, since chimeric viruses with ORFs3-4 were susceptible to anti-SDSU73 hyperimmune serum, even at low level (1:2). In the case of 1648-02, GP5 and M played an important role in virus neutralization; GP3 and GP4 were also important for virus neutralization, but to a less degree. The role of GP3-GP4
and GP5-GP6 of 17198-6 in virus neutralization were equally important. Chimeric viruses containing either ORFs3-4 or ORFs5-6 were similarly susceptible to the anti-17198-6 hyperimmune serum. Since GP3, GP5, and M proteins are not equally involved in virus neutralization, mixing ORFs3-4 and ORFs5-6 from heterologous strains does not always guarantee broad cross neutralization of chimeric viruses. For example, MN184-1648 was only susceptible to anti-1648-02 hyperimmune serum, so in order to obtain broad cross neutralization chimeric viruses should be constructed in an organized manner based on the significance of each heterologous ORFs3-4 and ORFs5-6 in neutralization rather than randomly combining them.

The ORFs3-4 of MN184 and SDSU73 were not involved in neutralization, while those of 1648-02 and 17198-6 were. Since GP4 of type 2 PRRSV has not been proven to be related to neutralization, the difference should result from the presence of GP3. One possible reason for this could be that neutralizing epitopes in ORFs3 of 1648-02 and 17198-6 were abolished in that of MN184 and SDSU73. The GP3 amino acid sequences of the four strains were compared as shown in Fig 5. Two amino acids at 151 and 250 of GP3 are identical in 1648-02 and 17198-6. The same two amino acids are also identically present in MN184 and SDSU73, but differ from those of 1648-02 and 17198-6. Amino acids at 134 of GP3 are identical in MN184 and SDSU73 but different in 1648-02 and 17198-6. These three sites could be related to virus neutralization, and mutations of these sites in MN184 and SDSU73 destroy the epitope. Another possible cause for the difference in the role of GP3 in neutralization would be the difference in glycosylation pattern since N-glycosylation has been reported to shelter neutralizing epitopes from the reaction with neutralizing antibodies [1, 10, 33]. Since GP3 of MN184 and SDSU73 are not involved in neutralization and those
of 1648-02 and 17198-6 are, if N-glycosylation is the reason for this difference, there should be amino acids in MN184 and SDSU73 GP3 N-glycosylated to block their neutralizing epitope, and meanwhile the same position in 1648-02 and 17198-6 GP3 are not N-glycosylated. The predicted N-glycosylation sites of GP3 by NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/) are listed in Table 3. No such site that is N-glycosylated in MN184 and SDSU73 GP3 but not glycosylated in 1648-02 and 17198-6 GP3 is observed; therefore N-glycosylation sheltering may not be the reason of the difference.

Among the donor strains, MN184 tends to induce more severe clinical symptoms and a lower level of neutralizing antibodies. Inoculation of MN184 in one of our previous studies killed 3 of 4 pigs (unpublished data). The neutralizing antibody titer in the remaining pigs was only 1:2 at 45 days post inoculation while pigs inoculated with other strains produced neutralizing antibodies higher than 1:8 against their homologous viruses. SDSU73 could be neutralized by both the anti-SDSU73 and the anti-17198-6 hyperimmune sera; however, the anti-SDSU73 hyperimmune serum could only neutralize SDSU73 rather than 17198-6, suggesting that 17198-6 may have more neutralizing epitopes than SDSU73. Moreover, those epitopes should be more important in neutralization of the 17198-6 strain compared to epitopes shared with SDSU73. The neutralization activity of 17198-6 ORFs3-4 and lack of neutralization of SDSU73 ORFs3-4 may be the reason for this, but a neutralizing epitope difference in the GP5 or the M protein may also be involved.

Three chimeric viruses, 17198-MN184, 17198-SDSU73, 17198-1648, were highly susceptible to hyperimmune sera generated against both donor strains. This observation implied that rendering broad cross neutralization to chimeric viruses by combining ORFs3-4 and ORFs5-6 from heterologous PRRSV strains could be widely applied; however, not all of
chimeric virus containing mixed ORFs3-4 and ORFs5-6 from any two PRRSV strains could obtain broader susceptibility to cross neutralization by antisera of both donor viruses. A careful combination of genes based on immunobiological property of each gene product is important for such reactivity. In addition, based on results of our previous animal challenge study [31] demonstrating that pigs inoculated with a chimeric virus containing ORFs3-4 from VR2332 and ORFs5-6 from JA142 were produced against both VR2332 and JA142 challenges, the three chimeric viruses 17198-MN184, 17198-SDSU73, and 17198-1648 may also be able to provide broad cross protection against heterologous PRRSV strains challenge. This possibility remains to be confirmed by animal study.

References


Table 1 Primers used to amplify each ORF of donor strains for gene swapping

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<td>ATGGGCTGTCCCTGACGACTTTGCAATGGAC</td>
</tr>
<tr>
<td>SDSU73-5R</td>
<td>TTAATTTGGCATATTAGACGACGAGTTACCACCCTCC</td>
</tr>
<tr>
<td>17198-6-3F</td>
<td>ATGGCTAATAGCTGTGACATTCCCTCCATATTCC</td>
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<tr>
<td>17198-6-3R</td>
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<tr>
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Table 2. Identities of ORF5 gene and GP5 protein among PRRSV strains 1648-02, 17198-6, MN184, SDSU73 and VR2332

<table>
<thead>
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<th>ORF5 identity (%)</th>
<th>1648-02</th>
<th>17198-6</th>
<th>MN184</th>
<th>SDSU73</th>
<th>VR2332</th>
<th>JA142</th>
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<tr>
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<td>91.4</td>
<td>89.7</td>
<td>88.9</td>
<td>89.4</td>
</tr>
<tr>
<td>17198-6</td>
<td>89.1</td>
<td>-</td>
<td>86.7</td>
<td>92.2</td>
<td>90.5</td>
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<tr>
<td>MN184</td>
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<td>86.1</td>
<td>-</td>
<td>87.4</td>
<td>86.6</td>
<td>87.4</td>
</tr>
<tr>
<td>SDSU73</td>
<td>88.6</td>
<td>89.6</td>
<td>84.1</td>
<td>-</td>
<td>90.0</td>
<td>92.7</td>
</tr>
<tr>
<td>VR2332</td>
<td>86.6</td>
<td>90.5</td>
<td>84.1</td>
<td>87.1</td>
<td>-</td>
<td>91.0</td>
</tr>
<tr>
<td>JA142</td>
<td>91.5</td>
<td>95.5</td>
<td>88.1</td>
<td>91.0</td>
<td>91.0</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 3 Likelihood of GP3 glycosylation of the four PRRSV donor strains 1648-02, 17198-2, MN184 and SDSU73

<table>
<thead>
<tr>
<th>Location</th>
<th>Virus</th>
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<th>17198-2</th>
<th>MN184</th>
<th>SDSU73</th>
</tr>
</thead>
<tbody>
<tr>
<td>29</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>42</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+++</td>
<td>+++</td>
<td>+++</td>
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</tr>
<tr>
<td>195</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td></td>
<td>++</td>
</tr>
</tbody>
</table>

* likelihood of glycosylation: -, not likely; +, less likely; ++, more likely; +++, most likely
Figure 1. Construction of twelve chimeric PRRS viruses. ORFs 3-6 of VR2332 in shuttle vector were replaced with those from different donor PRRSV strains (1648-06, 17198-2, MN184, SDSU73). Mutant structural genes were inserted back to the VR2332 cDNA infectious clone by utilizing endonuclease sites BsrG I and Hpa I.
Figure 2. ORF5 gene diversity of 3000 PRRSV strains. ORF5 genes of 3000 PRRSV strains were aligned and phylogenetic tree was constructed by MEGA software. The four selected donor strains 1648-02, 17198-6, MN184, and SDSU73 distinct from each other as well as the cDNA infectious clone backbone VR2332 were labeled with colored box.
Figure 3. Serum virus neutralizing (SVN) antibody titer of hyperimmune sera against homologous and heterologous PRRSV donor strains (1648-02, 17198-6, MN184, SDSU73)
Figure 4. Serum virus neutralizing (SVN) antibody titer of hyperimmune sera generated for PRRSV donor strains MN184 (A), SDSU73 (B), 1648-02 (C) and 17198-6 (D) against chimeric virus strains constructed by inserting ORFs3-4 or ORFs5-6 from each of the donor strains to VR2332 cDNA infectious clone. The 12 chimeric viruses listed on the X axis were tested for their susceptibility to hyperimmune sera generated by donor strains respectively. Results were compared with homologous virus of the hyperimmune sera. Asterisk (*) indicated viruses whose susceptibility were not significantly different from the homologous virus.
Figure 4. continued
Figure 5. Alignment of GP3 amino acid sequences of the four donor strains. Residues identical with those of 1648-02 were shown as “.”. Boxes at position 134, 151, 250 indicated amino acids that are identical between the two strains MN184, SDSU73 and the other two strains 1648-02, 17198-6.
CHAPTER 4. ROLES OF NON-STRUCTURAL AND STRUCTURAL GENE PRODUCTS
IN THE NEUTRALIZATION OF PRRS VIRUSES

Dong Sun, Won-il Kim, Chong Wang, Kyoung-Jin Yoon

Abstract

PRRSV has had great economic impact on the swine industry since its recognition. Modified live vaccine (MLV) has been widely applied on pig farms to control or prevent the disease. Even though MLV is more efficacious than killed virus vaccine, several issues related to its safety and efficacy, such as lack of cross protection against heterologous strains and reverting to virulent, has limited the use of MLV. In this study, eight different patterns of structural genes with mixed JA142 and VR2332 components were constructed and put into three cDNA infectious clone backbones to evaluate the roles of virus backbone (i.e., non-structural proteins) and several structural gene products in cross neutralization of the chimeric viruses by JA142 and VR2332 antisera. Chimeric viruses carrying the same structural gene(s), no matter which backbone the virus was constructed with, had almost the same susceptibility to the neutralizing activity of JA142 and VR2332 antisera, suggesting that a non-structural backbone has very limited impact on the specificity of viral immunity. On the other hand, chimeric viruses with different gene combination patterns displayed a great diversity in their susceptibilities to the JA142 and VR2332 antisera. The differences indicated that the determinants of VR2332 neutralization may be located in GP3 or GP4; in contrast, determinants of JA142 located in M protein, suggesting that careful combination of structural genes is critical to create a chimeric virus with broader cross neutralization.
Introduction

Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), which causes reproductive disorder in sows and boars and respiratory distress in pigs of all ages [2, 3, 14, 43], is classified as a member of the family *Arteriviridae* in *Nidovirales* order [1]. The virus was first isolated in the United States and the Netherlands in 1992 and 1991 respectively [4, 51] after the recognition of a PRRS outbreak in North America and Europe in 1987 and 1990 [21, 38]. Currently, it is commonly seen in most pig producing countries around the world and is continuously bringing huge economic losses to pig producers [16, 35].

The PRRSV genome is positive-sense single-stranded RNA with the size around 15.4 kb [5]. ORF1a and ORF1b, which are 12kb together at the 5’ end of genome, encode at least 14 non-structural proteins that are critical for virus replication. Following ORF1, the 3kb region at the 3’ end of genome contains 8 ORFs (ORF2a, ORF2b, ORF3, ORF4, ORF5, ORF5a, ORF6 and ORF7) encode structural proteins.

PRRSV has a much higher mutation rate ($10^{-2}$/site/year) than standard RNA viruses ($10^{-3}$ to $10^{-5}$/site/year) [15]. Since its recognition, the genetic diversity of PRRSV strains has been extensively observed [20, 40]. PRRSV is divided into two genotypes (European type, type 1 and North American type, type 2) that are distinct from one another both genetically and antigenically [11, 34]. In addition, virus strains in the same genotype have a high degree of variation. Based on ORF7 sequence type 1 PRRSV (PRRSV-1) strains have been divided to 3 subtypes whose distributions are skewed in various geographical locations [12, 45]. Similarly, type 2 PRRSV (PRRSV-2) strains have been grouped into 9 clusters according to the ORF5 gene [44]. Besides genetic variation, antigenic variation among heterologous strains has also been well documented [9, 23, 24].
PRRSV remains to be one of the most important viral pathogens in the swine industry. Since the discovery of PRRSV, vaccine candidates constructed based on multiple platforms have been developed and evaluated [8, 18, 31, 42]. Yet, among these, modified live vaccine (MLV) is currently believed to be the best option in terms of protection against disease. Commercial vaccines derived from several field isolates are available in the market, but the effects of MLVs are greatly reduced by a number of demerits. First, due to a high degree of genetic variation, vaccines can only provide efficient protection against their homologous strains [28, 32]. Second, MLVs are developed from virulent field isolates that have caused severe disease in the past through continuous passing in cell culture systems [52, 53]. The attenuation step takes a long time and the direction is not controlled. A virus may be over attenuated if it is passed too much [52]. Third, decreased virulence of virus during the cell adaption process has the potential of reverting back to virulent once vaccines are applied back into pigs [26, 33]. These drawbacks should be avoided in the next generation of PRRSV vaccine.

It has been well characterized that PRRSV structural proteins are closely related to protective immunity. Numerous neutralizing epitopes and T cell epitopes have been identified in structural proteins [6, 10, 41, 48, 50, 55]. Many studies illustrated that gene products of ORF3-4 play roles in virus neutralization; while the relatedness of gene products of ORF2 and ORF7 have not been reported. Broad cross neutralization could be achieved by mixing structural genes of multiple heterologous strains [25, 54]. Our previous animal study demonstrated that a chimeric virus replacing VR2332 ORF5-6 with the corresponding gene of JA142 could provide protection against both virus challenges [46]. On the other hand, several virus virulence determinants have been postulated in non-structural proteins [22, 27,
In this scenario, it was hypothesized that a vaccine virus constructed to contain non-structural protein gene from naturally avirulent field isolate and well-organized mixed structural protein genes from target strains should be able to greatly shorten the virus attenuation time and provide broader protection. It has already been reported that replacing non-structural genes of a virulent PRRSV strain with those of an avirulent strain could significantly reduce virus virulence [49], but it remains unknown if the immunogenicity of virulent structural genes will be affected by switching of the non-structural gene backbone. The current study aimed at illustrating the effect of changing the chimeric virus backbone in virus neutralization.

**Materials and materials**

**Infectious cDNA clones, Cells, Viruses and Antisera.** The VR2332 infectious clone pOK12-VR2332 [37] was obtained from Dr. Kay Faaberg at the National Animal Disease Center (NADC) through material transfer agreement with the University of Minnesota. The NVSL97 (the same as JA142) infectious clone pFL12 [47] was kindly offered by Dr. Fernando Osorio at the University of Nebraska. The VR2385 DNA-launched infectious clone pIR-VR2385 [36] was generously provided by Dr. Xiang-Jin Meng at Virginia Tech. The nucleotide identities of ORF5 gene and genome of the three isolates (VR2332, JA142, and VR2385) are listed in Table 1. Infectious clone plasmids were used as templates for structural gene recombination. MARC-145 cells were used both for virus rescue and virus propagation. Cells were cultured and maintained with RPMI-1640 media (Sigma-Aldrich Corporation, St. Louis, MO) containing 10% (v/v) fetal bovine serum (FBS, Atlas biological,
Fort Collins, CO) in a 37 °C incubator with 5% CO2 supplied. Antisera for three isolates were previously prepared in our lab [25].

**Modification of DNA-launched Infectious Clone Vector.** Plasmid pOptiVEC\textsuperscript{TM}-TOPO (Life Technologies, Grand Island, NY) was amplified (Fig. 1) with primers pOpti-F and pOpti-R (Table 2) by Platinum \textit{Pfx} DNA Polymerase (Life Technologies) following user manual with 60°C as annealing temperature. The 3221 bp product was digested by Rsrl II, separated by agarose electrophoresis and purified by QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The purified product was ligated by T4 DNA ligase (Promega, Madison, WI) at 4°C overnight. After transformation into One Shot TOP10 Chemically Competent \textit{E. coli} cells (Life Technologies), the plasmid was extracted by QIApREP Spin Miniprep Kit (Qiagen) and submitted to the Iowa State University Nucleic Acid Facility for sequencing. The constructed plasmid was named pOpti-PRRS, and was used in subsequent DNA-launched infectious clone construction.

**Construction of DNA-launched Infectious Clone.** To generate authentic 5’ end and 3’ end after transcription, a hammerhead ribozyme and a hepatitis delta virus ribozyme were added to the 5’ end and 3’ end of viral genome of infectious clone plasmid pOK\textsubscript{12}-VR2332 and pFL12 (Fig. 2) [17]. Each viral genome, together with inserted ribozymes was then cut off and inserted into vector pOpti-PRRS between a CMV promoter and a TK polyadenylation signal by using Sbf I and Pac I endonuclease sites. For pOK\textsubscript{12}-VR2332, the 5’ end was amplified by Platinum \textit{Pfx} DNA Polymerase (Life Technologies) using 5Rz-1F/VR5Rz-R (table 2) as primers and pOK\textsubscript{12}-VR2332 plasmid as template for the first step.
The first step PCR product was further amplified by primers VR5Rz-2F/VR5Rz-R. The second step PCR product was then used to replace the 5’ end of pOK12-VR2332 using Asc I and Fse I. The 3’ end of pOK12-VR2332 was similarly replaced with the two-step PCR product amplified by primer sets VR3Rz-F/3Rz-1R and VR3Rz-F/3Rz-1R by using endonuclease sites BsrG I and Acl I. Regarding pFL12, the 5’ end was replaced with the two-step PCR product of 5Rz-1F/JA5Rz-R and JA5Rz-2F/JA5Rz-R using restriction enzyme sites for Rsr II and Spe I. The 3’ end was amplified by JA3Rz-F/3Rz-1R for the first amplification. The product was then purified and further amplified by JA3Rz-F/3Rz-2R. The 3’ end was replaced by the product of the second amplification with sites BstB I and Pac I. The new infectious clone plasmids with 5’ end and 3’ end ribozymes added were named pOK12-VR2332-Rz and pFL12-Rz respectively. The sequences of 5’ and 3’ ends replaced region of both pOK12-VR2332-Rz and pFL12-Rz were sequenced for confirmation. After sequencing confirmation, the product was cut off by Sbf I and Pac I and inserted into vector pOpti-PRRS. The resulting DNA-launched infectious clones were named pOpti-VR2332 and pOpti-JA142, respectively.

Construction of chimeric viruses. Eight patterns of recombinant structural genes were constructed (Fig. 3) in the context of three infectious clone backbones. Overlap PCR was intensively utilized to construct recombinant structural gene region from ORF2 to ORF7 as shown in Fig. 4. Sequences of primers are listed in Table 3. Two fragments with an overlap region of about 20 bp were amplified and purified. Then the two fragments were added in the same PCR reaction mixture without primers added. A short PCR cycling of 5 cycles was initially performed, then the forward primer of the 5’ fragment and the reverse
primer of the 3’ fragment were added and another PCR cycling of 25 cycles was performed. Following this PCR strategy, recombinant structural gene regions were constructed.

To place all eight patterns of structural genes into three different backbones, larger fragments combining structural gene with 5’ fragment and 3’ fragments (Table 4) consistent with backbone and structural gene pattern were constructed. Primer sequences are listed in Table 5. Larger fragments were put into the pOpti-VR2332 backbone by digestion with Pme I and Pac I, and ligation. Similarly, Sac I and Xba I were used for the pOpti-VR2385 backbone, and AsiS I and Pac I were used for pOpti-JA142 backbone. All constructed recombinant plasmids were sequenced for confirmation.

**Rescue of chimeric viruses.** All wild-type and chimeric viruses were rescued by electro-transfection. MARC-145 cells were digested by trypsin and washed twice with DMEM (Sigma-Aldrich Corporation, St. Louis, MO). Washed cells were resuspended in ice cooled electroporation buffer (DMEM with 1.25% DMSO) to the concentration of 1.25×10^6 cells/mL. Subsequently, 400 μL of cell suspension was added to an ice cooled 4 mm cuvette and mixed with 1 μg plasmid DNA. Cells were shocked by Multiporator system (Eppendorf, Hauppauge, NY) using 1000V 40 μS and incubated on ice for 1 min. Then the cuvette was incubated in a 37 °C water bath for 8 min. After the water incubation, 1.6 mL RPMI-1640 containing 10% FBS was mixed with cells in the cuvette and the mixture was transferred to a 6-well plate. When CPE appeared (approximately 120 h post transfection), viruses were harvested and propagated four more times. All viruses were kept at -80 °C until use. Chimeric viruses were named based on infectious clone backbone and structural gene pattern; for example, VR2385 backbone with pattern 3 was named VR2385-3.
**Virus Neutralization Assay.** Susceptibility of chimeric viruses and the four wild-type viruses to hyperimmune serum against each of the four wild-type strains was measured *in vitro* by fluorescent-foci neutralization (FFN) assay. Briefly, serum was first heat inactivated at 56 °C for 30 min and 2-fold serially diluted with cell culture media (RPMI-1640 containing 10% FBS). Then 100 μL diluted serum was mixed with 100 focus forming units (FFU) of virus in 100 μL cell culture media. The mixture was incubated for 1 h at 37 °C and transferred onto monolayered MARC-145 cells in 96-well plates. After 1 h incubation at 37 °C, mixture was discarded and cells were replenished with 100 μL media per well. Then 20 h later incubating at 37 °C, cells were fixed with ice cold 80% (v/v) acetone-water solution and stained with 1:10000 diluted PRRSV N protein specific monoclonal antibody SDOW17 (Rural Technologies, Brookings, SD) and subsequently FITC-labeled secondary antibody (KPL, Gaithersburg, MD). After washing with PBS (0.01M, pH 7.2), the fluorescent foci in each well were counted. The VN titer of each serum was expressed as the reciprocal of the highest dilution of the serum in which 90% or greater reduction in the number of FFU as compared to that in control wells (i.e., without serum added) was observed. The virus neutralization assay was performed three times separately.

**Data Analysis.** JMP software (SAS Institute Inc., Cary, NC) was used for statistical analysis. To compare the difference in susceptibilities of viruses to neutralizing antibodies, Wilcoxon Rank Sum test was used.
Results

Cross neutralization of wild-type viruses. To assess the background reactivity of the three backbones, viruses were rescued from the three wild-type infectious clones and evaluated for their susceptibilities to VR2332 and JA142 antisera by means of virus neutralization assay. The JA142 antiserum could only neutralize JA142 with titer 1:16-32, while its cross neutralization activity against VR2332 and VR2385 was not observed (Fig. 5A). The VR2332 antiserum could neutralize VR2332 with titer as high as 1:64 (Fig. 5B). The cross neutralization activity against JA142 was not observed. VR2385 was susceptible to 1:2-4 diluted VR2332 antiserum, but the susceptibility compared to VR2332 was lower ($p=0.059$).

Effect of non-structural gene backbone on virus neutralization. Previous studies have clearly demonstrated that structural genes mediate PRRSV neutralization. To investigate the involvement of non-structural gene product(s) and UTR in PRRSV neutralization, chimeric viruses containing the several patterns (pattern 1-6) of recombinant structural genes were constructed in the context of three backbones. The susceptibility of each virus to JA142 and VR2332 antisera was assessed by means of FFN and compared with corresponding viruses with the same pattern in other backbones. Susceptibilities of 5 of the 6 patterns to JA142 antiserum were not changed at all. Only JA142 backbone with pattern 6 decreased its susceptibility to JA142 antiserum less than 2-fold (Fig. 6A). Similarly, only JA142 backbone containing pattern 1 and 2 decreased susceptibilities to VR2332 antiserum in a less than less than 2-fold level (Fig. 6B). Differences in susceptibilities of viruses to antiserum were observed in viruses with pattern 1 and pattern 2 to VR2332 antiserum and
pattern 6 to JA142 antiserum, but these differences were not significant (p>0.1). Even though there was a difference in susceptibility of pattern 3 and pattern 5 to JA142 antiserum, the difference was only 2-fold. On the other hand, in all three backbones, replacing ORFs2-7 of JA142 with those of VR2332 completely reversed the susceptibility of viruses to JA142 and VR2332 antisera and vice versa (Fig. 6, pattern 1 vs pattern 6).

**Role of ORF2 and ORF7 products in virus neutralization.** To confirm that ORF2 and ORF7 are indeed not related to PRRSV neutralization, chimeric viruses differing in their ORF2 and ORF7 region were compared for their susceptibilities to antisera. Since the VR2385 has a low level of cross neutralization with both VR2332 and JA142 antisera, the reactivity of VR2385’s ORF2 and ORF7 products with the VR2332 and JA142 antisera was expected to be low. Thus the ORF2 and ORF7 of VR2332 and JA142 were first replaced with those from VR2385 to demonstrate the role of VR2332 and JA142’s ORF2 and ORF7 products in neutralization. When ORF2 and ORF7 of VR2332 were replaced with VR2385’s ORF2 and ORF7, i.e., VR2385-1 vs VR2385-8 (Fig. 7A and 7B), no difference in the susceptibility of chimeric virus to both JA142 antiserum and VR2332 antiserum was seen. When ORF2 and 7 of JA142 were replaced with those of VR2385, i.e., VR2385-6 vs VR2385-7 (Fig. 7C and 7D), a less than 2-fold difference in the susceptibility to the JA142 antiserum was observed (p>0.1).

In the context of the JA142 backbone, JA142-1 and JA142-8 that differed only in their ORF2 and ORF7 were compared. Both viruses were susceptible to the VR2332 antiserum in the virus neutralization titers (Fig. 7F) (p>0.1). On the other hand, their susceptibilities to the JA142 antiserum were significantly (p<0.05) different from one another.
JA142-1 was not susceptible to the JA142 antiserum at all, while JA142-8 could be neutralized by 16-fold diluted antiserum.

In the VR2332 backbone, VR2332-6 and VR2332-7 were compared (Fig. 7G and 7H). There was no difference in their susceptibilities to the VR2332 antiserum, while their susceptibilities to the JA142 antiserum were greatly affected. Replacing JA142’s ORF2 and ORF7 with the corresponding regions of VR2332 greatly reduced the susceptibility of the chimeric viruses to the JA142 antiserum.

**Role of ORF5 and ORF6 products in neutralization.** ORF5 product has been believed to be the major protein in virus neutralization; however, in our study, when the ORF5 of VR2332 was replaced with JA142’s ORF5, susceptibility of the new virus to both VR2332 and JA142 antisera was not changed (pattern 1 vs pattern 2, Fig. 6). When VR2332’s ORF6 was further replaced with that of JA142, no difference in their susceptibilities to the VR2332 antiserum was observed, but the susceptibility to the JA142 antiserum was greatly affected (pattern 2 vs pattern 3, Fig. 6). Replacement of VR2332’s ORFs5-6 with those of JA142 did not change the susceptibility of the chimeric viruses to the VR2332 antiserum, but the susceptibility to the JA142 antiserum was greatly increased (pattern 1 vs pattern 3, pattern 4 vs pattern 5, Fig. 6).

**Role of ORF3 and ORF4 products in neutralization.** When the ORFs3-4 of VR2332 were replaced with those of JA142, the susceptibility of the chimeric viruses to VR2332 antiserum were greatly decreased, whereas the susceptibility to JA142 antiserum was not affected much (pattern 1 vs pattern 4, pattern 3 vs pattern 5, Fig. 6). To our surprise,
even though pattern 4 had ORFs from both JA142 and VR2332, no neutralization was observed, indicating that the JA142’s ORF3 and ORF4 products were not involved in virus neutralization and that ORF2, ORF5 and ORF6 products of VR2332 were not involved in VR2332 neutralization.

Discussion

In this study, we modified two RNA-launched PRRSV cDNA infectious clones pOK_{12}-VR2332 and pFL12 into DNA-launched clones pOpti-VR2332 and pOpti-JA142 by changing the vectors. The two viruses carried in the infectious clones are quite different, and their unique endonuclease sites (only one site is contained in the virus genome) are also not identical. Unique endonuclease sites in infectious clones are usually used for initial construction and downstream gene manipulation work when only a partial gene component needs to be mutated. Due to the importance of such unique sites, they should be maintained in newly constructed cDNA infectious clones, so they should not be contained in the vector plasmid. Several PRRSV strains were analyzed for their unique sites, and based on this analysis, commercially available vectors were screened. pOptiVEC^{TM}-TOPO was then selected and further modified. Several sites were added and constructed a new vector pOpti-PRRS. The new vector could be used not only for our two strains, but could also be used for other strains.

Adding hammer head ribozyme and hepatitis delta virus (hdv) ribozyme to generate authentic 5’ end and 3’ end have been utilized by many researchers. In their work, the sequence was not identical. The structure of hammer head ribozyme is important for the self-slicing function. Two different kinds of hdv ribozyme have been used. Both form the same
secondary structure although their sequences are different. The two different sequences were constructed in our infectious clones and their efficiencies were compared (data not shown). No major difference was observed, suggesting that the secondary structure is the most important thing for the function of hdv ribozyme.

The ORF1 region has been postulated to be related to the virulence of PRRSV. In the current study, all different structural gene pattern built in three different infectious clone backbones did not show any difference. The result is considered reasonable because virus neutralization is mediated by interaction between neutralizing antibodies and virion surface structural proteins. Since ORF1 product is related to virus virulence and virus replication, we can utilize the same backbone and insert different structural genes to generate a live virus vaccine with high safety, especially when a new virulent virus emerges as long as avirulent infectious clone backbone is used as virus vector. This approach may save the time to attenuate the virus through continuous cell passages.

The VR2385 strain had a low level of susceptibility to both VR2332 and JA142 antisera, so the involvement of its ORF2 and ORF7 products in virus neutralization with both antisera was expected to be unlikely. Replacing the ORF2 and ORF7 of VR2332 with those of VR2385 did not change the susceptibility of the chimeric virus to the anti-VR2332 antiserum, indicating that VR2332’s ORF2 and ORF7 products may not play a major role in neutralization. In contrast, when JA142’s ORF2 and ORF7 were replaced with those of VR2385, virus susceptibility to the JA142 antiserum decreased, suggesting that ORF2 and/or ORF7 products may have some role in virus neutralization of JA142. When ORF2 and ORF7 of VR2332 were replaced with those from JA142, the new virus became susceptible to anti-JA142 antiserum with SVN titer of 1:16. When JA142 ORF2 and ORF7 were replaced with
those of VR2332, the virus exhibited significantly reduced susceptibility to JA142 antiserum. Taken as a whole, the evidence suggests that the role of VR2332 ORF2 and ORF7 has little effect on virus neutralization, while those of the JA142 have a much more important role in virus neutralization. Considering that ORF7 encode N protein located inside of virion, the role of N protein in virus neutralization is very unlikely. It is then more plausible that virus neutralization activity would be more related to ORF2 product(s).

GP5 has been widely considered as the main protein involved in virus neutralization [13, 39]. However, viruses swapped their ORF5 between VR2332 and JA142 did not get affected on their susceptibility to the VR2332 and JA142 antisera, suggesting that ORF5 of both VR2332 and JA142 might not be critical for virus neutralization. Even though postulated NEs were present in GP5 of both JA142 and VR2332, they do not appear to be related to virus neutralization. Typically, ORF5 is used to compare the relationship of PRRSV strains and predict their degree of cross protection. Our finding suggests that this may be not a broadly applicable in a large scope. Further, replacing ORF6 did not change the susceptibility to VR2332 antiserum, suggesting that the ORF6 of VR2332 may not be related to virus neutralization. In contrast, the susceptibility of the virus to the JA142 antiserum was significantly increased from no susceptibility to 1:8 (JA142 backbone) or 1:16 (VR2332 and VR2385 backbone), suggesting that JA142’s ORF6 product should be related to virus neutralization. The dissociation of ORF5 and ORF6 of VR2332 from virus neutralization has also been reported by another group [30], further confirming our observations.

GP3 and GP4 proteins have been identified playing roles in virus neutralization [7, 19], even though such a role of GP4 of North American PRRSV strains is questionable. In the current study, we did not separate the exact roles of ORF3 and ORF4 but treated them as
a whole part. Replacing VR2332 ORF3-4 with JA142 ORF3-4 did not provide the resulting chimeric virus any susceptibility to the JA142 antiserum, but the chimeric virus totally lost its susceptibility to the VR2332 antiserum, suggesting that neutralization of VR2332 is totally mediated by ORF3-4 while the ORF3-4 of JA142 is not related to virus neutralization.

Taken all together, our findings indicate that non-structural gene products are not involved in virus neutralization and structural gene components of two genetically and antigenically different strains VR2332 and JA142 have totally different roles in virus neutralization. The role of each gene product in virus neutralization should be assessed case by case. Concerning future vaccine development, a well characterized avirulent virus could serve as backbone for timely vaccine development. To achieve a virus with the capability to induce broader cross neutralizing antibody, the gene component of the virus should be evaluated and designed carefully in an organized manner based on the immunobiological function of each gene product.

References


Table 1. Nucleotide identities of ORF5 and genome of the three isolates VR2332, JA142 and VR2385

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<th>ORF5 identity</th>
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Table 2. Primers used for construction of DNA-launched VR2332 and JA142 infectious clones

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</tr>
<tr>
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Table 4. amplification of 5’ fragment and 3’ fragment for recombinant virus construction

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Figure 1. Construction of vector plasmid pOpti-PRRS. Vector pOptiVEC™-TOPO was amplified with primers pOpti-F and pOpti-R. PCR product was digested by Rsr II, purified and ligated. Ligation product was transformed into *E. coli*. The new vector was sequencing confirmed and named pOpti-PRRS.
Figure 2. Construction of DNA-launched infectious clones. The 5’ end of RNA based infectious clones pOK12-VR2332 and pFL12 were amplified by PCR to insert hammerhead ribozyme. New fragments were used to replace the original 5’ end of pOK12-VR2332 and pFL12 by using endonuclease Asc I/Fse I and Rsr II/SpI. Similarly, hepatitis delta ribozyme was added to the 3’ end of virus genome in pOK12-VR2332 and pFL12 after polyadenine by using BsrG I/Acl I and BstB I/Pac I respectively. New plasmids with 5’ end and 3’ end ribozymes were named pOK12-VR2332-Rz and pFL12-Rz. Viral genomes with hammerhead ribozymes and hepatitis delta ribozymes of pOK12-VR2332-Rz and pFL12-Rz were cut off and inserted into plasmid pOpti-PRRS between cytomegalovirus immediate early promoter (P<sub>CMV</sub>) and Herpes simplex virus thymidine kinase polyadenylation signal (TK pA) by using Sbf I and Pac I. New plasmids were named pOpti-VR2332 and pOpti-JA142 respectively. Solid triangle indicated the T7 promoter and open arrow indicated CMV promoter.
Figure 2. continued

pFL12

Rsr II  Spe I  BstB I  Pac I

pFL12-Rz

Rsr II, Sbf I  Spe I  BstB I  Pac I

pOpti-JA142

Sbf I  Spe I  BstB I  Pac I
Figure 3. Schematic diagram of structural gene swapping patterns. Eight patterns of structural gene were constructed in three backbones. Gene components were labeled according to their origins.
Figure 4. Construction of the 8 patterns. Overlap PCR was used for gene recombination of structural gene. PCR primers used for each fragment were shown underneath each fragment diagram. Template of each fragment was shown as in legend. An around 20bp overlap region was used to connect two adjacent fragments.
Figure 5. Cross neutralization of JA142 and VR2332 antisera to wild-type viruses. Susceptibilities of three wild-type viruses were tested by means of FFN. Tests were done three times in three different days. (A) Susceptibilities of three wild-type viruses to JA142 antiserum. VR2332 and VR2385 were not susceptible to JA142 antiserum, while JA142 was susceptible to less than 1:16-32 fold diluted antiserum. (B) Susceptibilities of three wild-type viruses to VR2332 antiserum. JA142 was not susceptible to VR2332 antiserum. VR2332 was susceptible to no more than 1:64 fold diluted VR2332 antiserum. VR2385 was susceptible to less than 1:4 fold diluted antiserum.
Figure 6. Susceptibilities of chimeric viruses to JA142 and VR2332 antisera. Chimeric viruses were tested for their susceptibilities to JA142 and VR2332 antisera separately. Tests were done three times in three different days. (A) Susceptibilities of chimeric viruses to JA142 antiserum. (B) Susceptibilities of chimeric viruses to VR2332 antiserum.
Figure 7. Role of ORF2 and ORF7 in virus neutralization. Susceptibilities of chimeric viruses were compared side by side to demonstrate the role of ORF2 and ORF7 in virus neutralization. (A-D) ORF2 and ORF7 of VR2332 or JA142 were replaced with those of VR2385 which has low level of cross neutralization with JA142 and VR2332 antisera. (E-H) ORF2 and ORF7 of VR2332 or JA142 were replaced by their counterpart.
Numerous previous studies have demonstrated a high degree of genetic and antigenic variation that exists among PRRS viruses in the field. Protective immunity provided by a specific strain is normally limited to its homologous strains as a result of the variation. Vaccines derived from heterologous PRRS virus strains have been developed and used in different farms. However, providing broad protection by vaccination with multiple strains is not only laborious, but also a safety issue since recombination may generate new strains that can escape immunity or have high virulence. Therefore, alternative approaches were explored to find a way to construct a vaccine virus which can confer broader cross protection.

The first study focused on the possibility of providing broad cross protection against heterologous strains by vaccinating pigs with one virus strain. The chimeric virus JAP56, which was constructed by replacing VR2332 ORFs 5-6 with those of JA142, protected pigs against both VR2332 and JA142 represented by decreased viremia levels after challenges. Observations in this study illustrated that chimeric virus containing ORFs 5-6 from heterologous strain could provide broad cross protections against both PRRS virus strains. This strategy, therefore, can be used for vaccine development and provide vaccines able to protect pigs against a wide spectra of strains compared to currently available PRRS vaccines.

As the first study revealed that chimeric virus with mixed ORFs 5-6 from JA142 and other structural genes from VR2332 protection pigs against challenges with both strains, a following study was to explore the feasibility of applying this strategy to other strains. Since ORF2 and ORF7 have been demonstrated to be less involved in PRRS virus neutralization, the second study focused on mixing ORFs 3-4 and ORFs 5-6 from four distinct wildtype
strains SDSU73, MN184, 1648-06, and 17198-2. Serum virus neutralization was performed using antisera of the four strains with chimeric viruses. Low level of cross neutralization exists among the four wildtype PRRS virus strains, whereas several but not all chimeric viruses gained broader cross neutralizing reactivity. It was demonstrated that roles of ORFs 3-4 and ORFs 5-6 products in virus neutralization among the four strains varied and were more strain specific. This work further demonstrated the variation of structural gene products of different PRRS viruses in immunity induction. The findings emphasize that neutralization determinants should be well characterized on a strain-specific basis, and a well-organized chimeric virus containing neutralization determinants from multiple heterologous strains should be able to provide broader cross protection.

It is believed that structural gene products are related to virus neutralization. The first and second studies demonstrated that viruses with mixed structural genes could obtain broad cross neutralization. On the other hand, the role of genetic component besides the structural gene in virus cross neutralization has not been clearly identified. Focusing on the affection of UTR and ORF1 region to virus cross neutralization, the third study was conducted. Six structural gene patterns were constructed with mixed JA142 and VR2332 structural genes. Each pattern was built in three different infectious clone backbones. Regardless of structural gene pattern, no difference was observed in virus neutralization among the chimeric viruses possessing the same pattern. This study indicated only structural gene products are responsible for virus neutralization. Considering that non-structural gene contains main virus virulence determinants and play a key role in virus replication, our results laid an important foundation for future vaccine development to reduce the virus virulence in a timely manner.
ACKNOWLEDGEMENTS

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