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Immunological significance of genetic variation in structural proteins and the genetic determinants for cross protection of Porcine Reproductive and Respiratory Syndrome (PRRS) virus

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Immunological significance of genetic variation
in structural proteins and the genetic determinants for cross protection of
Porcine Reproductive and Respiratory Syndrome (PRRS) virus

by

Won-Il Kim

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

DOCTOR OF PHILOSOPHY

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2007

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ABSTRACT

The enormous genetic and antigenic diversity of PRRSV has become a diagnostic concern as it interferes with the accuracy of diagnostic tests and hampers the development of effective vaccines and the eradication of the disease. This study was conducted to assess the effects of genetic variation on serologic diagnosis and cross protection by antibody among different PRRS viruses and to identify the genetic elements critically associated with cross protection. Identification of the important genetic elements for cross protection would be useful not only to classify the viruses according to their immunologic relatedness but also to develop better disease-control methods including vaccines.

Three independent studies were designed to accomplish the stated objectives. The first study was conducted to determine if serologic data and the performance of serologic assays could be influenced by genotypic and/or biotypic differences of PRRS viruses and, if so, to assess the degree of effect. In the study, a comparative serologic study was conducted on five field and two cell-attenuated viruses to determine if serologic responses to PRRS virus infection could be influenced by biotypic and/or genotypic differences of the viruses. The isolates used for the study varied in their virulence to pigs and in genomic sequences. Ten pigs were inoculated with each isolate ($1 \times 10^3 \text{TCID}_{50}$) via the intranasal route. All inoculated animals became viremic during the study period. Some animals inoculated with the attenuated viruses remained seronegative until the end of the study (42 days PI), but all of the animals inoculated with field viruses developed ELISA- and IFA-detectable antibodies, regardless of the virus strain used in the IFA assay. In contrast, a great degree of variation in the onset and level of serum virus neutralization (SVN) antibody was observed by individual
pigs and by each virus. The reactivity of SVN antibody was highly specific for homologous viruses. Therefore, it was concluded that the biotypic differences among PRRS viruses may affect the kinetics of humoral immune response in infected pigs. In addition, the IFA test may be used as a confirmatory test when a false-positive ELISA result is suspected or vise-versa at least among North American strains (PRRS virus type 2), but SVN antibody titers are highly affected by antigenic variability.

The second study was to identify genetic determinants associated with cross protection in ORF5 that encodes the major envelop protein (GP5) since GP5 has been postulated to be the most important protein to induce SVN antibody. The genetic elements within ORF5 which affect cross-neutralization were determined by genetically comparing field isolates which were classified according to their relative susceptibility to SVN antibody raised against VR2332 strain (North American prototype PRRS virus). In addition, the mutants in which the amino acid sequences were substituted with those found in the viruses resistant to SVN antibody at specific sites in ORF5 were generated using a VR2332-backboned infectious cDNA clone and site mutagenesis to confirm the role of those identified sites. Five common sites/domains (I to V) were identified in ORF5 from the sequence comparison after sixty-nine field isolated were classified based on the result of in vitro SVN test and/or animal challenge after passive immunization of SVN antibody. This suggests that the changes in amino acid sequences at three sites (32-34, 38-39, and 57-59) located in the N-terminal ectodomain of ORF5 significantly affected the susceptibility of the viruses to SVN antibody.

Finally, the third study was performed to assess the role of other structural proteins besides GP5 in cross protection among PRRS viruses and to define the corresponding genetic
elements in each protein. In this study, chimeric mutants were generated by replacing ORF5 of an infectious clone constructed based on VR-2332 sequences with that of JA142, SDSU73, PRRS124, or 2M11715 to assess the role of ORF5 in cross neutralization. These viruses were genetically and antigenically distinct from VR-2332. In addition, chimeric mutants were constructed by substituting single or multiple structural genes of the VR-2332-infected clone with the corresponding gene(s) of JA142. Virus neutralization test was performed on all mutants to determine the affect of substitutions on the susceptibility or resistance of viruses to the neutralizing activity of antisera generated against VR-2332, JA142, SDSU73 and PRRS124. All ORF5-replaced mutants showed the level of susceptibility or resistance close to that of the donor strains against homologous or heterologous antisera but failed to achieve a complete reversion of cross neutralization. In contrast, substitution of ORFs 3-6 completely reversed the susceptibility of viruses to the neutralizing activity of anti-VR-2332 or JA142 antiserum. ORFs 3, 5, and 6 were additively responsible for such reversion between VR-2332 and JA142. These results indicate that the genetic similarity of ORFs 3 and 6 besides ORF5 should be taken into consideration to achieve the full-capacity of virus neutralization between two different PRRS viruses.

In conclusion, genetic variation of PRRSV negatively impacts cross neutralization among PRRS viruses. The similarity of specific amino acid determinants in GP3, GP5 and M proteins may significantly contribute to the level of cross protection between two viruses.
CHAPTER 1: GENERAL INTRODUCTION

Dissertation organization

This dissertation begins with an abstract and is followed by a general introduction, three research papers, a general conclusion, and an acknowledgement. References are listed at the end of the respective sections (The general bibliography is listed at the end of this dissertation). The general introduction (chapter 1) provides a broad overview of functional genomics of PRRSV in comparison with other arteriviruses to review the current knowledge of PRRSV life-cycle and to emphasize the research objectives. Chapter 2, “Effect of genotypic and biotypic differences among PRRS viruses on the serologic assessment of pigs for virus infection” is a research article that has been accepted to Veterinary Microbiology. Chapter 3, “Immunological significance of genetic variation in ORF 5 of porcine reproductive and respiratory syndrome virus (PRRSV)”, and chapter 4, “The requirement of structural proteins in cross-neutralization among different PRRS viruses”, are research papers which have been prepared to be submitted to the Journal of Virology. This dissertation contains experimental results obtained by the author during his graduate study under the supervision of his major professor, Dr. Kyoung-Jin Yoon.
Literature review: Comparative functional genomics of PRRSV and other arteriviruses

Introduction
Porcine reproductive and respiratory syndrome virus (PRRSV) causes reproductive failure in sows (increased stillbirths and mummification, and increased early or delayed return to heat) and boars (abnormality of semen, decreased libido) and respiratory distress and systemic infection (anorexia, lethargy and pyrexia) in all ages of pigs (2;22;37;164). It has been estimated that PRRSV infection costs the U.S. swine industry approximately 560 million dollars annually due to the reductions in the rate and efficiency of reproduction and growth during acute infection (141).

PRRSV was first isolated in the Netherlands in 1991, which was designated as “Lelystad” virus (LV) (216). Subsequently, the isolation of PRRSV was reported from the United States in 1992, which was designated as ATCC VR-2332 (22). Since the viruses identified in Europe and North America are genetically distinct from each other, PRRS viruses are categorized into two genotypes; European (type 1) and North American (type 2) types and these two genotypes share less than 70% sequence homology in the entire genome (50;214). More importantly, PRRS viruses of the two genotypes show minimal antigenic cross reactivity in serologic tests (91;120;140), and are not cross neutralized by antibody raised against each other (50;214).

PRRSV is an enveloped virion whose size ranges from 48 to 83 nm in diameter and contains the icosahedral nucleocapsid core of 25 to 35 nm in diameter (26). The buoyant density of the virus is 1.13 - 1.18 g/ml in CsCl gradients and 1.18 to 1.23 g/ml in sucrose
gradients (22;26;121). Taxonomically, the virus belongs to the family Arteriviridae in the order Nidovirales which is comprised of Coronaviridae, Roniviridae and Toroviridae (49) besides Arteriviridae (33;76). Viruses of these families possess similarity in both genomic structure and strategy of gene expression. Other members of Arteriviridae are equine arteritis virus (EAV), simian hemorrhagic fever virus (SHFV) and lactate dehydrogenase-elevating virus (LDV) of mice (33;182;195).

The genome of PRRSV is a single-stranded, 15kb-long, positive-sense RNA, which is capped at 5’ end and polyadenylated at 3’ end. As shown in Figure 1, the viral genome consists of at least nine open reading frames (ORFs) (8;51). ORFs 1a and 1b encode nonstructural proteins (Nsps) which are required for virus replication (8) and compose most of the viral genome (approximately 12 kb) (130). Two polyproteins, ORF1a and ORF1ab, are expressed by ribosomal frame shift at so-called “slippery sequences” (GCUUUAAAC in PRRSV type1 and GUUUAAAC in PRRSV type 2) and at least 12 Nsps are generated as a result of serial proteolytic cleavages of the two polyproteins by four viral gene-encoded proteinases [papain-like cysteine proteases (PCPα and PCPβ)(130), cystein protease (CP) (182), and 3C-like serine protease (SP) (182)].

The unique feature in the process of nidovirus replication is the generation of a co-3’-end nested set of subgenomic (sg) mRNAs from which open-reading frame (ORF) close to the 5’ end (i.e., non-overlapping portion) is expressed (76) (Figure 1). As shown in figure 2, three N-glycosylated minor envelope proteins (GP2a, GP3, and GP4) are translated from ORF 2a, 3 and 4 and form heterotrimers by disulfide linkages (222). ORF2b, which is completely embedded in ORF2a, encodes another non-glycosylated minor protein designated 2b protein which is equivalent to E protein in other arterviruses.
Figure 1. Genomic organization (A) and gene expression strategy (B) of PRRSV.

Open reading frames (ORFs) in the viral genome are indicated by a number. Leader sequence and leader transcription-regulating sequence (TRS) are shown in the orange- and blue- colored boxes, respectively, at the 5’ end of the genome. Each body TRS is indicated in blue-colored box at the upstream of each ORF or subgenomic (sg) mRNA. (A)_n represents polyadenylation at the 3’ end of the genomic or messenger RNAs. Nonstructural polyproteins (pp) and structural proteins (GP2a, 2b, GP3, GP4, GP5, M and N) are depicted at ORFs from which those are translated. The ribosomal frameshifting sequence (RFS) is located between ORFs 1a and 1b.
**Figure 2. Schematic structure of PRRS virus.** The virion contains a single-strand RNA genome encapsidated by nucleocapsid protein (N), forming an icosahedral core structure. Three minor glycosylated envelope proteins (GP2a, GP3, and GP4) form a heterotrimer while major envelope protein (GP5) and matrix protein (M) form a heterodimer. The GP3 that has been identified as an envelope-associated glycoprotein in PRRSV type 1 but a soluble glycoprotein in PRRSV type 2 is represented as a compromiser structure for both types of PRRSV. The 2b protein (also known as E protein in other arteriviruses), non-glycosylated minor envelope protein is also associated in the viral envelope.
In the case of EAV, ORF2a encodes E proteins; N-glycosylated GP2b protein is expressed from ORF2b and forms GP2b/GP3/GP4 heterodimer (219). For LDV and SHFV, E protein is expressed from ORF2a (156) and ORF4a (76), respectively. ORF5 codes the major envelope protein (GP5) that is glycosylated and forms a heterodimer with matrix (M) protein expressed from ORF6 (122). This heterodimer is known to be most abundant on the surface of virion and plays a pivotal role in virus assembly, recruiting heterotrimers of the minor envelope proteins and nucleocapsid (N) protein into the virion (222). The N protein is expressed from ORF7 and has been reported to contain nuclear localization signals (NLS) and nuclear export signals (NES) (173). The N protein forms homodimers which are known to be essential for virus infectivity (109) to a highly permissive clone (MARC-145) of African Monkey kidney cell line MA-104 (93). Detailed roles and functions of each protein product in infection, replication, immunobiology and pathogenesis of PRRSV are described below where appropriate.

PRRSV has several notable biological characteristics: a) preferential replication in macrophage-lineage cells (215); b) establishment of persistent infection resulting in chronic carriers (82;221); c) induction of weak and delayed innate or adaptive immune responses (118;134;137); d) induction of abundant and early non-neutralizing antibody (118;137); e) high degree of genetic and antigenic variation (10;90); f) a low infectious dose (81;234); g) variability in virulence (79); and h) shedding in bodily fluids such as boar semen (190) and milk (210). All of these characteristics are considered to be obstacles to the effective control and prevention of PRRS and are enigmas to be resolved for better understanding of the ecology and pathogenesis of PRRSV and building a better prevention and control strategy.
Details on each biological characteristic are well reviewed in 2003 PRRS Compendium (238).

Even still in its infancy, much knowledge related to the functional genomics of PRRSV has been accumulated since new technologies, such as reverse genetics, had been made available for studying PRRSV in details (39;109;129;142;197). This paper is to review current understanding on: a) the life-cycle of PRRSV (from cell attachment to virus release from the infected cells); b) the interaction between virus and host, such as host immune response against virus infection and virus strategies to evade host defense; and c) viral pathogenesis in animals. Special attention was given to the function or roles of each PRRS viral gene and protein in these processes as compared with other arteriviruses when necessary.

Virus replication and role of viral proteins/genes

Entry of PRRSV into target cells

Attachment PRRSV is reported to enter the target cell by receptor-mediated endocytosis via clathrin-coated pits and vesicles as shown in MARC-145 cells (monkey kidney cell line) and pulmonary alveolar macrophages (PAMs) of pig in vitro and then completes uncoating process in a pH-dependent manner (97;138). Three cellular receptors (heparin sulfate, sialoadhesin, and vimentin) have been postulated to be involved in the interaction between PRRSV and the target cell (94;205;206). Heparin sulfate proteoglycans has been shown to mediate the attachment of PRRSV to PAMs (88;205). This receptor molecule was reported to bind to the M protein or GP5-M heterodimer with a great affinity (55). However, heparin sulfate receptor seems not to be the prerequisite for virus internalization since binding of
PRRSV to this receptor did not initiate endocytosis of the virus and the addition of heparin did not abolish virus infection to PAM (53;55).

Sialoadhesin (Siglec-1), a 210kDa protein (p210), has been identified as a potential receptor molecule for PRRSV in PAMs (206). Initiation of an endocytosis process was demonstrated following virus attachment to this molecule. The N protein of PRRSV was detected with virus-specific monoclonal antibody in the cytoplasm of a non-permissive cell line (PK-15) expressing recombinant Siglec-1 on the surface 1 hour after inoculation, indicating that virus particles were internalized. In addition, Delputte et al. (2004) (54) demonstrated that sialic acids present on virus particles bound to Siglec-1 and that the virus infection to PAMs was inhibited by neuraminidase treatment which removes sialic acids from the viral envelope, suggesting that sialic acids are critical for PRRSV infection. Based on these observations, the following model was suggested for the attachment and internalization of PRRSV to PAMs (53): a) PRRSV first binds to heparin sulfate with GP5-M complex, which does not lead to virus internalization but mediates subsequent involvement of other receptors, such as Siglec-1; b) Siglec-1 then binds to sialic acids which are present on N-glycosylated major or minor envelope proteins of PRRSV and brings the virus into the cytoplasm.

Although binding of Siglec-1 and viral sialic acids induced successful virus internalization to PAMs, virus replication did not occur due to the failure of the uncoating process. The N proteins detected in the cytoplasm of Siglec-1-expressing PK-15 cells at 1 hour after inoculation were never disassembled or disappeared over time. In contrast, the N protein of PRRSV disappeared from the cytoplasm of MARC-145 cells, which is known to be highly permissive to PRRSV, at 3 hours after inoculation and reappeared at 12 hours after
inoculation as determined by immunofluorescence microscopy, indicating that internalized virus particles completed the uncoating process and replicated in the cells. Therefore, it was suggested that other cellular factor(s) or receptor(s) may be essential to the establishment of complete PRRSV infection and replication (53). Along with this speculation, vimentin complex which is composed of vimentin and vimentin-bound proteins, such as cytokeratin 8, cytokeratin 18, actin, and hair type II basic keratin, has been recently postulated to be responsible for the establishment of PRRSV infection (94). Vimentin and the vimentin-bound proteins were demonstrated on the surface and in the cytoplasm of PAMs and MARC-145 cells. This receptor seems to support internalization and replication of PRRSV since virus infection took a place in non-permissive cell lines, such as BHK-21 (baby hamster kidney) and CRFK (feline kidney), expressing recombinant vimentin as determined by the detection of N protein and viral genome 3 days after inoculation (94). However, PRRS viral protein interacting with vimentin has not yet been identified.

Since GP5-M heterodimers are predominant on the surface of virion, occupying 90-95% of virion structure with N protein (182) and are known to be important in inducing neutralizing antibody (75;155;213), the GP5-M heterodimers have been presumed to be critically involved in the interaction with cellular receptors. However, the role of minor envelope proteins (GP2a, GP3 and GP4) in the interaction with cells has been reemphasized after several studies demonstrated that ectodomain in GP5 and M of EAV were not essential for cell tropism, and heterotrimer structure of the minor envelope proteins was vital for the infectivity of EAV and PRRSV type 1 (61;209;219;222).
**Internalization and uncoating**  Attachment of PRRSV virions to cellular receptors is followed by endocytosis into clathrin-coated vesicles. The vesicles then enter the cytoplasm and fuse with endosomes (138). An acidic environment within the endosome is believed to be essential to trigger conformational changes of PRRS viral protein structures and mediates the fusion process between the viral envelope and the endosomal membrane, releasing the viral genomic RNA into the cytoplasm (138). Similar to the M2 protein of influenza A virus that functions as an “ion channel” to maintain the acidic pH in the endosome during membrane fusion, the 2b protein of PRRSV has been proposed to function as the ion channel and is essential for the uncoating process since: a) ORF2b-knockout mutant PRRSV which was generated by nullifying the start codon of ORF2b was unable to proceed to further steps of replication after internalization; and b) the expression of 2b protein in *E. coli* increased the permeability of the bacterial cell wall (113).

A fusion peptide hidden from the virion surface by neighboring constitutive proteins extrudes to the surface, following cleavage by proteinases and/or conformational change by lowered pH, and is inserted into the endosomal membrane to complete the fusion process (43). Although the fusion peptide has never been studied in arteriviruses, amphipathic sequences (a.k.a. heptad repeat, VSRIYQ), which form a highly stable helical structure so-called “coiled-coils” to bring the two membranes (i.e., viral envelope and endosomal membrane) in vicinity as a post-fusion conformation of fusion proteins, have been identified in ORFs 2 and 3 of PRRSV type 1 and in ORF2 of PRRSV type 2 (144). However, the actual role of those peptide sequences in viral fusion has not been confirmed yet.
Replication and gene expression of PRRSV

Successfully uncoated genomic RNA of PRRSV is instantly translated into polypeptide by cellular ribosomal machinery as the genome is already capped and polyadenylated. Most knowledge related to viral replication and gene expression of arterivirus comes from extensive studies of EAV and is believed to be common in all members of Arteriviridae (182). PRRSV ORF1a encodes a single polyprotein (pp1a), which is predicted to be cleaved at eight sites and form nine non-structural proteins: Nsp1α, Nsp1β, and Nsp2 through 8 (184;212). ORF1b polyprotein is expressed by −1 ribosomal frameshift which results in the nullification of the termination codon for ORF1a, hence generating a single ORF1ab fusion protein (pp1ab). The overlapping region between ORFs 1a and 1b contains two signals which are assumed to favor the frameshift event: a) slippery sequence [G(C)UUUAAAC in PRRSV or GUUAAAC in EAV], the actual frameshift site; and b) RNA pseudoknot structure immediately after the slippery sequences (28;85;139). The efficiency of frame shifting to produce pp1ab has been estimated to be 15-20% in EAV (57), suggesting that pp1a is expressed most of time instead of pp1ab. Four non-structure proteins designated Nsp 9 to 12 are generated from ORF 1b region of the ORF1ab polyprotein by proteolytic cleavage (184;212).

Four proteases are derived from ORF1a and are responsible for processing the other Nsp products. Nsp1 and Nsp2 contain cysteine autoprotease domains and are immediately cleaved away from polyprotein 1a after being synthesized from PRRSV genome. Nsp1 proteases were characterized as papain-like cysteine proteases (PCPα and PCPβ) (130) whereas Nsp2 protease is classified as cystein protease (CP) (182). Nsp2 is highly variable among PRRS viruses (8;66). A recent study (80) revealed that deletion of at least 200 amino
acids in the variable middle region of Nsp2 was dispensable for PRRSV infectivity while the region between 180-323 was critical for virus viability. Nsp4 protease, a member of 3C-like serine protease (SP), is responsible for processing 8 SP cleavage sites which are conserved (both sequence and location) among different arteriviruses (184). The SP cleavage sites could be variable due to two alternative pathways of SP cleavage (i.e., minor and major pathways) to yield a variety of cleaved products whose function remains to be elucidated (198). Those pathways are believed to be controlled based on the association of cleaved Nsp2 with the Nsp3-8 precursor. Therefore, when Nsp2 is associated with the Nsp3-8 precursor, Nsp4/5 site is cleaved by the Nsp4 proteinase (major pathway). In the absence of Nsp2, the Nsp5/6 and Nsp6/7 sites are processed and the Nsp4/5 junction remains uncleaved (minor pathway) (198).

Most of the Nsp products from ORF1b are postulated to be involved in virus replication (130;202). Putative RNA-dependent RNA polymerase (RdRp) and NTPase/RNA helicase motifs, which are part of the viral replicase complex, were reported to be associated with Nsp9 and Nsp10, (130;202). Nsp10 contains a putative zinc finger region which is implicated in the synthesis of sg mRNAs and replication of genomic RNA (57;130;202). Nsp11 contains uridylate-specific endoribonuclease (NendoU) domain which is conserved among all nidoviruses (163). The NendoU domain was reported to be essential for RNA synthesis and/or the production of virus progeny in arterivirus (163). Nsp12, the most C-terminal part of polyprotein 1ab, was found only in arterivirus but its function has not been characterized yet (76).

The replicase complex, which consists of RdRp, NTPase/RNA helicase motifs, and ORF1a-encoded cleavage products, is localized in intracellular membranes such as ER. The
hydrophobic domain of ORF1a-encoded cleavage products (especially, Nsp2, 3 and 5) was demonstrated to mediate the association of the replicate complex with those membranes (182;200). The replicase complex recognizes RNA sequences at the 3’ end of viral genome and initiates the synthesis of full-length or sg-length negative-strand RNA (151). In PRRSV type 1, it has been shown that a 34-nucleotide stretch within ORF7 (nucleotides between 14653-14686) is essential for the synthesis of negative-strand RNA and those 7 core nucleotides in the stretch interact with their complementary sequences resided in the region between 14996 and 15034 of the 3’ NTR region, which was described as “kissing interaction” (208). In addition, Choi et al (2006) (39) demonstrated that 911 nucleotides from the 3’ end of type 2 PRRS viral genome were involved in virus replication as a cis-acting element since luciferase-expressing PRRSV replicons containing those nucleotides were replication competent.

A synthesized full-length negative-sense RNA is, in turn, utilized as a template for the synthesis of full-length genomic RNA and sequences located in the 3’ terminus of the negative-strand RNA is believed to be essential for this process. Recently, it has been demonstrated that seven nucleotides (AUGACGU) in the 5’-NTR of viral genome play an important role in virus replication but can be functionally replaced with various sizes of AU-rich sequences (39).

The synthesis of a nested set of negative-sense sg RNAs is regulated by the Transcription-Regulating Sequences (TRS, 5 to 8 nt long in arteriviruses) located at the 3’ end of the common leader that is located at 5’ end of each mRNA, and the leader-to-body junction site in each of the sg mRNA. The important role of TRS in the regulation of transcription has been demonstrated by several mutational studies using an EAV infectious
cDNA clone. Introducing point-mutations into TRS decreased transcription level 100 fold. (151;203). It also has been shown that base pairing between positive- and negative-sense copies of TRS directs the co-transcriptional fusion of sg mRNA leader and body in the process of discontinuous transcription in arteriviruses (48;56;58;151). In brief, the synthesis of sg-length negative-strand RNAs is believed to be the outcome of the early termination of negative-strand RNA synthesis at different body TRS regions of the genomic template. The anti-body TRS at the 3’ end of the negative-strand sg-RNAs would base pair with the leader TRS. Then, anti-leader sequence is added to the 3’end of the negative-strand sg-RNAs by extending along the leader sequence in genomic RNA. At last, the complete negative-strand sg-RNAs that contain anti-leader sequences serve as templates for sg mRNAs (discontinuous extension of minus-strand RNA synthesis) (151) (Figure 3A).

There has been controversy as to whether the discontinuous step in the synthesis of sg RNA occurs during plus-strand synthesis commonly referred to as “leader-primed transcription” or minus-strand synthesis known as “discontinuous extension of minus-strand RNA synthesis”. In the ‘leader-primed transcription’ model (Figure 3B), full-length negative RNA is synthesized before transcription takes place, and then transcription is initiated from the 3’ end of full-length negative RNA to produce a leader primer of which the 3’-terminal leader TRS base pairs to the various anti-body TRS in the negative RNA. Subsequently, sg mRNA synthesis is completed with extension from the 3’ end of leader TRS (15;105). The leader-primed transcription model was initially postulated based on the inability to detect sg-length minus strands in coronavirus-infected cells (106). However, the presence of sg-length minus strand RNAs was later discovered for both coronaviruses and arteriviruses (56;177;178). Evidence from many recent studies supports a ‘discontinuous extension of
minus-strand RNA synthesis’ model (16;152;176;239). Nonetheless, both models are not mutually exclusive because the possibility that sg mRNAs could be produced by ‘leader-primed transcription’ from the sg-length minus-strand RNAs which were synthesized by ‘discontinuous extension of minus-strand RNA synthesis’ cannot be completely ruled out (201).

**Nuclear stage of PRRSV**

The N protein of arteriviruses is found in both nuclear and cytoplasmic compartments of infected cells even though positive RNA viruses are supposed to complete their entire replication cycle only in the cytoplasm of infected cells. Nuclear localization signal (NLS) and nucleolar localization signal (NoLS) sequences, which are enriched in basic amino acids, are involved in the translocation of N protein produced in the cytoplasm into the nucleus or nucleolus of infected cells. In contrast, nuclear export signal (NES) sequence, which is enriched in leucine residue, is involved in the translocation of N protein into the cytoplasm. Two NLS sequences, NLS-1 (a.a. 10-13) and NLS-2 (a.a. 41-47) (169), and one NoLS sequence (a.a. 41-72) have been identified in the N protein of PRRSV (171;173). In addition, one NES sequence (a.a. 106-117) has also been identified in the N protein (173). Those sequences are highly conserved in both types of PRRSV (173).

The function associated with the localization of arterivirus’ N protein into nucleus/nucleolus is not completely understood. Several possibilities have been suggested based on the previous studies of other viruses. Coronavirus N protein, Semliki Forest virus (SFV) capsid protein, and hepatitis C virus (HCV) core protein, which are equivalent to arterivirus N protein, are known to be translocated into the nucleus (34;67;231). The
Figure 3. Two opposite models for transcription of arteriviruses. It has been proposed that the discontinuous step in RNA synthesis operates during minus-strand synthesis (A, ‘discontinuous extension of minus-RNA model’) or during plus-strand synthesis (B, ‘leader-primed transcription model’). However, a base-pairing interaction between the sense copy of leader (L) transcription-regulating sequence (TRS) and the antisense copy of TRSs present at the 5’ end of each of the subgenomic (sg)-length RNA body segments (anti-body TRSs) is important in both models.
localization of those proteins in the nucleus was reported to be involved in the redistribution of host cell proteins and the inhibition of host cell protein synthesis by binding to nuclear proteins (34), blocking ribosome assembly in the nucleolus (63), or blocking nuclear import and export of host cell proteins and mRNAs (84). Ultimately the localization of those proteins into the nucleus results in the inhibition of cell division, which would be beneficial to the virus because it diverts cellular mechanism from the host cell division to the viral protein synthesis in the cytoplasm where the virus replicates. In contrast, Rowland and Yoon (2003) (173) suggested a somewhat different possibility for PRRSV. These investigators proposed that accumulation of N protein in the nucleus might be a host cell defense mechanism to inhibit viral assembly. The accumulation of a large portion of synthesized N proteins in the nucleus could inhibit the incorporation of N proteins into virions, which occurs in various cytoplasmic compartments (i.e. ER and Golgi complex). The virus could overcome this host defense by either overproducing N protein or incorporating NES sequences into N protein to get the protein back to the cytoplasm. In addition, nuclear/nucleolar localization of PRRSV N protein could be a result of molecular mimicry. For example, RNA binding domains of N protein which are typically enriched in lysine and arginine residues resemble nuclear and nucleolar localization signal sequences which might act as NLS or NoLS (103).

Interestingly, it was observed that the abolishment of the nuclear stage of PRRSV by substituting two lysine residues with glycine in NLS sequence of N protein decreased the viral replication in MARC-145 cell and resulted in virus attenuation as determined by demonstration of a shorter period of and a lower level of viremia when pigs were challenged with such a mutant virus. The substituted glycine residue quickly reverted to lysine or
arginine after \textit{in vivo} replication and N proteins of those reverted viruses regained the ability to enter the nucleus, indicating that the nuclear stage of PRRSV may not be essential for the virus viability but might be related to the pathogenesis of the virus (110).

\textbf{Assembly and exocytosis of PRRSV}

PRRSV particles are assembled in ER and Golgi apparatus where post-modifications (i.e. glycosylation, folding, and multimerization) of viral proteins also occur (51;52;122;222). GP5/M heterodimerization is essential to produce an infectious virus particle as GP5/M heterodimers have a pivotal role in virus assembly as a primary scaffold of virion structure. A study demonstrated that minor envelope proteins (GP2a, 2b, GP3 and GP4)-knockout PRRSV genomes still produced virus particles in the supernatant after transfection even though those were not infectious whereas no viral protein (i.e., no virus particle) was detected in the supernatant after the transfection of GP5 or M protein-knockout genome (222). In contrast, co-expression of ORFs 5, 6 and 7 was not sufficient to produce virus-like particles in a case of EAV (219). Therefore, the interaction between viral genome and those major structural proteins or other unknown components may be important during the process of virus packaging (183;219).

Minor envelope proteins of PRRSV can be released from ER by forming GP2a/GP3/GP4 heterotrimer; otherwise, individual minor envelope proteins remain in ER without being incorporated into the virion although GP3 can be released alone from the cells (222). The process of trimerization among minor envelop proteins has been studied for EAV. First, GP2b, which is equivalent to GP2a in PRRSV, covalently binds to GP4 through cystein 102 of GP2b to form a heterodimer, and then GP3 binds to GP2b/GP4 heterodimers
by a disulfide linkage to GP4 (217;218). Interestingly, the GP3 protein seems not to be covalently associated with the disulfide-linked GP2b/GP4 heterodimers when the virion leaves the cells even though the protein appears to non-covalently interact with the GP2b/GP4 heterodimers within the infected cells. Hence, disulfide linkage between GP3 and GP2b/GP4 heterodimers is believed to be formed only after virus particles are released from the infected cells. Although the 2b protein (equivalent to E protein of EAV) of PRRSV is not involved in the heterotrimerization with other minor envelope proteins (i.e., GP2a, GP3 and GP4), it is involved in the interaction between GP5/M heterodimer and GP2a/GP3/GP4 heterotrimer since 2b-knockout PRRSV or E-knockout EAV genome by nullifying the start codon failed to incorporate any of the minor envelope proteins into the virion (222).

Genomic RNA of PRRSV is incorporated into the virion through binding to the RNA-binding domain located over the residues between positions 34 and 51 in the N protein (46). In addition, N proteins form homodimers stabilized by both covalent and non-covalent bonds in ER. Covalent bonds are formed through disulfide linkages between conserved cysteines at position 27 in type 1 PRRSV and at position 23 in type 2 PRRSV while the region for non-covalent linkage was identified in the residues between positions 30 and 37 in the case of type 2 PRRSV (226). The homodimerization of N proteins is more likely essential for virus assembly since cysteine 23 of the N protein has been shown to be critical for virus infectivity (109). It was also proposed that PRRSV N protein may undergo heterodimerization with other structural proteins using cysteine 90 since this cysteine residue was not required for N protein homodimerization but essential for virus infectivity (109). Hence, the N protein was speculated to form a dimer with 2b protein through disulfide linkage between the cysteine 90 of the N protein and one of two cysteine residues at positions
49 and 54 of the 2b protein in the case of type 2 PRRSV (112). Even though it turned out that neither of the two cysteines of 2b protein was essential for viral infectivity, the interaction between 2b and N proteins through a non-covalent linkage was demonstrated (112). It was then postulated that the cluster of basic amino acid residues in the hydrophilic C-terminus of the 2b protein contribute to N-2b interactions since a single mutation in this cluster was lethal for virus infectivity (112).

The structure of PRRSV nucleocapsid has been recently revealed by X-ray crystallography (59;60). Truncated recombinant N protein (NΔ57) expressed in *E. coli* based on the sequences of VR-2332 indeed formed a homodimer even though N-terminal 57 residues were missing. The N dimers are assembled in parallel to form several layers of a ribbon structure through the end-to-end contacts between the dimers in the ribbon. These contacts are accommodated by the interactions between hydrophobic residues (valine at 112, isoleucine at 115 and arginine at 116) at the C-terminus of N protein and polar residues (serine and asparagine at position 93 and 94) in the loop structure between two β-sheet regions. The X-ray crystallography study proposed that α helix structure in the middle (α2, 69-85) of N protein would be on the surface of the nucleocapsid, which interacts with the cytoplasmic part of the M protein (60). Similarly, the important role of GP5/M dimers in the incorporation of N proteins into the virus particle was proposed in a case of EAV since GP5- or M-knockout EAV mutants resulted in the accumulation of N protein in the cytoplasm of transfected cells (219).

M protein may contain a late (L) domain which releases virus particles from the cytoplasmic membrane of infected cells during the budding process since the M-knockout EAV genome produced a viral core chain inside the lumen of ER-associated vesicles, instead
of releasing infectious virus into the supernatant. Such a domain also has been identified in Gag proteins of a number of retroviruses and matrix proteins of rhabdoviruses and filoviruses (71). All these proteins contain highly conserved motifs known to mediate protein-protein interactions between cellular proteins. Three classes of motifs have been defined in viral L domains: PTAP, PPXY, and YXXL (71). Unfortunately, none of those motifs could be demonstrated in EAV or PRRSV.

**Immunobiological roles of PRRS viral proteins**

**Brief overview of PRRSV immunobiology**

PRRSV is capable of modulating immune responses of infected pigs to the virus. In general, infection induces weak innate immunity followed by delayed and weak adaptive immunity [i.e., neutralizing antibody and interferon (IFN)-γ secreting cells] which seldom appears until after 3 to 4 weeks of infection (118;137). Although slow-reacting complement-requiring neutralizing antibodies (i.e., IgM) were demonstrated as early as 8 days after infection (89;192), early antibody response of infected pigs is predominantly directed against N protein of PRRSV which is detectable at 7 to 10 days after infection and is not protective against virus infection (118;137). Late induction of virus neutralizing (VN) antibody coincides with the disappearance of viremia. Passively acquired virus neutralizing (VN) antibody alone was proven to prevent viremia and reproductive failure in the animals challenged with virulent PRRS viruses (148), suggesting the critical role of VN antibody in the control of virus infection (232). Nonetheless, the development of cell-mediated immunity (CMI) appears to be required for the clearance of PRRSV since there have been a number of studies demonstrating the persistent detection of PRRSV in lung and lymph nodes
despite the presence of VN antibodies in serum or bronchoalveolar lavage fluid (3;42;102;221). PRRSV infection has been reported to stimulate the proliferation of both CD4+ and CD8+ T-cells in pigs (4;18;117;175;180). 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 (CTL) are known to be crucial in cell-mediated protective immunity against PRRSV (4;19;107;119;127;137;170;196).

Unlike other positive-sense RNA viruses, PRRSV does not elicit a high level of type I IFNs (i.e., IFN-α and β) or proinflammatory cytokines (TNF-α, IL-1, IL-6, IL-8 and IL-12) in vitro or in vivo (29;194;204). Such weak innate immune response is a significant loss to the anti-PRRSV protective immunity since it has been shown that IFN-α effectively inhibits PRRSV replication in vitro (29) and weak innate immune response would result in an inappropriate stimulation of adaptive immunity. It has been suggested that the down regulation of NF-κB, a key trigger for type I IFNs and proinflammatory cytokines would be responsible for the weak immune responses in PRRSV infection (137). However, a recent study reported a contradictory observation demonstrating that PRRSV activates the NF-κB pathway (114). Therefore, the mechanism of weak innate immunity after PRRSV infection is still vague and remains to be further studied.

B-cell epitopes and humoral immunity

Linear B-cell epitopes have been identified in both nonstructural and structural proteins of PRRSV by using phage display libraries or pepscan analysis. The Nsp2 region contains most of the identified B-cell epitopes (6 in PRRSV type 1 and 18 in PRRSV type 2) and most of the sera collected from pigs infected with PRRSV reacted with those epitopes
Besides Nsp2 protein, two epitopes in GP2 [roughly, 36-51 (ES10a) and 117-142 (ES10b)], two in GP3 (63-79 and 243-250), one in GP4 (48-76), which includes ORF4 neutralizing epitope (59-67), one in GP5 (roughly 178-199), and one in M (133-159) have been identified in PRRSV type 1 (144). All of the identified epitopes are highly conserved even between both types of PRRSV and cross-react with sera from pigs inoculated with PRRSV type 2 (144). For PRRSV type 2, two in GP2 (41-55 and 121-135), one in GP3 (61-105), one in GP4 (51-65), three in GP5 (1-15 and 31-45 and 187-200), one in M (151-174), and two in N (11-25 and 41-55) have been located (47). Recently, An et al. (2005) (9) also identified an additional epitope in the N protein (79-87), which is conserved between the two genotypes.

In addition, several immunologically reactive linear or conformational epitopes were identified with monoclonal antibodies (Mabs) generated against native or recombinant viral proteins. Epitopes in the N protein have been mapped in detail as compared to other proteins. Four linear epitopes (30-52, 37-52, 69-112 and 112 to 123) and one conformational epitope localized in the region 52-69 were identified for type 2 PRRSV and 11 utmost C-terminal amino acids have been shown to play a critical role in the formation of the conformational epitopes (224;225;229;230). Three linear epitopes (2-12, 25-30, and 40-46) and one conformational epitope (51-67 and 80-90) were found in the N protein of type 1 PRRSV. The site 25-30 was conserved in both genotypes (131). All Mabs which have produced against the N protein to date, however, lack virus-neutralizing activity. Besides N protein, two epitopes (67-74 and 74-85) in GP3 were recognized by Mabs generated against E. coli-expressed recombinant GP3 of type 2 PRRSV (237).
Two non-neutralizing epitopes reactive to Mabs have been identified in ORF5: N-terminus (27-30) (149) and C-terminus (170-201) (165). Three epitopes in GP5, two epitopes in GP3, and three epitopes in M were also identified with a variety of Mabs produced against type 2 PRRSV, but these epitopes have not been mapped for their locations yet (229).

The GP4, GP5 and M proteins have been shown to contain an epitope which induces VN antibody (31;51;229). GP3 may also have a neutralizing epitope (NE) since baculovirus-expressed GP3 conferred protection against infection by type 1 PRRSV (162) and one Mab against GP3 of PRRSV type 2 demonstrated statistically even stronger virus neutralization activity in PAMs than did a neutralizing Mab against GP5 (31). Nonetheless, GP5 is believed to be most important in inducing protective anti-PRRSV antibody among membrane-associated proteins of PRRSV since anti-GP5 VN antibody has shown to be most potent in virus neutralization (75;155;213). A neutralizing epitope of GP5 was identified in the N-terminal ectodomain (37-45 SHLQLIYNL) by mapping the epitope reactive to a GP5-specific neutralizing Mab, ISU25-C1, (229) using phage display (149). Another study determined the same site as a NE by testing PRRS-positive sera using an indirect ELISA coated with synthesized peptides, which represented the segments of GP5 ectodomain of PRRSV. Use of GP5 overlapping peptides of various lengths indicated that the epitope recognized by sera was located at the ectodomain between 36 and 52 of GP5 (161). The recognized epitope was considered as a NE because a similar site had been identified as a NE in LDV and one neutralizing anti-LDV Mab that is specific for the NE of LDV also neutralized both type 1 and 2 PRRS viruses (161). This epitope is highly conserved in both type 1 or type 2 PRRS viruses and even in LDV, implying its important function for the virus
Strain-specific NE also has been identified in GP5 of PRRSV type 1 (29-35) with a set of neutralizing Mabs which reacted with a subpopulation of Dutch isolates (223). Interestingly, this NE is overlapped with the predicted cleavage site for the signal peptide. The proline residue at position 24 in GP5 was demonstrated to be essential for binding of neutralizing Mabs, and it was speculated that the cleavage site might be changed by substitution of cysteine with proline at the position (223).

Neutralizing epitopes of EAV and LDV have also been shown located at the corresponding region in their major envelope protein (13;157;223). Four NEs were identified in the N-terminal ectodomain of GP5 in EAV (at position 49, 61, 67-90 and 99-106). It was observed that those determinants interacted with one another to form conformation-dependent epitopes and that the interaction between GP5 and M proteins was critical for the expression of some epitopes in neutralizing conformation (13). One additional neutralizing determinant was identified at position 98 from the analysis of EAVs which persisted in Hela cells (236). In contrast, the NEs identified in the major envelope proteins of PRRSV (GP5) or LDV (VP-3P) are the linear epitope. It is worthy to note though that synthesized peptides resembling those NEs failed to induce VN antibody, suggesting that additional residues might be necessary for the induction of VN antibody (118;157).

GP4 NE was identified at the position between 59 and 67 (SAAQEKISF), which is the most variable region in ORF4 of both types of PRRSV, by using a set of neutralizing Mabs generated against GP4 of PRRSV type 1. These Mabs didn’t react with PRRSV type 2 (132). Although NE in M protein hasn’t been mapped yet, it was assumed that the epitope should be located in the short N-terminal ectodomain consisting of 10 to 18 residues (51). GP5/M hetrodimer is expected to be important to induce neutralizing antibody since co-
expression of these two recombinant proteins was more effective in inducing VN antibody than was expression of individual recombinant protein (86;87).

**T-cell epitopes and cell-mediated immunity**

Host INF-γ production is a critical component of cell-mediated protective immunity in a variety of viral infections (137). IFN-γ mRNA has been detected in the lymph nodes, lungs and peripheral blood mononuclear cells from PRRSV-infected pigs (117;170). IFN-γ has been shown in vitro to inhibit PRRSV replication in PAMs (19) and MARC-145 cells (170). Pigs immunized with DNA plasmids encoding GP4, GP5, M or N of PRRSV induced virus specific IFN-γ production. In addition, T-cell proliferation was induced by individually purified structural proteins of PRRSV, such as GP5, M and N (20). When recombinant structural proteins (ORFs 2-7) in a vaccinia virus system were used for a T-cell proliferation test, the M protein was reported to be the most potent inducer of T-cell proliferation, followed by GP5, GP3 and GP2 while N protein was the weakest inducer (20). Moreover, co-expression of GP5 and M proteins seems to be more efficient to induce lymphocyte proliferation than expression of single protein as recombinant adenovirus co-expressing GP5 and M proteins produced stronger lymphocyte proliferation in mice than did adenovirus expressing individual protein (86). Collectively, a varying number of T-cell epitopes appears to be present in all membrane-associated proteins including M protein; however, those have not been mapped out yet.

IFN-γ and CTL have important functions in the clearance of persistent PRRSV infection. It has been observed that the primary sites of PRRSV persistence were tonsil, sternal lymph node, and inguinal lymph node, and especially tonsil had an extremely low
number of IFN-\(\gamma\) secreting cells or CD8\(^+\) cells (107;228). Moreover, T-cells isolated from the blood or lymphoid organs of pigs persistently infected with PRRSV were less responsive to mitogens such as concanavalin A (ConA) or phytohemagglutinin (PHA) (107), suggesting the possibility that T-cells are functionally impaired during persistent PRRSV infection. The important role of CTL in the protection against PRRSV infection also has been demonstrated by showing that the majority of T-cells recruited to the lungs, peripheral blood, and bronchoalveolar lavage fluid of surviving piglets infected \textit{in utero} with PRRSV were CD8\(^+\) cells that have the phenotype of CTLs (143;196). However, the action of CTL or IFN-\(\gamma\) secreting cells in PRRSV infection could be inefficient or limited to the clearance of local infection since no correlation was observed between viral load and the recruitment of T-cells in a variety of organs during either acute or persistent infection (228) and temporary depletion of CD8\(^+\) cells with the treatment of anti-CD8 Mab did not exacerbate PRRSV infection (116). To date, viral epitope associated with CTL response is not known. The decrease of \(\gamma\delta\) T-cell populations in a variety of tissues, especially lung and lymph nodes, was reported during PRRSV infection, which was speculated to be due to a low production of proinflammatory cytokines instead of direct viral effect on these T cells (228). The immunological function of \(\gamma\delta\) T-cell is known to be mainly regulatory for inflammatory immune responses and immune response progresses and swine contain a large percentage of \(\gamma\delta\) T-cells in peripheral blood circulation which is capable of responding to various microbiological challenges in both innate and adaptive immune responses (146;228).

Furthermore, it has been shown that \(\gamma\delta\) T-cell produced IFN-\(\gamma\) after PRRSV infection (146). Therefore, the reduction in the number of \(\gamma\delta\) T-cells in lung may contribute to the impaired CMI to PRRSV (228).
Protective function of immune responses to other arteriviruses is variable. The limited function of humoral or cellular immunity in resolving the infection has been reported in LDV infection in mice. LDV also infects macrophages and causes a life-long viremia after acute infection even though the infected mice have an active LDV-specific immune response (both antibody and CMI) (35;199). Furthermore, disruption of acquired humoral or cellular immunity did not affect the level or duration of viremia in LDV-infected mice, suggesting that anti-LDV immune responses may not play a critical role in controlling LDV replication (147). On the other hand, EAV produced a high level of humoral and cell-mediated immunity. Neutralizing antibody is detected 1 to 2 weeks after infection, peaks at 2 to 4 months and persists for years thereafter (14). EAV-specific CTL can be detected for periods of 4 months up to longer than 1 year after infection (32). Although there is little information related to the immune response to SHFV, persistent strains (P-248 and P-741) which were low virulent tended to induce minimal or no antibody response while virulent strains induced a good level of neutralizing antibody within 1 week after infection and viremia was cleared in 3 weeks (77). Genetic variation among SHFV seems to negatively affect the cross neutralization between different strains since neutralizing antibodies against one strain of SHFV do not completely neutralize the other strain. The GP7 (p54) protein encoded by ORF7, the counterpart of GP5 protein of other arteriviruses, is known to contain an important neutralizing determinant of SHFV (14;74).
Role of PRRS viral proteins in the pathogenesis of PRRSV

Apoptosis and cell death

PRRSV induces apoptosis and necrosis in PAMs and MARC-145 cells (133). A number of studies have shown that recombinant GP5 protein produced by a variety of expression systems can induce apoptosis (69;72;185) and that N-terminal 119 residues of GP5 were responsible for the apoptosis (69). However, apoptosis and the role of GP5 in the apoptosis appear to depend on virus strain as no cell death was observed in GP5 protein-expressing stable cells (111). Several studies have demonstrated that PRRSV-infected cells are degraded by necrosis directly due to the viral infection and that apoptosis is observed in bystander cells (95;133;181). Unlike PRRSV, EAV induces apoptosis directly in infected cells (12).

In PRRSV-infected pigs, widespread apoptotic cells were observed in a variety of affected tissues such as testes (186), lungs and lymph nodes (181;187). Similar to in vitro observation, the majority of apoptotic cells were not infected with PRRSV, indicating that PRRSV may not directly induce apoptosis but bystander cells experience apoptosis. It has been suggested that apoptosis observed in pigs after PRRSV infection is likely associated with the production of apoptogenic cytokines such as TNF-α (38;101). It is believed to be beneficial for PRRSV to suppress the apoptotic process in infected cells because such suppression ensures that PRRSV can replicate in the cells without being detected by the host immune system or infect the cell for a longer period of time (i.e., persistent infection). It remains to identify a viral factor inducing such modulation.
**Immunopathology**

Evidence of immunopathogenesis of PRRSV has been demonstrated. A greater increase in the level of serum antibodies (IgG, IgM and IgA) was shown in colostrum-deprived neonatal pigs 2 weeks after inoculation of PRRSV (115). Interestingly, less than 1% of the induced antibody was specific for PRRSV. Sera from the infected pigs appeared to have immune complexes which were disposed on glomerulous, basement membrane and vascular endothelium in the kidneys of infected piglets. Furthermore, autoantibodies specific for Golgi antigens and dsDNA could be detected 3-4 weeks after viral inoculation. These data suggest that PRRSV is able to induce B-cell hyperplasia which leads to immunological injury in colostrum-deprived neonatal pigs.

Consistent with the above observation, enlargement of lymph nodes (2-10 times normal size) is a common gross lesion observed in pigs infected with PRRSV (78). Renal lesions characterized by periglomerular and peritubular lymphohistiocytic aggregates (44;167) or segmental vasculitis in the pelvis and medulla (44) have also been reported. Similarly, LDV infection of mice appears to induce nonspecific polyclonal activation of B-cells. Immune complexes and autoantibodies also are prominent in the sera from LDV-infected mice (27;30;83;168). However, the mechanism for these events has never been studied.

**Cell tropism and virulence**

Modification of certain virus proteins has been postulated to affect the ecology of PRRSV infection. Although it has not been demonstrated in other arteriviruses, the number of glycosylation in the major envelope protein (VP-3P) of LDV could have an affect on cell
tropism of the virus since the strains that lost two N-glycosylation sites in the N-terminal ectodomain of GP5 (N to S or T mutation) became more susceptible to virus neutralization by antibody but obtained the ability to produce a paralytic disease in mice (36). Several cases of encephalitis characterized by segmental cuffing of blood vessels have been reported in pigs infected with PRRSV (79;193), but it remains to be further studied whether those neurological lesions are related to the different modification of viral envelope proteins.

The virulent mechanism of PRRSV has not been elucidated very well. However, it is plausible that PRRSV infection of alveolar macrophages causes massive cell death by necrosis of infected cells and apoptosis of bystander cells (101). In turn, such an event promotes the infiltration of immune cells into the damaged site of the lung resulting in the typical PRRSV-induced lung lesion, i.e., lymphohistiocytic interstitial pneumonia (78). Several chimeric viruses were constructed using a gene swapping technique between a highly virulent PRRSV infectious clone (FL12) and an attenuated vaccine virus (Prime-Pac®, Schering-Plough) to identify the virulent determinants of PRRSV. The generated chimeric viruses were inoculated to pregnant sows at day 90 of gestation to determine the regions responsible for reproductive failure. The study concluded that virulent determinants likely reside in none-structural regions (i.e., Nsp3 to 8) and structural genes (i.e., ORFs 2 and 5) since the substitutions of those regions or ORFs in the virulent infectious clone with those of the vaccine virus resulted in a decrease of the abortion rate (99). However, fine mapping of genetic markers for virulence has not been done.
Evasion of host defense

Several features of PRRSV hamper the effective control of virus infection and influence its clinical consequence. Some are the common characteristics of viral pathogens which can infect and replicate in immune effector cells or macrophages. Those pathogens (for example, lentiviruses and arteriviruses) are often capable of establishing persistent infection, tend to confer weak protective immunity, produce complicated systemic disease or syndrome, and predispose the host to secondary infections. In addition, PRRSV appears to utilize its own unique strategies to elude host defense.

Persistent infection

The persistence of PRRSV results as a “smoldering” infection in which the virus is continuously present at a low level within the animal (i.e., chronic carrier), eventually cleared with time (7,160). The varying duration of PRRSV persistent infection regardless of the route of infection, virus strains or the age of pigs has been documented in a number of studies (3,7,23,24,40,82,172,220,221). For example, Rowland et al. (1999) isolated virus from tonsil and lymph nodes up to 132 days post farrowing and detected viral RNA in one pig at 210 days post farrowing when 3 pregnant sows were inoculated with VR-2332 at 90 days of gestation (172). Wills et al. (1997) isolated virus from one out of 3 pigs inoculated with ISU-P at 4 weeks of age at 157 days after inoculation (221). In addition, virus was isolated from one boar sacrificed at 101 days after inoculation with VR-2332 (40). The huge range of duration of persistence is of great concern as it relates to isolation and acclimation practices in the production system when replacement animals are introduced to a farm as Horter et al. (2002) (82) were able to detect the presence of infectious virus by virus isolation or swine
bioassay in 51 of 59 pigs between 63 and 105 days after inoculation when those pigs were exposed to VR-2332 at 3 weeks of age.

The mechanism of PRRSV persistent infection remains unknown although weak and inefficient immune response to PRRSV should favor the event. Nevertheless, several plausible mechanisms have been proposed by recent studies. As discussed above, PRRSV appears to suppress the apoptosis of infected cells to maximize the viral replication without being detected by immune cells. Miller and Fox (2004) (133) proposed that apoptosis might be suppressed by the same mechanism as that inhibiting the synthesis of type 1 IFN mRNA since numerous previous reports suggested that virus-induced apoptosis and type 1 IFN synthesis are controlled via a common pathway although such a pathway has not been elucidated yet. These investigators also demonstrated that PRRSV infection significantly increased two potent suppressors (ATF-3 and Hsp70 genes) of the apoptotic pathway in MARC-145 cells 24 hours after infection even though the biological role of those genes has not been confirmed in cases of PRRSV infection. In addition, a recent study (45) demonstrated that viral protein was not detected on the plasma membrane of the infected cell. Instead, viral proteins which are not incorporated into PRRSV virion seem to be retained in ER or Golgi complex, which could make the virus infection invisible to the host immune surveillance and favor the persistent infection of PRRSV in infected cells. The study (45) demonstrated in vitro that macrophages infected with PRRSV were protected against antibody-dependent, complement-mediated cell lysis. In PRRSV type 1, one putative ER-retention motif was found in GP2 (LVXXXL, a.a. 23–28) and a putative ER-retention motif and ER lumen-retention motif each in GP3 [LVXXXL (a.a. 19–24) and HDEL (a.a. 87–90), respectively]. Additionally, putative Golgi-retention motif was also identified in GP3 of
PRRSV type 1 (CXXH, a.a. 144–147). In contrast, none of these motifs have been found in PRRSV type 2 yet. Hence, it may be interesting to confirm whether or not those identified retention motifs are indeed responsible for the localization of PRRSV proteins in the ER or Golgi.

**Induction of weak and late protective immunity**

The suboptimal immune response to PRRSV would be a direct consequence of virus infection in macrophages and dendritic cells, which are the major and first defense line of the body (7;160;211). However, several unique features observed during PRRSV infection could explain the inefficient host immune response to PRRSV. Recent studies demonstrated that PRRSV could not only modulate or suppress the host immune response (Immunomodulation) but also efficiently protect the critical region(s) of the virus vulnerable to the antiviral activity of antibody (i.e., glycosylation on NE) or lure the immune response away from the critical region(s) (i.e., decoy epitope).

**Immunomodulation** Viral immunomodulation has been well documented in other viruses; three major pathways to modulate the immune response have been identified in those viruses (62;73). First, viral pathogens, such as herpesvirus, adenovirus, and human immunodeficiency virus, produce viral proteins which can interfere with antigen presentation via the retention and/or internalization of MHC class I molecules in ER or Golgi complex or into the cytoplasm, respectively (1;21;154). Similar to this, the absence of PRRS viral proteins on the plasma membrane of infected cells is suspected to be attributed to the retention of individual viral proteins in ER or Golgi since there is no evidence that the viral
proteins are internalized into the cytoplasm when PRRSV-infected macrophages or MARC-145 cells were monitored by confocal microscopy over time after incubated with PRRSV-specific monoclonal or polyclonal antibodies (45). However, it is not known whether PRRSV modulates the surface expression of MHC molecules during the infection like other viruses.

Second, herpesvirus, poxvirus, and vaccinia virus modulate the host immune response by: 1) producing homologous viral proteins of host cytokines, i.e., mimicry (96;135); 2) producing specific viral proteins which bind to host cytokines or cellular cytokine-receptors (5;6;125;207); or 3) producing inhibitor molecules against chemokines (25;150). It is not known if PRRSV produces such viral proteins as described above, but the virus induces a significant level of IL-10, which inhibits macrophage activation and antigen presentation, the production of proinflammatory cytokines (IL-1 and TNF-α), and the synthesis of IFN-γ (41;68;188;189). Therefore, it is logical to assume that the induction of IL-10 production may be one of the strategies which PRRSV utilizes to modulate the host immune response.(188;189). Specific PRRS viral gene or protein involving upregulation of IL-10 has not been elucidated yet.

Third, vaccinia virus encodes a potent complement inhibitor which is referred to as vaccinia complement-control protein (VCP). This inhibitor promotes Factor I cleavage of C3b and C4b, which disrupts both the classical and alternative complement pathways (126;174). No information related to complement is available in PRRSV infection although exogenous complement was demonstrated in vitro to enhance virus neutralizing activity of sera from infected pigs.
**Heavy glycosylation of viral protein**  
GP5 of PRRSV contains a short N-terminal ectodomain (about 30 a.a.) which is heavily glycosylated and antigenically conserve. Most of the PRRS viruses contain 2 to 5 putative glycosylation sites in ORF5. Ansari et al. (2006) (11) have demonstrated that all putative sites were used for glycosylation. It has been speculated that the neutralizing epitope in the N-terminal ectodomain of GP5 would not be readily recognized by immune cells as heavy glycosylation can mask the epitope. When those glycosylation sites were removed by substituting asparagine (N) with alanine (A), mutants induced neutralizing antibody to a higher level than did a wild-type virus in challenged pigs (11). A similar result was demonstrated by other investigators (64). It remains to be further studied though if the removal of glycosylation from the GP5 NE of PRRSV increases the titer of serum virus neutralizing antibody to various PRRSV strains which antigenically vary when they maintain their native form (i.e., glycosylated protein).

**Decoy epitopes**  
Antigenic determinants in the N protein which induce abundant non-neutralizing antibody have been suspected to lure the immune response away from neutralizing epitopes. This mechanism is believed to be responsible for the weak and delayed induction of neutralizing antibody. In general, immunization of mice with intact PRRSV to generate Mabs ends up with N protein-specific Mabs since the predominant immune response is directed to the N protein (230). Yang et al (2000) (229) have successfully generated Mabs specific for GP3, GP5, and M by adopting an immunotolerization procedure (e.g., repeated treatment of cyclophosphamide) after the first immunization followed by the second injection of adjuvanted viral antigens (229). Ostrowski et al (2002) (149) postulated the presence of a decoy epitope in the N-terminal
ectodomain of GP5 of PRRSV type 2 (epitope A, 27-30) as the epitope was highly immunogenic and induced early non-neutralizing antibody under experimental conditions. When the Pan DR T-helper cell epitope (PADRE) was inserted between the NE and the decoy epitope in ORF5, the decoy effect of the non-neutralizing epitope was minimized and the immunogenicity of NE increased. As a result, the ORF5 DNA vaccine with such a modification induced a significantly higher level of VN antibody in the vaccinated mice than did naïve ORF5 (65). Although the advantage and validity of those approaches needs to be proven in pigs with virus challenge, those previous studies showed the possible benefit of directing the immune response toward the inefficient NE.

**Genetic and antigenic variability**

The existence of remarkable genetic and antigenic variability among PRRS viruses has been shown in numerous studies (10;90;229;230;235). The Nsp2, GP3 and GP5 have been reported as the most variable regions of the viral genome (8;51;166). Many studies have demonstrated that those genetic and antigenic diversities negatively affected the efficient cross protection among viruses (10;100;128;153). Similar field observations had been made prior to laboratory experiments, showing that pigs previously exposed to a PRRS virus can be re-infected if the incoming virus was significantly different (“heterologous”) from the previous virus (104). Since the linear NE identified in GP5 of PRRSV (149;161) is much conserved among both types of PRRSV, other determinants in GP5 need to be defined to explain the mechanism of the negative effect of genetic variation on the cross protection among PRRS viruses (158;159). Furthermore, since recent evidence (219;222) has revealed the critical involvement of minor envelope proteins (GP2, GP3, GP4) and M proteins in the
interaction with target cells and in protection against virus infection, the effect of genetic variation in ORFs corresponding to those proteins needs to be assessed (61;209).

**Antibody-dependent enhancement (ADE) of virus infection**

In general, virus-specific antibody executes or mediates antiviral activities. However, for some viruses, the presence of suboptimal neutralizing antibody or abundant non-neutralizing antibody from previous infection or of maternal origin in blood circulation can enhance virus infection to target cells, particularly immune cells via Fc-receptor mediated endocytosis (191;232). This phenomenon is known as ADE of virus infection. The clinical significance of ADE is exacerbating the severity of clinical manifestation in infected individuals (123).

For PRRSV, it has been shown that the presence of abundant non-neutralizing antibody induced after virus infection could be a benefit for the virus entry into the target cells such as macrophages, resulting in higher infection rates of the target cells and higher yield of progeny virus (51;179;232;233). It was postulated that viral proteins responsible for inducing ADE-mediating antibodies are N and GP5 proteins since PRRSV infection to PAMs was enhanced when the virus was treated with the Mabs generated against some epitopes on the N and GP5 proteins (31). However, such epitopes have not been mapped out yet. Hence, it would be of interest to identify epitopes related to ADE and study if the reduction of immunogenicity of those epitopes could prevent the ADE effect of PRRSV *in vitro* and *in vivo*. Furthermore, those epitopes could be removed from a vaccine virus by genetic engineering so that the vaccine does not induce any ADE-mediating antibody and yet still induce neutralizing antibody.
Conclusions

Many actual or potential biological functions of genes and proteins of PRRSV during its life-cycle have been unveiled or postulated since this virus was first recognized in the late 1980. As discussed in this paper, understanding the role of viral genes or proteins in entry, replication, assembly, and release from infected cells during PRRSV infection starts to increase although there is much more to add, prove and confirm. Other arteriviruses share with PRRSV many common features in those steps even though they have somewhat different genomic size, organization and identity. However, the immunobiology and pathogenesis of PRRSV and its infection still remain unclear. The unique features of PRRSV, such as inefficient induction of protective immunity, persistent infection, variable virulence, and strain-specific virus neutralization, have become obstacles to the effective prevention and control of PRRS. Since both inactivated and modified live vaccines that have been generated based on the conventional knowledge do not appear to be as effective as they should be in the control of virus infection, future studies need to be focused on those obstacles to build a new prevention and control strategy.
CHAPTER 2: EFFECT OF GENOTYPIC AND BIOTYPIC DIFFERENCES AMONG PRRS VIRUSES ON THE SEROLOGIC ASSESSMENT OF PIGS FOR VIRUS INFECTION

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Abstract

Genetic, antigenic, and pathogenic variability is known to exist among PRRS viruses and has garnered great attention for diagnostics and disease control/prevention. A comparative serologic study was conducted on five field and two cell-attenuated viruses to determine if serologic responses to PRRS virus infection could be influenced by biotypic and/or genotypic differences of the viruses. The isolates used for the study varied in their virulence to pigs and in genomic sequences. Ten pigs were inoculated with each isolate (1 x 10^3 TCID_{50}) via the intranasal route. All inoculated animals became viremic during the study period. Some animals inoculated with the attenuated viruses remained seronegative until the end of the study (42 days PI), but all of the animals inoculated with field viruses developed ELISA- and IFA-detectable antibodies, regardless of the virus strain used in the IFA assay. In contrast, a great degree of variation in the onset and level of SVN antibody was observed by individual pigs and by each virus. The reactivity of SVN antibody was highly specific for homologous viruses. Cross neutralization titers were better correlated with sequence
homology of ORFs 4 and 5 among the viruses than any other structural genes. We conclude that the biotypic difference among PRRS viruses may affect the kinetic of humoral immune response in infected pigs. The IFA test may be used as a confirmatory test when a false-positive ELISA result is suspected or vise-a-versa, but SVN antibody titers are highly affected by antigenic variability.

**Introduction**

Porcine reproductive and respiratory syndrome (PRRS) continues to impose a significant economic burden on the swine industry worldwide. In the United States, the economic impact of PRRS to swine producers has been estimated to be approximately $560 million in loss per year (Neumann et al., 2005). Because of its economic significance, a great deal of resource has been invested in developing effective prevention and control strategies but protocols providing consistent success have been elusive.

The syndrome is caused by porcine arteriviruses, PRRS virus, a small enveloped RNA virus belonging to the family *Arteriviridae*. The virus has several distinct biological characteristics, including preferential replication in macrophage-lineage cells (Wensvoort et al., 1991), the ability to establish persistent infection (Wills et al., 1997; Horter et al., 2002), a high degree of genetic variation (Kapur et al., 1996; Andreyev et al., 1997), a low infectious dose (Yoon et al., 1999; Hermann et al., 2005), variability in virulence (Halbur et al., 1996), and shedding in boar semen (Yaeger et al., 1993; Swenson et al., 1994) and milk (Wagstrom et al., 2001).

All of these are obstacles to the effective control and prevention of PRRS, but the high rate of genetic change and resulting antigenic variability has been of particular
diagnostic concern because genotypic and phenotypic variations can interfere with the accuracy of diagnostic tests (Christopher-Hennings et al., 1995; Yoon et al., 1995a; Yang et al., 1999; 2000). The detrimental effect of antigenic diversity on the serodiagnosis of PRRS virus infection has been documented between two extreme genotypes of PRRS virus (i.e., European and North American types); viruses that share less than 70% nucleic acid homology in their entire genome (Wensvoort et al., 1992; Murtaugh et al., 1995). However, the effect of lesser degrees of genotypic heterology on the performance of PRRS serology has not been investigated. Thus, the following study was conducted to determine if serologic data and the performance of serologic assays may be influenced by genotypic and/or biotypic differences of PRRS viruses and, if so, to assess the degree of effect.

**Materials and Methods**

**Viruses, cells and media.** Seven PRRS viruses were used for the study. All challenge viruses replicated readily on MARC-145 cells, producing varying titers of progeny viruses. All viruses were prepared and treated in the identical manner. The virus inoculums represented 1 to 5 passages on cell culture, with the exception of the registered vaccine seed viruses (i.e., Ingelvac PRRS™, Ingelvac ATP™). The designation, origin and biological characteristic of each virus are summarized in Table 1 and have been previously described (Johnson et al., 2004). Genomic sequences (ORFs 2 to 7) of the all viruses had been deposited to GeneBank (accession # EF442771 to EF442777).

**Animal trial.** The animal study was conducted as part of a previous study (Johnson et al., 2004). The study consisted of a total of nine treatment groups (i.e., 7 different viruses,
a pool including all viruses, and one negative control group). Three- to four-week-old specific pathogen-free pigs were purchased from a commercial herd known to be free of PRRS virus and housed in an animal facility at the compliance of Biosafety level 2. The animals were allowed ad libidum access to feed and water. Ten pigs each were randomly selected and assigned to each treatment. Each group was intranasally inoculated with the corresponding PRRS virus at a titer of approximately $1 \times 10^3$ TCID$_{50}$/ml in Eagle’s Minimum Essential Medium (EMEM, JRH Bioscience, Lenexa, KS) supplemented with 4% fetal calf serum (FCS, JRH Bioscience). A low dose of the virus was given to the animals to mimic perceived field conditions and has been proven to be as effective as a high dose challenge for stimulating the immune response (Yoon et al., 1999). Each pig received 2 ml of the virus inoculum. The sham-inoculated control group was given 2ml of EMEM supplemented with 4% FCS. After inoculation, the animals were monitored for overt clinical signs throughout the study period. Serum samples for virologic and/or serologic assays were collected from each pig on days 0, 1, 3, 7, and every 7 days thereafter until day 42 post inoculation (PI). All tested sera were heat-inactivated for 45 min at 56° C.

**Indirect fluorescent antibody (IFA) test.** Antigens of each virus were prepared by inoculating MARC-145 cell monolayers in alternate rows of 96-well plates with 100 µl/well of the virus at the titer of $10^4$ TCID$_{50}$/ml and fixing infected cells with cold acetone-ethanol (8:2) mixture after a 20-hour incubation at 37° C. Uninfected cell monolayers used as cell control antigens were prepared in the identical manner using virus-free cell culture medium. All plates were dried and stored at -20° C until use. The presence of PRRS viral antigen in each plate was confirmed by immunofluorescence microscopy using PRRS virus-
specific monoclonal antibody, SDOW17 (Nelson et al., 1993) labeled with FITC (Rural Technologies, Brookings, SD, USA).

For testing, a series of 2-fold dilutions was made for each serum sample with 0.01M phosphate-buffered saline (PBS, pH 7.2). Fifty µl of each diluted sample was applied to each pair of 2 wells containing viral or cellular antigens and incubated for 1 hour at 37° C. After the samples were washed off with 0.01M PBS (pH 7.2), the antigen-antibody reaction was visualized by applying optimally-diluted goat anti-swine IgG antibody conjugated with FITC (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) and incubating for another hour at 37° C. Each plate was observed under a fluorescent microscope. The PRRS virus-specific IFA antibody titer of each serum was determined as the reciprocal of the highest dilution in which specific fluorescence was detected.

**Serum-virus neutralization (SVN) test.** All tested sera were heat-inactivated for 45 min at 56° C before testing. Each serum was then diluted using a 2-fold serial dilution technique in RPMI-1640 (Sigma, St. Louis, MO, USA) supplemented with 10% FCS (Sigma), 20 mM L-glutamine (Cellgrow, Herdon, VA, USA), and an antibiotic-antimycotic mixture (Sigma) which consisted of 100 IU/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamicin and 0.25 mg/ml amphotericin B (hereafter, RPMI growth medium). One hundred µl of each diluted sample was mixed with an equal volume of each virus at a rate of 10³ TCID₅₀/ml. Mixtures were incubated for 1 hour at 37° C and then each mixture was inoculated onto MARC-145 cell monolayers prepared in 96-well plates 24 hours earlier. Each sample was run in duplicate. After a 1 hour incubation at 37° C, all inoculums were removed and replaced with 200 µl of RPMI growth medium. Thereafter, cells were incubated at 37° C and monitored daily for cytopathic effect (CPE). The titer of inoculated
virus was verified by the back titration of the inoculum. The presence of virus-specific CPE in each well was recorded after incubating for 5 days. The presence of virus in wells without CPE was further determined by immunofluorescence microscopy using SDOW17-FITC conjugate. The SVN titer of each serum was determined as the reciprocal of the highest dilution in which no evidence of virus growth was detected.

**Enzyme-linked immunosorbent assay (ELISA).** Serum samples were assayed by ELISA (IDEXX HerdChek® PRRS 2XR, Westbrook, Maine, USA) following the procedures recommended by the manufacturer. Samples with S/P ratios ≥0.4 were considered positive for antibody against PRRS virus.

**Virus assays.** Two cell types were used for virus titration: MARC-145, a clone of the African Monkey kidney cell line MA-104 (Kim et al., 1993) and porcine pulmonary alveolar macrophages (PAMs). PAMs were collected from 3- to 4-week-old pigs by lung lavage technique (Yoon et al., 1996) and stored at -80° C until used. Both cells were cultured in RPMI growth medium. For virus titration, 10-fold dilutions were prepared in RPMI growth media for each virus. One hundred µl of each diluted sample was inoculated onto MARC-145 cell monolayers and PAMs which had been prepared in 96-well plates 24 hours earlier. Each sample was run in triplicate. After a 1 hour absorption, all inoculums were removed and cells were replenished with RMPI growth medium, then incubated at 37° C in a humid 5% CO₂ atmosphere. Cells were observed for CPE daily. At day 5 PI, the presence of CPE and/or viral antigen was evaluated by immunofluorescence microscopy for each well. The virus titer was calculated using the Reed and Muench method (Reed and Muench, 1938) and expressed as TCID₅₀ per ml.
Virus titers were also measured by a quantitative fluorogenic reverse transcription-polymerase chain reaction (real-time RT-PCR) using TaqMan® chemistry. Using sequences deposited in GenBank, virus-specific oligonucleotide primers and a fluorescent probe were engineered to target a highly-conserved region of PRRS virus ORF6. The forward primer (ORF6F: 5’-GCCATAGAAACCTGGAAATTCATC-3’) and reverse primer (ORF6R: 5’-GCGGCCTAGCAAGCACA-3’) were synthesized by a commercial vendor (Integrated DNA Technologies, Coralville, IA, USA). An TaqMan® MGB probe with a 5’ reporter 6-carboxyfluorescein (FAM) and a 3’ non-fluorescent quencher (ORF6P: 5’-6FAM-CCTCCAGATGCGG) was synthesized by Applied Biosystems (Foster City, CA, USA). To conduct the assay, viral RNA was first extracted from 140 µl of serum using the QIAamp® viral RNA mini kit (Qiagen, Valencia, CA, USA). Real-time RT-PCR was then carried out with the QuantiTect® Probe RT-PCR Kit (Qiagen) in a 25 µl reaction volume using 2.5 µl of extracted template. Primers were added at a final concentration of 0.4 µM each and the probe was at a final concentration of 0.2 µM. The PCR amplification was performed on the ABI 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Cycling conditions were as follows: a) reverse transcription for 30 min at 50° C; b) a 15 min activation step at 95° C, and c) 35 cycles of 15 sec at 94° C and 60 sec at 60° C. A set of PRRS virus preparations, each with a known virus titer, were used to generate a standard curve. Samples with threshold cycles (Ct) ≤35 were considered positive. The amount of PRRS virus in each sample was calculated by converting the Ct value to a virus titer using the standard curve.

**Sequencing.** All viruses were sequenced for open-reading frames (ORFs) 2 to 7. Viral RNA was extracted using QIAamp® viral RNA mini kit (Qiagen) according to the
manufacturer’s recommendation. Each target gene was then amplified using QIAGEN® One-Step RT-PCR kit (Qiagen). PCR products were purified using QIAquick® PCR purification kit (Qiagen) and submitted to the Iowa State University Nucleic Acid Facility for sequencing. PCR and sequencing primers were as follows:

- **ORF2 to 4 Forward (P2-4):** 5’-AAACGGGTGAGGACTGGGAGGATTA-3’;
- **ORF 2 to 4 Reverse (P2-4):** 5’-CAAAACAGAACGGCAGCATCACACC-3’;
- **ORF5 Forward (P5F):** 5’-CCTGAGACCATGAGG-3’;
- **ORF5 Reverse (P5R):** 5’-TTTAGGGCATATATCATTACACTGG-3’;
- **ORF6 Forward (P6F):** 5’-GCCCTAGCTGCTGCTAG-3’;
- **ORF6 Reverse (P6R):** 5’-GGCTGGCCATCCCCCTTTCTTG-3’;
- **ORF7 Forward (P7F):** 5’-TCGTGTTGGGTGG-3’; and
- **ORF7 Reverse (P7R):** 5’-GCCATTCACCACACCTTCCCTT-3’.

**Data analysis.** Sequence data were aligned and homologies evaluated using Lasergene™ (DNASTAR Inc., Madison, WI, USA) software. Analysis of variance (ANOVA) was used to compare the serologic responses of pigs within and among treatments. Least squares means Tukey HSD (Dawson-Sanders and Trapp, 2001) was used to evaluate the level of serum antibody against PRRS viruses. Correlation between antibody responses and sequence homology was assessed. All statistical analysis was conducted by using JMP (SAS Institute Inc., Cary, NC, USA).

**Results**

**Genetic comparison of challenge viruses.** Relative to VR-2332, the amino acid homologies of ORFs 2 to 6 of the other 6 PRRS viruses ranged from 84.1 to 100 % (Table 2).
ORFs 3 and 5 were the most variable and ORFs 6 and 7 were the most highly conserved among the viruses. Based on ORFs 2 to 7, the overall amino acid sequence divergence of cell-attenuated viruses from their parental field strains was less than 3%. Phylogenetically, each of the field viruses formed its own branch whereas cell-attenuated viruses branched along with their parental strains (Fig. 1). Overall the MN184 isolate was the most divergent from VR-2332 and the other viruses used.

**Antibody responses of pigs to infection.** All of the inoculated animals became viremic by 3 days PI (Johnson et al., 2004), demonstrating that the animals were infected with the virus. However, some of the animals inoculated with attenuated viruses were negative for anti-PRRS virus antibodies in ELISA by the end of the study (day 42 PI). Two and 4 out of 10 each inoculated with MLV and ATP, respectively, remained seronegative, probably because of the challenge dose under the manufacturer’s recommendation and poor replication of vaccine viruses in the pigs. All of the other challenged animals seroconverted to PRRS virus as determined by ELISA. The animals inoculated with wild-type PRRS viruses developed earlier and higher levels of ELISA-detectable antibody than did pigs inoculated with attenuated viruses based on S/P ratio (Johnson et al., 2004). The level of antibody induced by wild type viruses was still higher than those by attenuated viruses even at 42 PI ($p < 0.0167$) even though antibody responses to wild type viruses were at the declining phase and those to attenuated viruses were still increasing (Fig. 2). Pigs inoculated with MN 184 produced the highest level of antibody detectable by ELISA during all sampling time points and S/P ratios ranged from 1.84 to 3.98 at 42 PI. On average, the S/P ratios in pigs inoculated with other wild-type PRRS viruses ranged between 1.3 and 2.8,
while pigs inoculated with attenuated PRRS viruses showed the ELISA S/P ratios between 1.1 and 1.2 at 42 PI.

IFA testing demonstrated the pattern of antibody response to PRRS virus infection similar to that revealed by ELISA. Pigs that were seronegative by ELISA for PRRS virus also remained seronegative by IFA test at day 42 PI when the study was terminated. All other animals were seropositive by IFA test for PRRS virus at day 42 PI regardless of the virus strain used in the test (Fig. 3). In addition, when sera collected at day 7, 15 and 28 PI from the pigs challenged with VR-2332 were tested by the IFA test using various viruses as viral antigen, similar level of PRRS virus-specific antibody was detected at all sampling points (Fig. 4). As observed in ELISA, the IFA titers of the pigs inoculated with attenuated viruses were significantly ($p < 0.0001$) lower than those of the pigs inoculated with wild type viruses at 42 PI (Fig. 3). The pigs inoculated with wild-type PRRS viruses produced IFA titers of 1:640 or higher while those inoculated with attenuated viruses did between 1:40 and 1:1280.

Since it has been generally demonstrated that SVN antibody starts to be detected at 3 to 4 weeks after a PRRS virus challenge and it persists for long periods, up to 5 months (Murtaugh et al, 2003; Lopez and Osorio, 2004; Diaz et al, 2005), the SVN test was done on sera collected at day 0 and 42 PI. There was a great degree of variation in the induction of SVN antibody by individual pig and by each virus regardless of virus types, i.e. wild or attenuated (Fig. 5). Among pigs inoculated with wild-type PRRS viruses, all pigs inoculated with JA142 and SDSU73 produced relatively high levels of SVN antibody (1:8 to 1:256) against homologous virus, whereas only 4, 1, and 6 of 10 pigs inoculated with VR2332, MN184 and 17198-6, respectively, developed SVN antibody ranging between 1:8 to 1:32,
against homologous virus at day 42 PI, although these viruses induced similar or even higher level of ELISA and IFA antibodies than the other 2 viruses (JA142 and SDSU73) in all seroconverted pigs. Among pigs inoculated with attenuated viruses, most of the ATP-inoculated pigs (n=6), which seroconverted to the virus as determined by ELISA and IFA, developed SVN antibody (1:8 to 1:32) against the homologous virus. In contrast, no SVN antibody was detectable in any of the sera collected at day 42 PI from pigs inoculated with MLV.

**Cross serologic reactivity.** ELISA and IFA antibody titers were not affected by genetic variation among the challenge viruses. Regardless of the implied antigenic difference among the viruses based on deduced amino acid sequences of ORF5, a relatively high and constant level of virus-specific antibody which cross reacted with the homologous and heterologous viruses was detected in pig sera collected at day 42 PI by the IFA test as well as the commercial ELISA (Fig. 2 and 3). A similar observation was also made on sera collected from pigs at days 7, 15 and 28 PI by the IFA test and at all sampling points by the ELISA (Johnson et al., 2004). Yet, the IFA test appeared to be more sensitive than ELISA since IFA titers of day 7 PI sera which were negative by ELISA ranged from 1:10 to 1:80 (Fig. 4).

In contrast, the SVN antibody titers of pig sera were affected by genetic diversity among the challenge viruses. Significant virus neutralizing activity of sera was apparent only against the homologous or related viruses although a low level of cross neutralization was observed between a few viruses (Fig. 5). It should be noted, however, that the SVN antibody against the ATP strain could not cross-neutralize its parental strain JA142 while the SVN antibody against JA142 effectively cross-neutralized ATP even though these viruses were
perceived antigenically related based on the obvious parent-progeny relationship and high 
degree of deduced amino acid homology. The same comparison could not be made though 
for the pair of VR-2332 and MLV strains since MLV-inoculated pigs did not develop SVN 
antibody by day 42 PI. Sera collected at day 42 PI from pigs inoculated with the pooled 
virus showed broad and uniform SVN antibody titers against all the challenge viruses.

**Relationship between serologic profiling and genetic variation among PRRS**

viruses. In general, there was no significant correlation between IFA titers against the 
homologous and heterologous viruses and deduced amino acid sequence similarity among 
the challenge viruses. Even so, the IFA titers were significantly higher when the sera from 
pigs inoculated with JA142, ATP, SDSU 73 or 17198-6 were tested against these four 
viruses, especially JA142 and 17198-6, compared to the rest of the viruses (Fig. 3). In 
sequence analyses, these four viruses showed a closer phylogenetic relationship among 
their own for ORF3 and then ORF4 amino acid sequences than to other viruses. Hence, 
some degree of correlation ($r^2 = 0.63$ and 0.54, respectively) between IFA titer and amino 
acid sequence homology of ORFs 3 and 4 was observed.

In comparison, a good correlation was observed between SVN titers to the 
homologous and heterologous viruses and amino acid sequence homology of ORFs 2 to 5 
among the challenge viruses. Amino acid sequence homologies of ORFs 4 and 5 among the 
viruses showed a higher correlation ($r^2=0.69$ and 0.68, respectively) with the degree of cross 
neutralization than did amino acid sequence homologies of ORFs 2 and 3. In contrast, no 
significant correlation was observed between the degree of cross neutralization and amino 
acid sequence homology of ORFs 6 and 7 among the viruses.
In vitro replication characteristic of PRRS viruses. Both wild type and attenuated PRRS viruses replicated equally well in the MARC-145 cell line. However, wild-type PRRS viruses tended to replicate better in PAMs than attenuated viruses such as MLV and ATP strains (Table 3). Wild-type PRRS virus, MN184, which induced the highest level of viremia (Johnson et al., 2004) and ELISA antibody, showed significantly better growth in PAMs than MARC-145 cells, whereas attenuated viruses, MLV and ATP, replicated to a significantly higher titer in MARC-145 cells than PAMs. In each case, the virus titer assessed by the quantitative real-time RT-PCR was closer to the higher titer of each virus measured by cell culture-based infectivity assay regardless of cell type.

Discussion

Serology is an important tool for assessing the infection/exposure or immune status of animals for a virus, which in turn plays a critical role in controlling the disease. However, the accuracy of serologic profiling can be affected by the antigenic differences between the virus infecting the animals and the virus used in the test. Since remarkable genetic and antigenic variability among PRRS viruses has been shown in numerous studies (Andreyev et al., 1997; Christopher-Hennings et al., 1995; Kapur et al., 1996; Yang et al., 1999; 2000; Yoon et al., 1995a), this study was conducted to evaluate the relationship between genetic variation among PRRS viruses and serologic assessment of antibody responses of pigs to those viruses after infection.

The ELISA kit that is commercially manufactured by IDEXX Laboratories has been most widely used to monitor the exposure of pigs to PRRS virus in North America (Yoon et
Test results (i.e., S/P ratios) are considered to correlate with the level of virus-specific antibody in individual animals (Yoon et al., 1995b). As the nucleocapsid protein is believed to be the antigenic basis for the test, ELISA has been perceived to be highly sensitive in detecting pigs exposed to PRRS virus since the nucleocapsid protein (ORF7 product) of PRRS virus is antigenically the most conserved among PRRS viruses of both North American and European types (Murtaugh et al., 1995). Our observations that most of the virus-infected pigs became seropositive by ELISA for PRRS virus by day 21 PI, regardless of the challenge virus, supports such a perception even though several pigs inoculated with a relatively low titer (i.e., $10^3$ TCID$_{50}$/ml) of cell-attenuated viruses remained seronegative at termination of the study (day 42 PI).

Although ELISA provides several merits such as built-in quality control by the manufacturer, high throughput and broad spectrum of detection, the occurrence of suspect false positive results has been reported and become a challenge to both diagnosticians and practicing veterinarians for interpretation of the results and confirmation (Horter et al., 2002; Keays et al., 2002; O’Connor et al., 2002; Torremorell et al., 2002). In the present study, the IFA test showed the diagnostic performance similar to ELISA on samples collected during the study period with respect to detecting animals seropositive for various antigenically distinct PRRS viruses. It is not surprising that the IFA test diagnostically performs similar to ELISA since the IFA test can detect antibody to the nucleocapsid as does ELISA, and a previous study in our laboratory revealed a similar kinetics of IFA- and ELISA-detectable antibody development in pigs experimentally infected with a PRRS virus (Yoon et al., 1995b). However, such a broad spectrum of detection capability of the IFA test is somewhat contradictory to the general perception that results of the IFA test would be variable.
depending upon the PRRS virus used in the test as compared to one infecting the animals (Christopher-Hennings et al., 2002; Yoon et al., 2003). Observations in this study clearly indicate that the IFA test using a strain of PRRS virus can detect pigs exposed to antigenically distinct PRRS viruses and is able to detect the exposed animals earlier than ELISA. Therefore, the IFA test may be a good alternative test for confirmation when ELISA results are questionable.

One of the drawbacks of both the IFA test and ELISA is that these tests cannot differentiate functional antibodies, such as neutralizing or complement-fixation antibody, from the general population of virus-specific antibodies. Detection of virus neutralizing antibody is important to estimate the immune protection of animals for PRRS as efficient protection from infection and/or disease by only virus-specific antibody was demonstrated (Yoon et al., 1996; Osorio et al., 2002). SVN antibody to PRRS virus has been reported to be strain-specific so that serum antibody effectively neutralizes only antigenically homologous viruses (Yoon et al., 1997). Consistent with the previous report, three features of SVN antibody response were observed in this study.

One, results of the cross SVN test was greatly affected by genetic variation among the challenge viruses. SVN antibody was efficacious only for the homologous or its attenuated virus. Yet, it is interesting to note that there was only one-way cross neutralization between the cell-attenuated (ATP) and the parental strain (JA142). That is, sera produced by JA142 can abrogate the infection of ATP to MARC-145 cells but not the other way around even though these 2 viruses show less than 3% difference in deduced amino acid sequence homologies of ORFs 2 to 7. Although this could be attributed to a relatively low SVN
antibody production against cell-attenuated viruses (Fig. 5), it remains to be further studied why antibody against ATP was so inefficient in neutralizing JA142.

Two, the cross neutralizing activity of serum antibody was found to correlate with amino acid sequence homology of genes encoding for the envelope-associated proteins (e.g., ORFs 2 to 5) among PRRS viruses than genes for internal proteins (i.e., ORFs 6 and 7). The degree of cross neutralization between 2 PRRS viruses could be better predicted based on the amino acid sequence homology of ORFs 4 and 5. These observations are in agreement with previous reports of the presence of neutralizing epitopes in these gene products (Meulenberg et al., 1997, Pirzadeh and Dea, 1997, van Nieuwstadt et al., 1996; Yang et al., 2000, Yoon et al., 1995b). It is interesting though that no amino acid substitution was observed in an antigenic domain identified in ORF5 which has been postulated to be the major neutralizing epitope (Ostrowski et al., 2002; Plagemann et al., 2002). This might explain why a low degree of cross neutralization was observed among different PRRS viruses in this study. Therefore, other epitope(s) antigenically divergent among viruses should be present and involved in the virus neutralization process since significant differences in cross neutralization were observed in this study.

Three, the level of SVN antibody produced was more affected by virus strain and host rather than by virus biotype (i.e. virulent or avirulent). Among virulent viruses, JA142 and SDSU 73 induced a high level of SVN antibody whereas VR2332, MN184 and 17198-6 induced no to a low level of SVN antibody against each homologous virus. These results are unexpected because those three wild-type viruses produced a similar or higher level of ELISA and IFA antibodies when compared to JA142 and SDSU73. Furthermore, while ATP, attenuated virus of JA142, induced even a higher level of SVN antibody than those
three wild-type viruses, MLV did not induce any neutralizing antibody even against the homologous virus. It should be noted, however, that the animals in this study did not receive the full licensed immunization dose of either of these vaccine strains; hence, the lack of SVN antibody production in these animals does not mean the inability of the vaccine viruses to produce neutralizing antibody in vaccinated pigs.

Some of the pigs inoculated with cell-attenuated viruses (MLV or ATP) remained seronegative throughout the study while all those inoculated with field viruses seroconverted. Apparently the presence of non-seroconverted animals in the groups infected with the cell-attenuated viruses negatively affected the average antibody level determined by ELISA, IFA or SVN tests. In turn, it appeared that wild type viruses induced a higher level of antibody response than attenuated viruses did. Nonetheless, the lack of seroconversion in some of the pigs inoculated with attenuated PRRS viruses, even though viremia was apparent, raises the question of whether or not biotypic difference influences replication behavior of the virus and antigen presentation to the immune system. In support, our study demonstrated that cell-attenuated viruses replicated better in MARC-145 cells whereas field viruses tended to replicate better in PAMs, natural host cells, or replicated similarly in both cell types (Table 3). As the challenge dose of each virus was adjusted based on virus titer (TCID$_{50}$/ml) measured in MARC-145, it could be speculated that the pigs inoculated with field viruses might have been exposed to a higher level of PRRS virus than the pigs inoculated with attenuated viruses so that the pigs exposed to the attenuated viruses developed a lower level of antibody response. However, since a previous study reported that the level of viremia and antibody response is independent of challenge dose and route (Yoon et al., 1999), other
reasons may also account for inferior antibody response of pigs to the attenuated virus, which remains to be determined.

Knowing that a high degree of genetic, antigenic and pathogenic variability exists among PRRS viruses, it is logical to assume that a monovalent vaccine might not be able to provide vaccinated animals with the immune protection against a variety of PRRS viruses. In this study, pigs inoculated with the virus pool produced a broad range of IFA and SVN antibodies against different viruses. It is worthwhile to note that JA142, ATP and SDSU73 that induced higher SVN antibody in individually challenged pigs still raised a higher level of SVN antibody in the pigs challenged with a pooled virus when compared to the rest of the viruses (Fig.5), indicating that the induction of SVN antibody may be associated with virus strain rather than the biotype or replication rate of virus in the pigs. The induction of broad ranged SVN antibodies by inoculation with a pool of multiple strains would be an intriguing idea for the development of better efficacious vaccines. However, care must be taken in the selection of strains since: a) different PRRS viruses vary in their replication ability and fitness (Johnson et al., 2004) and capability of inducing immunity as demonstrated in this study; b) more severe clinical signs were produced in pigs by injection of a multi-strain attenuated vaccine (Mengeling et al., 2003); and c) sub-optimal level of antibody could favor the viral infection (Yoon et al., 1997).
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### Table 1. Designation and biotypes of PRRS viruses used for study

<table>
<thead>
<tr>
<th>Name</th>
<th>Year of Isolation</th>
<th>Origin</th>
<th>Virulence</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>VR2332</td>
<td>1991</td>
<td>Minnesota</td>
<td>Moderate</td>
<td>Parental strain of MLV</td>
</tr>
<tr>
<td>MLV</td>
<td>N/A(^a)</td>
<td>N/A</td>
<td>Avirulent</td>
<td>Licensed vaccine seed (Ingelvac PRRS(^{™}))</td>
</tr>
<tr>
<td>JA142</td>
<td>1997</td>
<td>Iowa</td>
<td>High</td>
<td>Parental strain of ATP</td>
</tr>
<tr>
<td>ATP</td>
<td>N/A</td>
<td>N/A</td>
<td>Avirulent</td>
<td>Licensed vaccine seed (Ingelvac ATP(^{™}))</td>
</tr>
<tr>
<td>SDSU73</td>
<td>1996</td>
<td>Iowa</td>
<td>High</td>
<td>Field isolate</td>
</tr>
<tr>
<td>MN184</td>
<td>2001</td>
<td>Minnesota</td>
<td>High</td>
<td>Field isolate</td>
</tr>
<tr>
<td>17198-6</td>
<td>1997</td>
<td>Oklahoma</td>
<td>High</td>
<td>Field isolate</td>
</tr>
</tbody>
</table>

\(^a\)Not applicable
<table>
<thead>
<tr>
<th>Virus strain</th>
<th>ORF2</th>
<th>ORF3</th>
<th>ORF4</th>
<th>ORF5</th>
<th>ORF6</th>
<th>ORF7</th>
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<tbody>
<tr>
<td>VR2332</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>MLV</td>
<td>98.8</td>
<td>98.8</td>
<td>99.4</td>
<td>99.0</td>
<td>99.4</td>
<td>100.0</td>
</tr>
<tr>
<td>JA142</td>
<td>94.2</td>
<td>87.5</td>
<td>89.4</td>
<td>91.0</td>
<td>97.7</td>
<td>94.4</td>
</tr>
<tr>
<td>ATP</td>
<td>93.4</td>
<td>86.3</td>
<td>88.8</td>
<td>90.5</td>
<td>97.1</td>
<td>94.4</td>
</tr>
<tr>
<td></td>
<td>(99.2)*</td>
<td>(97.3)</td>
<td>(98.9)</td>
<td>(98.5)</td>
<td>(99.4)</td>
<td>(100)</td>
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<tr>
<td>SDSU73</td>
<td>93.8</td>
<td>88.6</td>
<td>91.1</td>
<td>87.1</td>
<td>99.4</td>
<td>96.8</td>
</tr>
<tr>
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<td>86.3</td>
<td>89.9</td>
<td>84.1</td>
<td>96.0</td>
<td>95.2</td>
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<tr>
<td>17198-6</td>
<td>93.8</td>
<td>87.8</td>
<td>89.4</td>
<td>90.5</td>
<td>98.9</td>
<td>97.6</td>
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</tbody>
</table>

*Sequence identity of ATP with its parental strain, JA142
Table 3. Growth of PRRS viruses in African Monkey kidney cell line (MARC-145) and porcine alveolar macrophages (PAM) as measured by virus infectivity assay in comparison to the estimate of virus amount by a quantitative fluorogenic reverse transcription polymerase chain reaction (Q-PCR)

<table>
<thead>
<tr>
<th>Virus</th>
<th>Virus titer (TCID$<em>{50}$/ml in log$</em>{10}$)$^a$</th>
<th>MARC-145</th>
<th>PAM</th>
<th>Q-PCR$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>VR2332</td>
<td>3.16 ± 0.80</td>
<td>3.30 ± 0.87</td>
<td>3.70 ± 0.14</td>
<td></td>
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<tr>
<td>MLV</td>
<td>3.24 ± 0.46$^c$</td>
<td>1.30 ± 0.15</td>
<td>3.25 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>JA142</td>
<td>3.03 ± 0.26</td>
<td>3.78 ± 0.28</td>
<td>3.85 ± 0.21</td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>3.10 ± 0.74</td>
<td>1.67 ± 0.76</td>
<td>3.25 ± 0.35</td>
<td></td>
</tr>
<tr>
<td>SDSU73</td>
<td>3.14 ± 0.49</td>
<td>3.90 ± 0.87</td>
<td>4.45 ± 0.35</td>
<td></td>
</tr>
<tr>
<td>MN184</td>
<td>2.92 ± 0.64</td>
<td>4.50 ± 0.36</td>
<td>4.80 ± 0.42</td>
<td></td>
</tr>
<tr>
<td>17198-6</td>
<td>3.10 ± 0.63</td>
<td>3.70 ± 0.50</td>
<td>3.60 ± 0.57</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Virus titer is expressed as mean ± standard deviation.

$^b$PCR estimate of virus titer was made in comparison to standards with known infectious virus titer (TCID$_{50}$/ml)

$^c$Viruses with significantly different titers between MARC-145 and PAM were marked bold.
Figure 1. Phylogenetic relationship among challenge PRRS viruses based on nucleic acid sequence of open-reading frames (ORFs) 2 to 7. Bar indicates that one nucleic acid change per 100 sequences.
Figure 2. PRRS virus-specific ELISA antibody responses of pigs at day 42 post inoculation. The X axis indicates the inoculum to each group. The solid line across the figure is the cut-off S/P ratio (0.4) for positives. Each box represents 25 to 75% of observation. Whiskers above and below of each box represent the 90 and 10% of observations, respectively. Solid and dash lines within each box are median and mean, respectively. Dots above or below the whiskers on each box represent outliers not included between 10 and 90% of observation. Asterisk indicates the groups that induced a significantly higher level of ELISA antibody compared to the rest of the groups as determined by Tukey HSD ($p<0.05$).
Figure 3. IFA antibody response to the homologous and heterologous strains of PRRS virus at day 42 post inoculation (40 x log₂). Label at the left side and bottom indicate the virus that pigs were inoculated with and the virus used in test, respectively. Black and gray bars represent antibody titers against homologous virus and its parental or attenuated virus, respectively. Asterisk indicates the group that induced a significantly higher level of IFA antibody compared to the rest of the groups as determined by Tukey HSD (p<0.05).
Figure 4. IFA antibody response of VR2332-challenged pigs to homologous and heterologous viruses at day 7, 15 and 28 post inoculation. Each box represents 25% to 75% of observations. Solid and dash line within each box indicate median and mean, respectively.
Figure 5. Neutralizing antibody response (log2) of pigs to the homologous (solid black bar) and heterologous (gray bars) strains of PRRS virus at day 42 post inoculation. Label at the left side and the bottom indicate the virus that pigs were inoculated with and the virus used in test, respectively. MLV inoculum was excluded because of the absence of neutralizing antibody. Gray bar represents the antibody titer against its attenuated virus. Asterisk indicates the groups that induced a significantly higher level of SVN antibody compared to the rest of groups as determined by Tukey HSD ($p<0.05$).
CHAPTER 3: IMMUNOLOGICAL SIGNIFICANCE OF GENETIC VARIATION IN ORF 5 OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (PRRSV)

(A paper to be submitted to Journal of Virology)

Won-Il Kim, Jae-Jo Kim, Sang-Ho Cha, Wai-Hong Wu,
Rich Evans, Kay Faaberg, Kyoung-Jin Yoon

Abstract

A high rate of genetic and antigenic variability among porcine reproductive and respiratory syndrome (PRRS) viruses is an outstanding characteristic of the virus, which hampers effective prevention and control of the disease. The major envelope protein encoded by ORF5 is known to have a critical role in inducing neutralizing antibody and conferring protective immunity. Although a conserved linear neutralizing epitope (NE) was identified in the N-terminal ectodomain of ORF5 (37-45), poor cross-neutralization among viruses is still an issue. This study was conducted to determine the sequence elements within ORF5 which affect cross-neutralization by genetically comparing 69 field isolates collected between 1999 and 2002 and were classified according to their relative susceptibility to virus neutralizing (VN) antibody raised against the VR2332 strain (North American prototype PRRS virus) \textit{in vitro} and \textit{in vivo}. In addition, mutants whose amino acid sequences were substituted with those found in the viruses resistant to VN antibody at specific sites in ORF5 were generated using a VR2332-backboned infectious cDNA clone and site mutagenesis to confirm the role of those identified sites in cross neutralization. Five common sites/domains
were identified in ORF5 from the sequence comparison between susceptible and resistant viruses to VN activity of anti-VR2332 sera. The changes of amino acid sequences at three sites (32-34, 38-39, and 57-59) located in the N-terminal ectodomain of ORF5 significantly affected the susceptibility of the viruses to VN antibody, indicating that genetic variation at specific regions of ORF5 affects cross neutralization between PRRS viruses.

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is the causative agent of PRRS which causes significant economic loss to the swine industry due to reproductive disorder in breeding animals, respiratory illness and systemic diseases in adults and younger pigs (1;8;12;48). The virus is a member of the family Arteriviridae that belongs to the order Nidovirales with Coronaviridae, Roniviridae and Toroviridae (10;14). Other members of this family are equine arteritis virus (EAV), simian hemorrhagic fever virus (SHFV) and lactate dehydrogenase-elevating virus (LDV) of mice (10;54;55).

PRRSV is a single-stranded, positive-sense RNA virus that replicates primarily in macrophage-lineage cells (1;8). The PRRSV genome, approximately 15kb in length, contains at least nine open reading frames (ORFs) (3;16). ORF 1a and 1b encodes non-structural proteins required for virus replication (3). Three N-glycosylated minor envelope proteins (GP2a, GP3, GP4) are translated from ORF 2a, 3 and 4 and form a heterotrimer, which is reported to be critically involved in the transport of those proteins to Golgi complex; otherwise, the individual protein remains in endoplasmic reticulum (ER) (61). ORF2b completely embedded in ORF2a encodes another non-glycosylated minor protein designated 2b (62). ORF5 codes the major envelope protein (GP5) that forms a heterodimer with
membrane (M) protein expressed from ORF6 (33). This heterodimer is known to be most abundant on the surface of virion and plays a pivotal role in virus assembly recruiting other minor envelope proteins and nucleocapsid (N) protein into the virion (61). N protein is encoded by ORF7. It contains nuclear localization and export signals (NLS and NES) and forms a homodimer, which is demonstrated to be essential for virus infectivity (30;53).

PRRSV induces weak and delayed virus-specific neutralizing antibody and cellular immunity. In general, neither neutralizing antibody nor interferon-gamma producing cells are detected until 4 weeks after infection (31). Nonetheless, the critical role of neutralizing antibody in the control of virus infection was demonstrated in pregnant sows with passively transferred virus neutralizing (VN) antibody which prevented the development of viremia and reproductive failure from challenge with a virulent PRRSV (40;65). The role of cellular immunity in the virus clearance also has been shown (7;32;35;51). Even though N protein induces a high level of antibody production at an early stage after the virus infection, anti-N protein antibody is not protective (9). It has been reported that recombinant proteins or plasmid DNA of ORFs 4, 5 and 6 have an ability to induce VN antibody and confer protective immunity to vaccinated pigs (16;22;26;38;44); however, antiviral activity or protection mediated by GP5 has shown to be most efficient to control the virus infection among those proteins (6;20;43;63). Accordingly, the presence of a conserved linear neutralizing epitope in GP5 has been reported (41;47).

The remarkable genetic and antigenic diversity of PRRSV hampers the development of effective vaccines and the eradication of the disease (19;36;64). There are two most discrete genotypes: North American- (type 2) and European-type (type 1) PRRS viruses. Those two genotypes of PRRSV have less than 70% homology in the entire genome and are
not cross neutralized by antibody raised against each other even though some level of cross reactivity has been reported (15;58). Moreover, many field-based studies have also demonstrated that a great deal of genetic and antigenic diversity exists within the same genotypes and negatively affects the efficient cross-protection among different viruses (4;27;36;42). Similar field observations have been made indicating that pigs previously exposed to PRRSV can be re-infected if the incoming virus is different enough (i.e., heterologous) from the previous virus (28). Therefore, sequencing and restriction fragment length polymorphism (RFLP) analysis of ORF5, which is known to be the most variable among PRRS viruses (16), have been applied to classify PRRS viruses based on the genetic relatedness among the viruses (4;59). However, results of those genetic methods have not always correlated well with immunological cross protection between the viruses. Since not only is overall sequence homology important but also specific amino acid changes should be important for the immunological property of the virus, identification of sequence elements that determine the degree of cross protection among the viruses is necessary to classify the viruses according to immunological similarity. The following study was conducted to identify genetic elements in ORF5 which are immunologically significant for virus cross neutralization.

Materials and Methods

Experimental design. The study was conducted to assess the immunological significance of genetic variation in ORF5 among PRRS viruses (n=69) and to determine the amino acid sequences responsible for evading neutralizing antibody. First, the ability of field
isolates of PRRSV to replicate in cells in the presence of VN antibody raised against the VR-
2332 strain and in pigs passively immunized with such VN antibody was assessed. The
viruses were then classified into several groups (n=6) based on their susceptibility to VN
antibody in vitro and in vivo. Second, genetic comparison was performed among the groups
to identify the potential sequence elements pertinent to such a difference in the susceptibility
to VN antibody. Third, site-directed mutagenesis was conducted on a PRRSV infectious
cDNA clone that was produced based on VR-2332 to generate mutants with amino acid
substitution(s) at the identified site. Changes in the susceptibility of those mutants to the VN
activity of anti-VR-2332 antibody were assessed to determine the immunobiological role of
the identified genetic determinants in cross neutralization.

Viruses and cells. Sixty-nine field isolates of PRRSV that were collected from
submissions to the Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL)
between 1999 and 2002 were employed for the study. In addition, VR-2332 (North
American prototype PRRSV and parental strain of Ingelvac® PRRS MLV vaccine virus),
JA142 (parental strain of Ingelvac® ATP vaccine virus) and SDSU73 (one of the field
isolates from severe PRRS outbreaks) were used for the study (23).

All of the viruses were prepared in MARC-145 cells, a highly permissive clone of the
African Monkey kidney cell line MA104 (25) which represented 4 cell-culture passages. The
viruses were used after their titers were adjusted to $10^3$ TCID$_{50}$/ml.
**Antisera.** Antisera against the VR-2332 strain were produced in 22 PRRS-naive pigs experimentally injected with the virus at a rate of $10^3$ TCID$_{50}$/ml. Sera were collected from those animals after 2 to 3 months post inoculation. Sera with serum virus neutralizing antibody (SVN) titer of 1:64 or higher were combined together and used for VN assays after being adjusted to 1:64 SVN titer. Antisera against JA142 and SDSU73 were also included in this study, which were generated from a previous study in our laboratory (23). In brief, the JA142 or SDSU73 strain was intranasally inoculated into ten pigs at a rate of $10^3$ TCID$_{50}$/ml. The antisera were collected at 42 days after inoculation, combined together for each virus, and used for VN assays.

**Virus neutralization assay.** Florescent focus neutralization (FFN) assay was performed as previously described (62) to assess the susceptibility of field isolates or rescued mutants to the neutralizing activity of antisera raised against VR-2332, JA142 or SDSU73. After heat inactivation at 56°C for 60 min, a pair of PRRSV antibody-positive and -negative serum was serially diluted 2-fold in RPMI-1640 (Sigma, St. Louis, MO, USA) supplemented with 10% FCS (Sigma), 20 mM L-glutamine (Cellgrow, Herdon, VA, USA), and an antibiotic-antimycotic mixture (Sigma) which consisted of 100 IU/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamicin and 0.25 mg/ml amphotericin B (hereafter, RPMI growth medium) and mixed with an equal volume of each virus suspension at a rate of 100 florescent focus forming unit (FFU) per 0.1 ml. Mixtures of virus-antisera were then incubated for 1 hr at 37°C. The mixtures were inoculated onto MARC-145 cell monolayers prepared in 96-wells plates 24 hrs in advance and incubated for another 1 hr at 37°C. The inoculum was
removed from the plates and the cells were replenished with 0.2 ml/well of RPMI growth media. The plates were incubated for 20 hrs at 37° C in a humid 5% CO₂ atmosphere. At the completion of the 20-hr incubation the supernatant was removed. The cell monolayers were washed once with 0.01M phosphate-buffered saline (PBS, pH 7.2) and fixed with cold acetone-water mixture (8:2) for 10 min at ambient temperature. The plates were dried for 1 hr at ambient temperature, washed once with PBS, and then stained with PRRSV-specific monoclonal antibody SDOW-17 that was conjugated with FITC (Rural Technologies, Brookings, SD). After 1-hr incubation the wells were washed three times with PBS and the number of virus-specific fluorescent foci in each well was counted. VN antibody titer of each antiserum against each virus was expressed as reciprocal of the highest dilution in which 90% or higher reduction in the number of FFU was observed. The assay was repeated at least 3 times on 3 different days.

Preparation of immunoglobulin. The sera with VN antibody titer of > 1:16 against VR-2332 were used for the preparation. Immunoglobulin (IgG) was fractionated from those sera using a salt precipitation method as previously described (65). Each serum was mixed slowly with an equal volume of cold saturated ammonium sulfate solution (Sigma) by dropping the solution while stirring. The mixture was incubated for 30 min at 4° C and then centrifuged at 9000 x g for 30 min (Optima™ L-90K Ultracentrifuge, SW28, Beckman Coulter, Fullerton, CA, USA). The pellet was suspended in the minimum volume (1/10 of the original volume) of 0.01M PBS (pH 7.2) and dialyzed in 5 volume of PBS until no residual ammonium sulfate was detected in the dialysis buffer (PBS) by adding 1%
barium chloride (Sigma). Each IgG preparation was tested for the cytotoxicity and VN titer. IgG solutions with at least 1:128 VN titer without any cytotoxicity were selected, adjusted to 1:128 VN titer with PBS, and mixed together thoroughly before aliquoted in a small quantity for storage at -20°C until used. Normal serum globulin (NSG) was prepared in the same manner as above from sera taken from age-matched PRRSV-free pigs. Total protein concentration of IgG and NSG preparation were determined with DC™ Protein Assay (Bio-Rad, Hercules, CA, USA) and then the protein concentration of NSG was adjusted equally to that of the IgG preparation before being stored.

**In vivo assessment of cross neutralization among PRRS viruses.** All pigs were purchased from a commercial specific-pathogen-free (SPF) herd known to be free of PRRS by virologic and serologic monitoring over time. Besides PRRSV, the animals were also tested to be free of porcine circovirus type 2, swine influenza virus and *Mycoplasma hyopneumoniae* before and on arrival. Pigs were of similar genetic background and of comparable health status and individually housed in HEPA-filtered isolation units during the study period to prevent cross contamination of viruses between pigs or infection by other pathogens of extraneous origin. Each of the isolation units was equipped with air influx and ventilation, automated flush system, feeder and drinking water supply. All units were set for constant temperature and relative humidity during the entire study.

Twenty isolates were selected based on their classification by *in vitro* VN test and prepared for the challenge to assess their susceptibility to VN antibody *in vivo*. The 20 isolates included four viruses from R1, three viruses each from R2 and R3, and all
susceptible viruses (n=10). These selected viruses represented the rest of the viruses in the same group. In order to obtain a highly homologous virus inoculum, each selected isolate and the control virus was plaque-cloned two times in MARC-145 cells. After that, 15 viral isolates were collected from each of the final plaque purified viruses by plaque assay and sequenced for ORF5 to confirm its homogeneity as described in a previous study (11). Then, each of the final plaque-purified viruses was propagated once in MARC-145 cell to a large quantity.

For each run ten 2-week-old littermates were obtained. Five of these pigs were selected and injected intraperitoneally with anti-VR-2332 IgG at a rate to yield VN antibody titer of 1:16 in the blood circulation of the animal based on body weight as previously described (40;65). The remaining five pigs were injected with NSG at the same protein concentration of IgG and served as shame controls. One day after injection each pair of two pigs (one with anti-VR-2332 IgG and the other with NSG) were randomly selected and inoculated intramuscularly with one of the 4 plaque-cloned isolates at approximate $10^3$ FFU/ml in 2 ml of RPMI-1640. The remaining pair of two pigs were inoculated with VR-2332 in the same way as the other pairs and served as the homologous virus control. All of the pigs were kept in the isolation units for 14 days after challenge. This procedure was repeated until all viruses were evaluated. All animal handling and care was done under the guideline and supervision of the Institutional Animal Use Committee of Iowa State University.

After the primary animal challenge described above, five viruses of the twenty challenged viruses were retested by following the same procedure as above to confirm the reproducibility of viremic patterns in the pigs injected with anti-VR-2332 IgG or NSG. In
addition, three viruses that showed a complete inhibition of replication by passively transferred anti-VR-2332 IgG in the primary animal challenge were retested with the same procedure as above but using a different challenge route (i.e., intranasal) to test the effect of different challenge route on the assessment.

**Sampling and assays.** Sera were collected at 0, 3, 7, 10 and 14 days post challenge (DPC) and assayed for viremia by a real-time quantitative RT-PCR and antibody response by ELISA and SVN tests. Viruses obtained from each of the virus-positive serum samples were sequenced for ORF5 and compared to the inoculum virus in order to further document the homogeneity of each inoculum and lack of cross contamination during *in vivo* replication. All of the pigs were euthanized at the end of the study (14 DPC) and necropsied. A variety of tissues (tonsil, lung, spleen, and lymph nodes) were collected from each pig and submitted to ISU-VDL for pathological examination and immunohistochemistry (IHC) for PRRSV.

**Quantitative PCR.** The level of viremia in the serum was measured by a quantitative fluorogenic reverse transcription-polymerase chain reaction (i.e., real-time RT-PCR) using TaqMan® chemistry. Virus-specific oligonucleotide primers and a fluorescent probe were engineered to be within a highly conserved region of PRRSV ORF6 using sequences deposited in GenBank. Forward primer (ORF6F) was 5’-GCCATAGAAACCTGGAAATTCATC-3’ and reverse primer (ORF6R) 5’-GCGGCCTAGCAAGCACAA-3’ which were synthesized by a commercial vendor (Integrated DNA Technologies, Coralville, IA, USA). MGB probe with a 5’ reporter 6-
carboxyfluorescein (FAM) and a 3’ non-fluorescent quencher (ORF6P: 5’-6FAM-CCTCCAGATGCCG) was synthesized by Applied Biosystems (Foster City, CA, USA).

For assay, viral RNA was extracted using QIAamp® viral RNA mini kit (Qiagen, Valencia, CA, USA) from 140 µl of each virus material. Real-time RT-PCR was then carried out with the QuantiTect® Probe RT-PCR Kit (Qiagen) in a 25 µl reaction volume using 2.5 µl of extracted template. Primers were added at a final concentration of 0.4 µM each; the probe was at a final concentration of 0.2 µM. The PCR amplification was performed on the ABI 7900HT Sequence Detection System (Applied Biosystems). Cycling conditions were as follows: a) reverse transcription for 30 min at 50° C; b) a 15 min activation step at 95° C; and c) 35 cycles of 15 sec at 94° C and 60 sec at 60° C. A set of PRRS virus preparations each of which had known virus titer was used to generate a standard curve. Samples with a threshold cycle (Ct) of 35 cycles or less were considered positive. The amount of PRRSV in each serum sample was calculated by converting Ct value to virus titer (FFU/ml) using the standard curve made of viruses with known titers.

**Serology.** Antibody response was evaluated by the commercial ELISA kit (IDEXX HerdCheck® PRRS 2XR, Westbrook, Maine, USA). The VN test was conducted as described above to confirm the titer of transferred VN antibody in pigs.

**Sequencing.** The target gene (ORF5) was amplified using QIAGEN® One-Step RT-PCR kit (Qiagen). PCR products were purified using QIAquick® PCR purification kit
(Qiagen) and submitted to the ISU Nucleic Acid Facility for sequencing. PCR and sequencing primers were as follows:

ORF5 Forward (P5F): 5’-CCTGAGACCAGGTTGGG-3’; and
ORF5 Reverse (P5R): 5’-TTTAGGGCATATATCATCAGC-3’.

**Generation of mutant virus.** An infectious cDNA clone constructed based on the sequence of VR-2332 (39) was kindly provided by Dr. Kay Faaberg at the University of Minnesota (St. Paul, MN) and used to generate mutant viruses. Mutagenesis was done using QuikChange® II site-directed mutagenesis kit (Stratagene, West Cedar Creek, TX, USA) as per the manufacturer’s instruction. Shuttle vector (TOPO® XL PCR cloning vector, Invitrogen, Carlsbad, CA, USA) containing the entire structural genes (ORF 2 to 7) of VR-2332 was constructed and used as a template for mutagenesis to prevent incorporation of any unexpected mutation into the infectious clone during the process. The mutation was confirmed by sequencing the whole structural genes in the shuttle vector and the whole fragment of structural genes in the shuttle vector was substituted for that in the plasmid of the infectious clone using BsrGI and HpaI enzyme sites. The infectious clone containing a mutation in the correct position was selected by sequencing after purifying the plasmid from competent cells (XL10-Gold® Ultracompetent cells, Stratagene). The plasmid of the infectious clone was linearized by AclI and purified using DNAclear™ kit (Ambion, Austin, TX, USA). The linearized plasmid was transcribed using T7 promoter by mMESSAGE mMACHINE® T7 kit (Ambion). The transcribed RNA was purified by MEGAclear™ kit (Ambion), and 1-10 µg of the purified RNA was transfected into MARC-145 cells (5 × 10⁶
cells/ml) prepared in chilled Dulbecco’s Modified Eagle’s Medium (DMEM, Sigma) containing 1.25% Dimethyl sulfoxide (DMSO, Sigma) by electroporation at 250 volts and 950 µF (Gene Pulser Xcell Electroporation System, Bio-Rad) as previously described (56). The transfected cells were plated on a 6-well plate and the media was changed with RPMI growth media 16-18 hrs after plating. At 48 hrs, 200 µl of the supernatant was inoculated on MARC-145 cells prepared in a 24-well plate. After incubation for 1 hr at 37°C the inoculum was replaced with RPMI growth media and the plate was kept under observation until the cytopathic effect became visible and evident. Rescued mutants were grown one time in a 25-cm² flask, titrated, and stored at -80°C until used.

Data analysis. All data were statistically analyzed with JMP (SAS institute Inc., Cary, NC, USA). The repeated measurements of viremia and antibody response in IgG- or NSG-transferred pigs were analyzed with multivariate analysis of variance (MANOVA) to determine the overall difference among the six groups classified based on the susceptibility of challenged viruses to the transferred VN antibody. The effect of the transferred IgG on the level of viremia was determined by the differences between the mean values of viremia at each sampling point in the IgG-and NSG-transferred pigs per each group and analyzed with MANOVA to determine the overall differences among the groups. A contrast was constructed where direct comparisons between the groups were necessary. The effect of transferred VN antibody in each group was also assessed by subtracting the area under the viremic curve of IgG-transferred pigs from that of NSG-transferred pigs (i.e., the area between 2 viremia curves) and analyzed with Wilcoxon Rank Sum test (21) to define the differences among the groups. In addition, the VN titers of field isolates and mutant viruses
measured by repeated VN tests were analyzed with Wilcoxon Rank Sum test (13) to determined the difference in the susceptibility to VN activity of antisera among the viruses.

Deduced amino acids sequences of ORF5 were aligned using Lasergene® MegAlign software (DNASTAR Inc., Madison, WI, USA) and compared between virus groups. In addition, protein analysis software, Lasergene® Protean was used to analyze GP5 (i.e., deduced amino acid sequence) of the viruses used in the study.

**Results**

*In vitro* assessment of cross neutralization among PRRS viruses by immunoglobulin raised against VR-2332. VN titer of VR-2332 IgG against VR-2332 (i.e., homologous neutralization) was 1:64. Replication of 10 of 69 field isolates in MARC-145 cells was substantially reduced by the anti-VR-2332 IgG (1:16 to 64 VN titer) and those viruses were classified into S (susceptible) group. Five isolates were neutralized by antiserum at a lower dilution (1:8) of the IgG, showing moderate resistance against neutralizing antibody. The infectivity of the remaining 54 isolates to MARC-145 cells was not or poorly affected by the neutralizing activity of the anti-VR-2332 IgG (i.e., VN titer <1:8) (Table 1).

Consistent amino acid changes between susceptible and resistant viruses were identified at three sites in ORF5 as compared to VR-2332 (Table 2). Sites I and II were located in the N terminal ectodomain of GP5 (H38L39 and A57N58K59, respectively) and site III (A137) was located between two transmembrane regions (64-130 and 170-190) of GP5 (16). Resistant viruses were classified further into three groups (R1, R2 and R3) based on the
number of site(s) where they had an amino acid change. R1 group (n=6) had the amino acids change only in site II while R2 group (n=11) had changes in two sites (sites I and III or II and III). R3 group (n=42) had changes in all three sites.

The effect of amino acid substitutions in the identified sites of ORF5 on the efficiency of anti-VR-2332 neutralizing antibody in inhibiting infection of other viruses to MARC-145 cells is illustrated in Figure 1. Susceptible field viruses that had no changes in any of the three identified sites as compared to VR-2332 were inhibited by significantly high dilutions [1:24.6 ±1.0 (Mean±SEM)] of anti-VR-2332 IgG which showed 1:64 VN titer to the VR-2332 strain. The susceptibility of R1 group to VN antibody (1:7.1±1.3) was significantly 

\( p=0.036 \) or 

\( p=0.002 \) higher than that of R2 (1:4.3±0.9) and R3 groups (1:3.4±0.5), respectively. There was no significant difference in the susceptibility to the neutralizing activity of anti-VR2332 IgG between R2 and R3 viruses or within R2 group viruses regardless of the difference in changed sites (i.e. sites I and III or II and III).

**In vivo assessment of cross protection from PRRS viruses by passively transferred immunoglobulin raised against VR-2332.** Pigs with passive transfer of IgG specific for VR-2332 at a rate yielding 1:16 SVN titer in the blood circulation did not develop detectable viremia until 14 DPC when they were exposed to VR-2332, whereas pigs with NSG developed viremia as early as by 3 DPC and remained viremic at the end of study (14 DPC). As shown in Figure 2, all pigs challenged with the viruses that were resistant to the VR-2332 VN antibody in vitro became viremic by 3 DPC even though levels of viremia in IgG-transferred pigs were lower than that in NSG-transferred pigs at all sampling points. In contrast, the ten viruses susceptible to the VN antibody in vitro were classified further into
three groups: a) completely susceptible group (Sc, n=3) whose replication was completely inhibited by passively transferred IgG; b) partially susceptible group (Sp, n=4) that could not develop at 3 or 7 DPC but became viremic intermittently after that; and c) Svt group (n=4) that was susceptible to the VN activity of VR-2332 antisera *in vitro* but resistant in pigs with passive transfer of anti-VR2332 IgG as evidenced by the development of viremia. In addition, when susceptible viruses were categorized based on this classification, the Sc and Sp groups showed higher susceptibility to VR-2332 VN antibody (1:25.3±1.5 and 1:28.7±1.3 respectively) than the Svt group (1:19.5±1.5) *in vitro* even though it was not statistically significant (Figure 1).

The animal challenge study was repeated for eight selected isolates. Infection by all three viruses classified into the Sc group was still completely inhibited by passively transferred IgG when those viruses were challenged via the intranasal route. In addition, two viruses each from the Sp and Svt groups and one virus from the R1 group demonstrated the same viremic patterns when those viruses were retested by the same procedure.

The level of viremia in IgG- or NSG-transferred pigs varied for each challenged virus (Figure 2). In NSG-transferred pigs, no significant difference in the level of viremia by any of the PRRS viruses was observed over all sampling points (p=0.2449) or at each sampling point (p>0.1, by sampling day; 3, 7, 10 and 14 DPC) although the mean level of viremia by R3 group appeared to be the highest among all viruses inoculated. In contrast, a significant difference in viremia pattern by groups was observed in IgG-transferred pigs (p=0.0119) since no or a low level of viremia was observed in the IgG-transferred pigs challenged with PRRS viruses of the Sc or Sp group. Overall the level of viremia in IgG-transferred pigs, if developed, was lower than that in NSG-transferred pigs for all challenged viruses.
Nonetheless, the degree of reduction of viremia in pigs passively immunized with IgG varied by group. Much greater reduction in the level of viremia (determined by the differences between the mean of viremia level in the IgG-and NSG-transferred pigs for each virus) was observed in pigs with IgG when challenged with PRRS viruses of the Sc or Sp group, as compared to that by PRRS viruses of the Svt group or R groups using a contrast ($p=0.0181$). Consistent with this, the area between two viremic curves (i.e. viremia curves for IgG- and NSG-transferred pigs) of the Sc and Sp groups (16.1-45.3) was larger than that of the remaining groups (1.4-32.7), but it was not statistically significant ($p=0.1322$) by the Wilcoxon Rank Sum test.

ELISA and VN antibody responses in NSG- or IgG-transferred pigs before and after challenge are shown in Figure 3. In NSG-transferred pigs, there was no significant difference in ELISA antibody titers among all groups ($p=0.1467$); however, R2 and R3 group viruses tended to induce a higher and earlier ELISA antibody response (0.81±0.21 and 1.47±0.21, respectively at 10 DPC) compared to the remaining groups (0.31±0.18 and 0.32±0.12 for R1 and S, respectively at 10 DPC). No significant difference in the level of ELISA antibody (i.e., S/P ratios) was observed between S (Sc, Sp and Svt) and R1 groups. No VN antibody for VR-2332 and challenge viruses was detected in those pigs until the end of study. In IgG-transferred pigs, the S/p ratio of ELISA varied between 0.78 and 1.28 (0.93±0.11) after passive immunization (-1 DPC) and between 0.30 and 0.77 at 14 DPC (0.48±0.08). No significant difference in the level of ELISA antibody was observed among the groups. VN titer constituted by passive transfer gradually decreased from 1:16 or 32 before challenge to 1:2 or 4 at 14 DPC (Figure 3).
The G residue at 151 in ORF5 of Sc group was substituted with R, I or K in that of the Sp group and the rest of the viruses and constituted site V (Table 2). PRRS viruses of the Svt group developed viremia in both pigs injected with IgG or NSG. The viruses that were composed of Svt and R (i.e. R1, R2, and R3) groups had another consistent amino acid change at 34 or 32 to 34 of ORF5, compared to those in the Sc and Sp groups. This site was designated as site IV (S^{32}N^{33}D^{34}) and Svt and all R groups were found to have various amino acid sequences at positions 32 to 34, commonly S or N at 34 (Table 2 and Figure 4).

**Role of ORF5 amino acid changes in cross neutralization among PRRS viruses.**

*Generation of mutant viruses.* Twelve mutants were generated from a VR-2332 based infectious cDNA clone (39) in this study (Table 3 and Figure 5). The VR-2332 infectious clone was manipulated by site-direct mutagenesis to have the sequence(s) found in the majority of resistant viruses at each of the five identified sites (I to V) in order to determine the effect of an individual site on the susceptibility of the virus to the VN activity of anti-VR2332 IgG. The sequences substituted at all sites except site I happened to be similar to those of JA142 (Figure 5). Mutants containing amino acid changes at all sites (VRall) and except one particular site (VRall-37KF and VRall-137S) were also generated to assess the combinational effects of the identified sites and to determine the specific site responsible for the entire resistance of the virus to VN antibody. In addition, the entire ORF5 of the VR-2332 infectious clone was substituted with that of ‘atypical’ PRRS viruses (JA142 or SDSU73) which were known to antigenically not cross react with VR-2332 to assess the role of the ORF5 product in virus resistance to VN antibody. Back-mutant viruses designated as JAP5BM and SDP5BM (whose ORF5 sequences at sites I to IV were restored
to the original sequences in the VR-2332 infectious clone) were also generated to determine if the restoration of those sequences results in restoration of the susceptibility of the virus to VN antibody to the similar level of VR-2332. It was confirmed that no changes other than the intended mutations were incorporated to the mutants by sequencing entire structural genes.

**Susceptibility of single or multiple sites-mutated viruses to VN antibody specific for VR-2332.** Five mutants that have (an) amino acid change(s) at each of the five identified sites of the VR-2332 infectious clone were tested for their susceptibility to VN antibody (Figure 6). The 32NSN and 57KDH mutants showed a significant reduction in their susceptibility ($p=0.0117$) to anti-VR2332 VN antibody compared to VR2332E which was rescued from the original infectious clone. The 37KF and 137S mutants also showed significant reduction in the susceptibility to the VN antibody ($p=0.0418$) but at a lesser degree than did 32NSN and 57KDH. The 151G mutant showed the susceptibility similar to the VR2332E to the VN antibody; hence, this mutation (R151G) was not included in further analyses.

The VRall mutant showed the greatest reduction (1:13.0±3.5) in the susceptibility to the anti-VR-2332 VN antibody. It was similar to how JA142 (1:10.0±3.5), heterologous virus control, responded to anti-VR2332 VN antibody *in vitro*. Using VRall-37KF and VRall-137S mutants, the contribution of amino acid changes at position 37 and 38 to the overall resistance of PRRSV to VN antibody appeared to be bigger than that of the amino acid change at position 137 as the susceptibility of VRall-37KF (i.e., restoration of amino acids at positions 37 and 38 to those of VR2332) to the anti-VR2332 VN antibody was
increased about 2-fold \((p=0.0407)\) whereas VRall-137S showed susceptibility similar to VRall mutant to the anti-VR2332 VN antibody, suggesting that the amino acid change at 137 is not significant in cross neutralization (Figure 6).

**Relationship between ORF5 genetic profile and degree of cross neutralization among PRRS viruses.** Results of two-way VN tests on JAP5, JAP5BM, SDP5 and SDP5BM mutants against VR-2332, JA142 and SDSU73 are shown in Figure 7. JAP5 in which whole VR-2332 ORF5 was replaced with that of JA142 showed significant reduced susceptibility to anti-VR-2332 VN antibody. The antibody had 1:18.7±4.0 VN titer to JAP5 while it had 1:48.0±3.5 VN titer against VR2332E (Figure 7A). When sequences at all three sites were restored back to those of VR-2332, the susceptibility of the virus (JAP5BM) to VR-2332 antiserum was restored by 2-fold (1:32.0±4.4) even though it was not completely recovered to the same level of VR-2332E. Similar results were observed when SDP5 and SDP5BM were tested against the VR-2332 antiserum (Figure 7A).

In addition, all mutants were tested with JA142 or SDSU73 antiserum which showed 1:64 VN titer against the homologous virus (JA142 or SDSU73) in MARC-145 cells (Figure 7B). The JA142 antiserum (that showed <1:2 VN titer against VR2332E) had substantially lower VN titer (1:16) against the JAP5 mutant as compared to that against the homologous virus (1:64). On the other hand, JAP5BM in which amino acids at all three identified sites in ORF5 were restored back to those of VR-2332 completely escaped from the neutralizing activity of the JA142 antiserum (i.e., <1:2 VN titer). Moreover, the VRall-37KF mutant that had the similar amino acid sequences of JA142 at the three identified sites showed enhanced susceptibility to the VN activity of the JA142 antiserum (i.e., VN titer of 1:6.7±3.5).
Interestingly, the VRall mutant that contained KF at 37 and 38 sites instead of HL of JA142 restored the similar resistance as VR2332E against the JA142 antiserum (Figure 7B). Similar observations were made with the SDP5 and SDP5BM mutants. The SDSU73 antiserum that could not inhibit infection of VR2332E to the MARC-145 cells (i.e., <1:2 VN titer) showed 1:16 VN titer against SDP5. However, the susceptibility of the mutant (i.e., SDP5BM) to the SDSU73 antiserum was decreased to 1:4 as illustrated in Figure 7C when amino acids at identified sites were reverted to those of VR-2332.

**Discussion**

This study was conducted to determine the relationship between genetic similarity of ORF5 and the degree of cross neutralization among PRRS viruses and to identify genetic elements associated with virus neutralization. The ORF5 was selected for the study because: a) it encodes for the major envelope protein of PRRSV; b) it is highly variable region of the viral genome; and c) it was reported to contain epitope(s) inducing VN antibody. The ability of field isolates of PRRSV to replicate in cells (MARC-145) and pigs in the presence of VN antibody was compared to classify the viruses into several groups according to their susceptibility against VN antibody raised to VR-2332. The idea of the animal study was grounded in previous studies in which it was demonstrated that pigs with passively transferred neutralizing antibody titers of ≥1:16 did not become viremic when they were challenged with homologous PRRSV (40,65). Thus, PRRS viruses were not expected to replicate in passively immunized pigs unless genetic differences between the challenge virus and the virus used to produce antiserum resulted in significant antigenic differences. Since two viruses whose ORF5 sequences differ more than 6% are considered as unrelated viruses (49), most of the viruses that had a homology higher than 94% to VR-2332 were selected for animal studies to increase the chance for identifying genetic changes significantly effecting...
cross virus neutralization. In general, two classification systems (in vitro VN test in MARC-145 cells and in vivo bioassay) correlated well with each other particularly for identifying resistant viruses as all resistant viruses determined by the in vitro VN test were also resistant to passively transferred VN antibody in pigs. However, the range of susceptible viruses became even narrower in the animal study since replication of the three viruses, which were classified in vitro into the susceptible group, in inoculated pigs was not affected by the presence of passively transferred VN antibody. These observations suggest that overall sequence homology of ORF5 between viruses may not always be a good indicator for immunological relatedness for VN antibody and that other gene products or other host factors influence cross neutralization among viruses.

One disadvantage of animal trials in this study was the lack of experimental replication due to the fact that a relatively large number of viruses needed to be studied and each virus challenged in a pair of pigs (IgG- or NSG-transferred pigs). Nonetheless, data (from the animal trials analyzed by groups in which the viruses have changes at the same sites, rather than the individual virus) could be used for the analysis since the main purpose of the animal study was to evaluate the consistency between two virus-classification systems and to assess the effect of the sequence change(s) determined by the in vitro test on the virus neutralization by transferred VN antibody in pigs. Furthermore, for some viruses of different groups, the animal trials were repeated. For example, pigs passively immunized with anti-VR2332 IgG were intranasally inoculated with one of three PRRS viruses of the Sc group. Infection of all three viruses was completely blocked by the presence of passively transferred IgG even though the animals were challenged via a different route (i.e., intranasal) as compared to the primary challenge study which was conducted through intramuscular inoculation (data not shown). In addition, a similar viremia pattern was observed in pigs with passively transferred IgG specific for VR-2332 after being challenged with one or two
PRRS viruses each from the Sp, Svt and R1 groups although the level of viremia varied between animal studies (data not shown). Therefore, the influence of transferred VN antibody on the overall viremia pattern of each virus in pigs was reproducible despite some degree of variation in the level of viremia.

Based on the classification determined by in vitro and in vivo assays, five candidate sites in ORF5 were determined to be associated with virus neutralization. A linear neutralizing epitope (NE) had already been identified in the N terminal ectodomain of ORF5 by other investigators (41;47). Since this NE (a.a. 37-45) is genetically conserved among North American PRRS viruses, it was hard to explain poor cross-protection among PRRS viruses if this linear NE were to be the major epitope inducing VN antibody (47). Many viruses resistant to the VN activity of VR-2332 antiserum had sequence substitutions (K or N at 38 and F or I at 39) for H^{38}L^{39} in ORF5 of VR-2332 (Site I). This site is located within the region (37-45) where the linear NE is also located. The HL sequences were postulated to be the most critical sites for binding to VN antibody (41) and the substitution with TY which was observed in PRRSV type I and LDV blocked neutralizing antibody recognition of this epitope (46). Consistent with these previous observations, this study demonstrated the negative effect of amino acid substitution of HL at 37 and 38 with KF on the susceptibility of viruses to anti-VR2332 VN antibody. The importance of such substitutions was reciprocally confirmed by VN test on the VRall-37KF mutant against VR-2332 antiserum and the VRall-37KF and VRall mutants against JA142 antiserum.

Sites IV and II are located right before or after the linear NE, respectively (Figure 4 and 5). SND residues at 32-34 (Site IV) in ORF5 of the Sc and Sp groups were replaced with SNS or SNN in the Svt group or with various sequences in the R groups. These sequences are located in the hypervariable sequence (32-39) of the N-terminal ectodomain (24;29). Our study showed that the substitution of the residue at 34 with N resulted in the addition of a
new N-glycosylation site to this region, which could mask the access of VN antibody to the conserved linear NE. Other investigators reported the sequence change at 34 as a possible marker for VN antibody-escaping viruses by other investigators (52) but failed to demonstrate a significant increase of resistance to VN activity although the virus with such a change gained some degree of resistance to VN antibody. It is suspected that such a failure may have been attributed to the use of a mixed virus population (i.e., some had D while others had N at 34) to generate antisera. This possibility is plausible since the amino acid (D) at this particular site was demonstrated to rapidly change to N or S during sequential pig-to-pig passages of a plaque-purified VR-2332 clone (11). In contrast, our present study was successful in identifying this particular site as an important neutralizing determinant because the homogeneity of PRRSV inoculum for antiserum production was confirmed by plaque purification and sequencing, not to mention that not one but three amino acids were replaced using a reverse genetic system. Similar sequence change (D to N) at the corresponding position in the major envelope proteins of other arteriviruses such as EAV and LDV was reported to be involved in the resistance of the virus to neutralization (5;45).

A$^{57}$N$^{58}$K$^{59}$ residues (Site II), which were found to affect the degree of virus neutralization in this study, reside in the region that has been recognized as another hypervariable region (57-61) by other investigators (24;29). The predicted antigenicity and surface probability was dramatically changed with amino acid substitution in this site between resistant and susceptible viruses (Figure 8). These changes were much greater than those by amino acid substitutions in the other two sites (i.e., I and IV) in the N-terminal ectodomain. Even though it is a computer-aid prediction, it is feasible that a great change in the antigenicity or surface probability with sequence substitution could result in conformational modifications of epitope, thus reducing its recognition by neutralizing antibody.
With the availability of the VR-2332 based infectious cDNA clone, our study demonstrated using a site-directed mutagenesis technique that amino acid sequence change in 4 of the 5 sites identified from sequence comparison affected virus neutralization of PRRSV by VR-2332 antiserum. Furthermore, the study also revealed the more critical role of sites I, II and IV than sites III and V in virus neutralization using mutants containing single or multiple amino acid substitutions at the identified sites (32NSN, 57KDH, VRall, VRall-137S), ORF5-swapped mutants (JAP5, SDP5) or back-mutated viruses (JAP5BM, SDP5BM). These three sites in the N-terminal ectodomain of PRRSV ORF5 are very similar to the reported four NEs in the N-terminal ectodomain of EAV ORF5 that were designated site A to D (5). Sites A, C and D of EAV are similar to site IV, I, and II of PRRSV, respectively, with respect to their location and substituted sequences. It was found that all three sites in EAV ORF5 were located near site C (linear neutralizing epitope) and affect each other not only in a linear but also conformational relationship. Therefore, it is plausible that the sites II and IV of PRRSV ORF5 that surround the linear NE and site I located within the NE region can affect each other in a similar way as demonstrated with EAV. However, it is not certain that sites II and IV comprise independent epitope as shown in EAV because no monoclonal antibodies specific for these sites have been produced, while site I is known to be involved in forming a NE. In addition, the N-terminal ectodomain of PRRSV ORF5 is much shorter (33 amino acid long) than that of EAV (120 amino acid long). Therefore, it would be logical that all three sites (I, II and IV) may constitute a single neutralizing epitope, together of which the core region is located between 37 and 45.

Sites III (A\textsuperscript{137}) and V (G\textsuperscript{151}) were originally used for RFLP analysis to discriminate VR-2332 or the vaccine virus (Ingelvac\textsuperscript{®} PRRS MLV) from other field strains (60). Also, G\textsuperscript{151} has been postulated as a possible marker for cell attenuation (2;11). These sites are located between two putative transmembrane (TM) regions (65-130 and 170-190).
topology of this domain (131-169) and C-terminus (191-201) has been controversial. Early studies (4;16) recognized the domain 131-169 as an endodomain. Rodriguez et al (2001) (50) reported that the C-terminus (170-201) of ORF5 was recognized by sera from PRRSV-infected pigs on ELISA and immunoblotting, but the peptide expressed by E. coli for amino acids at 130-170 was not recognized by any of those sera. Based on this observation, it was concluded that the C-terminus (191-201) sequence after TM region would be located at the outside of the virion. However, a different analysis (18) recognized the domain 130-170 between two TM regions as additional ectodomain of ORF5. In addition, Verheije et al (2002) (57) reported the importance of the last 7 residues at the C-terminus of ORF5 in the viability of PRRSV. It was, therefore, concluded that the C-terminus of ORF5 is exposed to the inside of the virion and interacts with other viral proteins to incorporate those proteins into the virion during virus assembly. These two undetermined domains are genetically conserved among different PRRS viruses (Figure 4). The amino acid substitutions at A137 and G151 may change the antigenicity of the site drastically but were found not to be so critical for virus neutralization by testing the 137S, 151R and VRall-137S mutants in this study.

As suspected previously, our observations concur that the ORF5 product is a critical element for virus neutralization. However, it is unlikely that the ORF5 product is the only element involved in virus neutralization since JAP5 and SDP5 mutants, even though the entire ORF5 was replaced, failed to: a) show the complete resistance against VR-2332 antiserum as did JA142 or SDSU73 and b) restore the full susceptibility of JA142 or SDSU73 against JA142 or SDSU73 antiserum. Therefore, it remains to study the role of other minor envelope proteins of PRRSV in virus neutralization as they could interact with cell receptors. The ORF6 product should also be important because ORF5-M heterodimer or M protein have been demonstrated to bind to one of the cellular receptors for PRRSV.
infection (17). Nonetheless, the sites identified in ORF5 in conjunction with virus neutralization in this study should aid in further virus classification potentially related to cross protection among PRRS viruses particularly which share sequence homology higher than 94%.

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Table 1. The susceptibility of field isolates to the neutralizing activity of antiserum raised against the VR-2332 strain of PRRSV and their sequence homology to VR-2332

<table>
<thead>
<tr>
<th>Susceptibility</th>
<th>Number of Isolates</th>
<th>VN titer</th>
<th>Deduced Amino Acids</th>
<th>Sequence Homology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1</td>
<td>1:64</td>
<td></td>
<td>100.0</td>
</tr>
<tr>
<td>Susceptible</td>
<td>10</td>
<td>1:16 - &lt;1:64</td>
<td>95.0 - 99.5</td>
<td></td>
</tr>
<tr>
<td>Moderate resistant</td>
<td>5</td>
<td>1:8 - &lt;16</td>
<td>88.1 - 96.5</td>
<td></td>
</tr>
<tr>
<td>Resistant</td>
<td>54</td>
<td>1:&lt;8</td>
<td>84.1 - 94.5</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>70</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
Table 2. Location and sequence of consistent amino acids changes in ORF5 between field
viruses susceptible and resistant to the neutralizing activity of antisera raised against the VR-2332 strain of PRRSV

<table>
<thead>
<tr>
<th>Location</th>
<th>Sc (n=3)\textsuperscript{a}</th>
<th>Sp (n=4)\textsuperscript{b}</th>
<th>Svt (n=3)\textsuperscript{c}</th>
<th>R (n=59)\textsuperscript{d}</th>
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</thead>
<tbody>
<tr>
<td>32-34</td>
<td>SND</td>
<td>SND</td>
<td>SNN or SNS</td>
<td>Various</td>
</tr>
<tr>
<td>38, 39</td>
<td>HL</td>
<td>HL</td>
<td>HL</td>
<td>Various</td>
</tr>
<tr>
<td>57-59</td>
<td>ANK</td>
<td>ANK</td>
<td>ANK</td>
<td>Various</td>
</tr>
<tr>
<td>137</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>S</td>
</tr>
<tr>
<td>151</td>
<td>G</td>
<td>R, I or K</td>
<td>R, I or K</td>
<td>R, I or K</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Sc: completely susceptible to VN antibody;

\textsuperscript{b}Sp: partially susceptible

\textsuperscript{c}Svt: susceptible \textit{in vitro} but resistant \textit{in vivo}

\textsuperscript{d}R: resistant
Table 3. Mutant viruses generated from VR-2332 based infectious clone in this study.

<table>
<thead>
<tr>
<th>Category</th>
<th>Name of Mutants</th>
<th>Location in ORF5 (a.a.)</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single site mutation</td>
<td>32NSN</td>
<td>32-34</td>
<td>SND → NSN</td>
</tr>
<tr>
<td></td>
<td>38KF</td>
<td>37, 38</td>
<td>HL → KF</td>
</tr>
<tr>
<td></td>
<td>57KDH</td>
<td>57-59</td>
<td>ANK → KDH</td>
</tr>
<tr>
<td></td>
<td>137S</td>
<td>137</td>
<td>A → S</td>
</tr>
<tr>
<td></td>
<td>151G</td>
<td>151</td>
<td>R → G</td>
</tr>
<tr>
<td>Multiple sites mutation</td>
<td>VRall</td>
<td>All four sites except R151G</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VRall-37KF</td>
<td>VRall without mutation at 37-38</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VRall-137S</td>
<td>VRall without mutation at 137</td>
<td></td>
</tr>
<tr>
<td>ORF5-replaced mutation</td>
<td>JAP5</td>
<td>Entire ORF5 was replaced with that of JA142</td>
<td></td>
</tr>
<tr>
<td></td>
<td>JAP5BM</td>
<td>Sequences at 32-34, 37-38, 57-59 and 137 were restored as those of VR2332</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SDP5</td>
<td>Entire ORF5 was replaced with that of SDSU73</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SDP5BM</td>
<td>Sequences at 32-34, 37-38, 57-59 and 137 were restored as those of VR2332</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1. Susceptibility of different groups of PRRSV field isolates to neutralizing antibody (1:64) raised against the VR-2332 strain of PRRSV. The horizontal line indicates the cut-off value (1:16) for the virus classification: resistant and susceptible group to antibody. Each box represents the 25 to 75% of observation. Error bars on each box represent the 10% (bottom) and 90% (top) values of observations, respectively. Dots above or below the error bars on each box represent outliers not included in the range between the 10 and 90% of observation. Solid and dash lines in each box are median and mean, respectively. When both values were identical, only median value is shown. Where only mean value is indicated, median value is either of the box lines (bottom or top) closer to the mean value. The name of groups is shown at the top and bottom of the plot. Asterisk represents the significant reduction ($p<0.05$) in the susceptibility to the antiserum in comparison with Sc group.
Figure 2. Viremia pattern of various groups of PRRSV field isolates in pigs passively immunized with PRRSV-specific immunoglobulin (circle) or normal serum globulin (square). The viruses were categorized to Sc (n=3), Sp (n=4), Svt (n=3), and R (n=4, 3, and 3 for R1, R2, and R3, respectively) by *in vitro* and *in vivo* assays using antisera raised against VR-2332. Dots are the mean of virus titers and bars represent standard variation. The numbers show the area between two curves.
Figure 3. Decay of neutralizing antibody (dotted line) in IgG-transferred pigs and ELISA antibody response (Solid line) of NSG-transferred pigs after virus challenge. ELISA antibody was measured in NSG-transferred pigs after virus challenge whereas serum-virus neutralizing (SVN) antibody was measured in IgG-transferred pigs to monitor the level of the transferred VN antibody. Horizontal line represents the cutoff S/P ratio (0.4) for positive in ELISA test. DPC represents the day post challenge.
Figure 4. Aligned deduced amino acid sequence of ORF5 of selected viruses. All amino acid sequences are compared to VR-2332. Dots indicated the same amino acid as in VR-2332. Letters represent the amino acid substitution at each site. The domains are identified based on the previous studies (16;34;37;50). S, Ecto, and TM represent signal peptides, ectodomain, and transmembrane regions of GP5, respectively. The domains that have not been determined are indicated as ND. Conserved linear neutralizing epitope is shaded and underlined. ▼ indicates identified sites (site I-V) that have consistent amino acid changes by the comparison between defined groups based on in vitro and in vivo tests.
Figure 5. Aligned deduced amino acid sequence of ORF5 of mutants. ▼ indicates identified sites (site I-V).
Figure 6. The susceptibility of PRRSV mutants generated by site-directed mutagenesis of VR-2332 infectious cDNA clone to the neutralizing activity of VR-2332 antiserum. Each box represents the 25 to 75% of the observations obtained from 6 independent repeats of VN test. Solid and dash lines in each box are median and mean, respectively. When both values were identical, only median value is shown. Where only mean value is indicated, median value is either of the box lines (bottom or top) closer to the mean value. The name of mutants is shown at the bottom of the plot. Asterisk represents the significant reduction ($p<0.05$) in the susceptibility to the antiserum in comparison with VR2332E rescued from the original infectious clone.
Figure 7. The susceptibility of PRRSV mutants generated by site-directed mutagenesis or gene swapping of VR-2332 infectious cDNA clone to the neutralizing activity of VR-2332 (A), JA142 (B), and SDSU73 (C) antisera. Each box represents the 25 to 75% of the observations obtained from 6 independent repeats of VN test. Solid and dash lines in each box are median and mean, respectively. When both values were identical, only median value is shown. Where only mean value is indicated, median value is either of the box lines (bottom or top) closer to the mean value. The name of mutants is shown at the bottom of the each plot. Asterisk represents the significant reduction or increase in the susceptibility to the antisera in comparison with VR2332E rescued from the original infectious clone ($p < 0.05$).
Figure 8. Predicted protein profile of ORF5 product (GP5) of susceptible and resistant viruses. Panel A is hydrophilicity plot of ORF5. Panels B and C are antigenicity and surface probability plot of VR-2332 (susceptible virus) and JA-142 (resistant virus, R3), respectively. ▼ indicates identified sites (site I-V).
CHAPTER 4: THE REQUIREMENT OF STRUCTURAL PROTEINS IN CROSS-NEUTRALIZATION AMONG DIFFERENT PRRS VIRUSES

(A paper to be submitted to Journal of Virology)

Won-Il Kim and Kyoung-Jin Yoon

Abstract

An in vitro study was conducted to comparatively assess the role of each structural protein (ORFs 2 to 6) of type 2 porcine reproductive and respiratory syndrome virus (PRRSV), porcine artervirus, in cross-neutralization of viruses. Chimeric mutants were generated by replacing ORF5 of an infectious clone constructed based on the VR-2332 strain (prototype PRRSV type 2) with that of strain JA142, SDSU73, PRRS124 or 2M11715 which were genetically and antigenically distinct from VR-2332. Chimeric mutants were also constructed by substituting one or more other structural genes of the VR-2332 infectious clone with corresponding gene(s) of JA142. Neutralization assays were performed in vitro on all mutants to determine the effect of substitutions on the virus neutralizing (VN) activity of antisera generated against VR-2332, JA142, SDSU73 or PRRS124. All ORF5-replaced mutants showed the level of susceptibility or resistance similar to that of the donor strains against homologous or heterologous antisera but failed to achieve a complete reversion of cross neutralization. In contrast, substitution of ORFs 3-6 completely reversed the susceptibility of the virus to the VN activity of VR-2332 or JA142 antiserum. Changes in ORFs 3, 5, and 6 were additively responsible for such reversion of the susceptibility between
VR-2332 and JA142, suggesting that the genetic similarity of ORFs 3 and 6 besides ORF5 should be considered for better cross neutralization between two different PRRS viruses.

**Introduction**

Porcine reproductive and respiratory syndrome (PRRS) is one of the most economically important viral diseases in the swine industries and is characterized by reproductive failure in adult pigs and respiratory illness and systemic infection in all ages of pigs (1;8;37;44). The causative agent is an arterivirus named PRRS virus (PRRSV) which is a member of the family *Arteriviridae* in the order *Nidovirales* with *Coronaviridae*, *Roniviridae* and *Toroviridae* (7;11;18). In general, PRRS viruses are classified into two genotypes; European (type 1) (51) and North American (type 2) PRRSV (5). The two genotypes of the virus share less than 70% homology in the entire genome (12;50) and are serologically distinct (30).

PRRSV contains a single 15kb-long single-stranded, positive-sense RNA genome, which is capped at the 5’ end and polyadenylated at the 3’ end. The viral genome encodes at least 9 open reading frames (ORFs) (13), each of which is expressed via the generation of a 3’-coterminal nested set of subgenomic (sg) mRNA’s (2;18). ORFs 1a and 1b, which take almost 2/3 of the genome, encode nonstructural proteins (Nsps) required for virus replication (2). At least 12 Nsps are generated as a result of serial cleavages of two polyproteins expressed from ORF1a and ORF1ab, respectively, which are encoded by ribosomal frame shift (45). Three N-glycosylated minor envelope proteins (GP2a, GP3, and GP4) are translated from ORF 2a, 3 and 4 and form heterotrimers by disulfide linkage (55). ORF2b, which is completely embedded in ORF2a, encodes another non-glycosylated minor protein
named as 2b (56). ORF5 codes the major envelope protein (GP5) that forms a heterodimer with membrane (M) protein expressed from ORF6 (32). This heterodimer structure dominates the virion surface and GP5 is believed to be the most important protein in the induction of virus neutralizing antibody (17;40;49). Nucleocapsid (N) is expressed from ORF7 and is the antigenic basis for a high level of non-neutralizing antibody which is induced in pigs during the early stage of PRRSV infection (13).

Numerous studies have demonstrated remarkable genetic and antigenic variability among PRRS viruses, emphasizing its negative effect on the accuracy of diagnostic test and on the efficient cross-protection among different viruses (3;29;39). Moreover, “atypical” PRRS, which was characterized by a high rate of abortion and mortality, has emerged even from vaccinated herds. Such an event is strong impediment to the development of effective vaccine and the eradication of the disease (33;57). Although the critical role of GP5 in the induction of neutralizing antibody is undeniable, recent studies have reported the incomplete protection conferred by ORF5-based vaccines (21;22), suggesting the potential involvement of other structural proteins in the interaction with target cells and protection against virus infection (14;48;53;55). Therefore, the following study was conducted to assess and compare the role of each PRRS viral structural protein encoded by ORFs 2 to 6 in cross-neutralization between genetically and antigenically distinct viruses.

**Materials and Methods**

**Experimental design.** The study was conducted using a gene-swapping technique to assess the contribution of each structural gene product of PRRSV to cross-neutralization
between two genetically and antigenically different viruses. Since GP5 has been postulated to be the major component for viral neutralization (17), ORF5 of an infectious clone constructed based on the sequence of the VR-2332 (38) was replaced with that of genetically much diverse PRRSV strains, such as JA142, SDSU73, PRRS124 and 2M11715, to generate chimeric mutants. The VR-2332 infectious cDNA was kindly provided by Dr. Kay Faaberg at University of Minnesota (St. Paul, MN). The virus neutralization (VN) test was then conducted on those mutants to evaluate the role of the ORF5 product in cross-neutralization among different PRRS viruses. In addition, each of the other membrane-associated structural genes (i.e., ORFs 2-4 and ORF6) of the VR-2332 infectious clone was replaced with the corresponding gene of JA142 to assess the role of each structural gene product in cross-neutralization between VR-2332 and JA142 by assessing the change in their susceptibility or resistance to the neutralizing activity of VR2332 or JA142 antisera (i.e., two-way analysis). Growth competence and equivalence of wild and mutant viruses in MARC-145 cells was checked by multi-step growth curve analysis to make sure that: a) the lack of or less growth of any virus in the presence of antiserum was not due to the inability of the virus to grow in the cell; or b) increased resistance to virus neutralization was not due to the enhanced adaptation of the virus to the cell.

**Viruses and cells.** Five field isolates of North American PRRSV were used for this study: VR-2332 (prototype PRRSV type 2), two PRRS viruses (JA142 and SDSU73) from ‘acute’ PRRS cases (23) and two recent field isolates (PRRS124 and 2M11715) (23;26). These viruses were genetically very distinct from each other (Figure 1). Their antigenic divergence from the VR-2332 strain were well documented elsewhere (25;26). Sequence
homologies among the viruses are summarized in Table 1. All of the viruses were propagated in MARC-145 cells, a highly permissive clone of the African Monkey kidney cell line MA104 (24) and represented 4 cell passages. The PRRS124 strain was kindly provided by Dr. Fernando Osorio at the University of Nebraska in Lincoln, NE.

**Gene-swapping mutagenesis.** Each ORF was replaced by PCR-based mutagenesis developed in our laboratory based on the concept of random mutagenesis (36). Briefly, each target ORF was amplified with RT-PCR using QIAGEN® One-Step RT-PCR kit (Qiagen, Valencia, CA, USA). Amplification primers were listed in Table 2. PCR products were purified after electrophoresed using QIAquick® Gel Extraction kit (Qiagen). For gene-swapping mutagenesis, a shuttle vector, which contained the whole structural genes (ORFs 2-7) of the VR-2332 infectious clone in TOPO® XL PCR cloning vector (Invitrogen, Carlsbad, CA, USA), was used as a template and the purified PCR products were used as primers. The mutagenesis was performed using the QuikChange® II site-directed mutagenesis kit (Stratagene, West Cedar Creek, TX, USA). The PCR reaction was prepared in a final volume of 25 µl as following: 2.5 µl of 10x reaction buffer, 0.5 µl of dNTP mix, 125 ng of primer (purified PCR product), 25 ng of template (shuttle vector), 1 µl of DNA polymerase, and water (molecular biology grade) up to 25 µl. The cycling conditions were 95°C for 1 min and 25 cycles of 95°C for 50 sec, 60°C for 50 sec, and 68°C for 16 min (2 min per kb). After amplification, 0.5 µl of Dpn I restriction enzyme (Stratagene) was added to the reaction and the mixture incubated for 2 hrs at 37°C. Then, 2 µl of the reaction was used for transformation using XL10-Gold® Ultracompetent cells (Stratagene) as per the
manufacturer’s protocol. Gene swap was confirmed by sequencing the whole structural genes in the shuttle vector before the structural genes were replaced back into the infectious clone.

**Generation of mutant virus.** The infectious clone which was constructed based on the sequence of VR-2332 was used for this study (38). The whole fragment of structural genes in the shuttle vector was substituted for that in the infectious clone using BsrGI and HpaI enzyme sites. The infectious clone containing a mutation in the right position was selected by sequencing after purifying the plasmid from competent cells (XL10-Gold®). The plasmid of the infectious clone was then linearized by AciII and purified using DNAClear™ kit (Ambion, Austin, TX, USA). The linearized plasmid was transcribed using T7 promoter by mMESSAGE mMACHINE® T7 kit (Ambion). The transcribed RNA was purified by MEGAClear™ kit (Ambion) and 1-10 µg of purified RNA was transfected into MARC-145 cells (5 × 10⁶ cells/ml) prepared in chilled Dulbecco’s Modified Eagle’s Medium (DMEM, Sigma, St. Louis, MO, USA) containing 1.25% Dimethyl sulfoxide (DMSO, Sigma) by electroporation at 250 volts and 950 µF (Gene Pulser Xcell Electroporation System, Bio-Rad, Hercules, CA, USA) (46). The transfected cells were plated on 6-well cell-culture plates (Fisher, Pittsburgh, PA, USA) and the media (DMEM) was changed with RPMI-1640 media (Sigma) supplemented with 10% fetal bovine serum (FBS, Sigma), 20mM L-glutamine, 100 IU/ml penicillin, 100µg/ml streptomycin, 50µg/ml gentamicin and 0.25µg/ml amphotericin B (hereafter RPMI growth media) 16-18 hrs after plating. At 48 hours after transfection, 0.2 ml of the cell culture supernatant collected from each well of the plates containing transfected
cells were inoculated on MARC-145 cells freshly prepared in 24-well plates. After incubation for 1 hr at 37°C, the inoculum was replaced with RPMI growth media. The inoculated cells were kept under observation until cytopathic effect (CPE) became evident. Each of the rescued mutants was propagated once in a 25-cm² cell culture flask (Fisher) and stored at -80°C until used.

**Antisera.** Antisera against VR-2332 were collected from 22 PRRSV-naive pigs after 2 months post inoculation of the virus at a rate of 10³ TCID₅₀/ml via intramuscular route. Ten sera with virus neutralizing (VN) antibody titer of 1:64 or higher were combined together and used for the study after SVN titer of the serum was adjusted to 1:64. One (VN titer = 64) of the 10 antisera was obtained from Dr. Fernando Osorio at the University of Nebraska in Lincoln.

Antisera against JA142 and SDSU73 were generated from the previous study in a similar manner (23). In brief, JA142 and SDSU73 were intranasally or intramuscularly inoculated in each 10 pigs at a rate of 10³ TCID₅₀/ml. The sera collected at 42 days after inoculation were combined together for each inoculated virus (VN titer = 1:64 for both antisera). An antiserum against PRRS124 (VN titer = 1:32) was kindly supplied from Dr. Osorio’s laboratory at the University of Nebraska-Lincoln.

All sera were tested by Western immunoblotting and immunofluorescence microscopy for their representation of antibody repertoire against each PRRS viral structural protein.
Virus neutralization assay.  Florescent focus neutralization (FFN) assay was performed as previously described (56) to assess the susceptibility of reference field viruses or rescued chimeric mutant viruses to the neutralizing activity of the antisera raised against VR-2332, JA142, SDSU73 and PRRS124 as described above. After heat inactivation at 56°C for 60 min, a pair of PRRSV antibody-positive and -negative sera was serially diluted 2-fold in RPMI growth media and mixed with an equal volume of each virus at a rate of 100 florescent focus-forming units (FFU) per 0.1 ml. Mixtures of virus and antiserum were then incubated for 1 hr at 37°C. The mixtures were inoculated onto MARC-145 cell monolayer prepared in a 96-well plate approximately 48 hrs and incubated for another 1 hr at 37°C. The inoculums were removed from the plates and the cells were replenished with 0.2 ml/well of fresh RPMI growth media. The inoculated cells were further incubated for 20 hrs at 37°C in a humid 5% CO₂ atmosphere. After cell culture media was removed, the cells were washed once with 0.01M phosphate-buffered saline (PBS, pH 7.2) and fixed with cold acetone-water (8:2) mixture. The plates were dried at room temperature, washed once with PBS, and then stained with PRRSV-specific monoclonal antibody SDOW-17 that was conjugated with fluorescein isothiocyanate (FITC) (Rural Technologies, Brookings, SD). After 1 hr incubation at 37°C, the wells were washed three times with PBS, and the number of virus-specific fluorescent foci in each well was counted. VN titer was expressed as reciprocal of the highest dilution in which 90% or higher reduction in the number of FFU was observed. The assay was repeated six times on six different days.
**Sequencing.** All of the viruses were sequenced for ORFs 2 to 6. PCR and sequencing primers were as follows:

- **ORF2 to 6 Forward (PSF):** 5’- CCACTGCCACCAGCTTGAAGTT -3’;
- **ORF2 to 6 Reverse (PSR):** 5’- CAGACACAATTGCGCTCACTAGG -3’.

Viral RNA was extracted using the QIAamp® viral RNA mini kit (Qiagen) according to the manufacturer’s recommendation. For sequencing full-length of ORFs 2-6, reverse transcription (RT) was conducted using Superscript™ III (Invitrogen) according to the manufacturer’s recommended protocol and cDNA was amplified with the GeneAmp® XL long PCR kit (Applied Biosystems, Foster City, CA, USA). The cycling conditions were 94°C for 1 min, 16 cycles of 94°C for 15 sec and 68°C for 4 min followed by 14 cycles of 94°C for 15 sec and 68°C for 4 min with time increased by 15 sec per cycle, and then extension at 68°C for 10 min. The PCR products were purified using QIAquick® PCR purification kit (Qiagen) and submitted to the ISU Nucleic Acid Facility for sequencing. Consensus sequence for each virus from raw sequence data was formulated using Lasergene® DNA analysis software (DNAsStar, Madison, WI, USA). Sequencing was repeated at least once more for each virus as proof-reading.

**Multi-step growth curve analysis.** To characterize growth competence and equivalence of mutants in cell culture as compared to the wild type viruses, a multi-step growth curve was determined for each virus. Viruses (0.01 m.o.i) were inoculated on the confluent monolayer of MARC-145 prepared in 25-cm² cell culture flasks. After 1 hr incubation at 37°C, the inoculum was discarded and the cells were replenished with RPMI
growth media. Five hundred µl of supernatant were taken from each flask at 0, 12, 24, and every 24 hrs thereafter until 4 days post inoculation. Each supernatant was subjected to virus titration using a microtitration infectivity assay (19). Virus titers were recorded as TCID_{50}/0.1ml (27). This procedure was repeated three times on three separate days for each virus.

**Production of recombinant PRRSV structural proteins.** To construct baculovirus expression vectors containing ORFs 2 to 6, the specific cDNA of each ORF was first amplified from the genomic RNA of VR-2332 by RT-PCR with amplification primers which contain EcoRI and XbaI sites for further manipulation. The sequences of the PCR primers were as follows:

\[
\begin{align*}
\text{P2EcoF} & : 5' - \text{GAATTCGAATTCTCCCCCGGGCCCTGTCATT} - 3' \\
\text{P2XbaR} & : 5' - \text{TCTAGATCTAGACCTACGGGTTCGTAGA} - 3' \\
\text{P3EcoF} & : 5' - \text{GAATTCGAATTCATGGTTAATAGCTGTACATT} - 3' \\
\text{P3XbaR} & : 5' - \text{TCTAGATCTAGAATTCTCATCTGTCACATTTGGG} - 3' \\
\text{P4EcoF} & : 5' - \text{GAATTCGAATTCATGGCTTCGTCCCTTCTTT} - 3' \\
\text{P4XbaR} & : 5' - \text{TCTAGATCTAGACCTACGGGTTCGTAGA} - 3' \\
\text{P5EcoF} & : 5' - \text{GAATTCGAATTCATGGCTTCGTCCCTTCTTT} - 3' \\
\text{P5XbaR} & : 5' - \text{TCTAGATCTAGACCTACGGGTTCGTAGA} - 3' \\
\text{P6EcoF} & : 5' - \text{GAATTCGAATTCATGGCTTCGTCCCTTCTTT} - 3' \\
\text{P6XbaR} & : 5' - \text{TCTAGATCTAGACCTACGGGTTCGTAGA} - 3'.
\end{align*}
\]
Each PCR product was sequenced for verification and, if correct, then cloned into a plasmid vector pGEM-T-easy (Promega, Madison, WI, USA). Each PCR product in pGEM-T-easy vector and the baculovirus protein expression plasmid pFastBac1 (Invitrogen, Carlsbad, CA, USA) were digested by EcoRI and XbaI (New England BioLabs, Beverly, MA, USA), purified after electrophoresed, and ligated to each other using the restriction enzyme sites. After transformation, pFBP2, pFBP3, pFBP4, pFBP5, and pFBP6 were constructed and the authenticity of each construct was confirmed again by sequencing. The pFBP2, pFBP3, pFBP4, pFBP5, and pFBP6 were then individually transformed into DH10Bac™ to generate recombinant baculoviruses (AcP2, AcP3, AcP4, AcP5, and AcP6) by transposition into AcMNPV genomic DNA according to the manufacturer’s instructions ("BAC-TO-BAC™ manual, Invitrogen). Recombinant viruses were inoculated in Sf-9 insect cells (Invitrogen) and propagated for 4 days. Rescued recombinant viruses in the supernatant were confirmed by sequencing. Cells infected with such viruses were used as antigen for western immunoblotting and/or immunofluorescence microscopy to confirm the presence of each recombinant protein. Uninfected Sf-9 cells and the cells infected with wild baculovirus were used as controls for both assays.

**SDS-PAGE and Western immunoblotting.**

**Sample preparation.** Sf-9 cells infected with baculovirus-expressed recombinant proteins of PRRSV or wild baculovirus (virus control) and uninfected cells (mock control) were pelleted by centrifugation for 10 min at 2000 x g. Each of the resulting cell pellets was lysed in a Tris lysis buffer (10mM Tris, 0.04% CHAPS, 0.15M NaCl, 0.0001% aprotinin and 1mM EDTA, pH 8.0) and mixed with 5X sample buffer containing 0.6M Tris-HCl (pH 6.8),
25% glycerol, 2% SDS, 10% β-mercaptoethanol and 0.1% bromophenol blue, and boiled for 5 min at 100°C.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).** Twenty µl of each sample was loaded on each well of a vertical 12% polyacrylamide slab gel and electrophoresed for 80 min at the constant voltage of 100 along with pre-stained molecular weight markers (Bio-Rad). After electrophoresis, polypeptides and the markers separated on each gel were electrotransferred onto a 0.2 µm nitrocellulose membrane (Bio-Rad) overnight at the constant voltage of 30 under a refrigerating condition. Each membrane was blocked with a 1% skim milk solution by submerging the membrane in the solution for 30 min at ambient temperature.

**Western immunoblotting.** Each serum sample (VR-2332 antiserum, JA142 antiserum, or PRRS-negative serum) was diluted 1:50 in 20mM Tris-buffered saline solution with 0.05% Tween 20 (TBST, pH 7.5) which contained 1% of uninfected or wild baculovirus-infected Sf-9 cell lysates to absorb non-specific antibodies. The diluted sera were incubated for 30 min at 37°C and spun down for clarification for 10 min at 2000 x g. The membrane with electrotransferred polypeptides and markers was incubated with each of the clarified sera for 1 hr at ambient temperature with slow and gentle rocking, and then rinsed 3 times using TBST. The membrane was incubated with optimally diluted goat anti-swine IgG labeled with peroxidase (Kirkegaard-Perry Laboratories, Gaithersburg, MD, USA) for 1 hr at ambient temperature. Then, antigen-antibody reactions were visualized by adding TMB substrate (Kirkegaard-Perry Laboratories) to the membrane and incubating for up to 5 min at ambient temperature. Colorimetric reaction was stopped by rinsing the membrane with distilled water. The presence of antibodies specific for PRRS viral proteins in each
serum was confirmed by comparison of band patterns against virus and cellular antigens and molecular discrimination of specific bands.

**Indirect florescent antibody (IFA) test.** Antigens of recombinant baculoviruses were prepared by inoculating Sf-9 cells prepared in 96-well plates with 50 µl/well of each of the recombinant baculoviruses AcP2, AcP3, AcP4, AcP5, or AcP6 and then fixing infected cells with a cold acetone-water (8:2) mixture after a 4 day incubation at 37°C. Uninfected Sf-9 cells and wild baculovirus-infected cells were prepared in the identical manner and used as negative and virus controls, respectively. All plates were dried and stored at -20°C until use.

For testing, each serum sample was first prepared in the same manner as described for western immunoblotting. Fifty µl of each diluted sample was applied to 3 wells: one with recombinant antigen and the others with control antigens (i.e., uninfected Sf-9 cells and wild baculovirus-infected cells). The plates were incubated for 1 hr at 37°C and cells washed with 0.01M PBS with 0.05% Tween 20 (PBST, pH 7.2). The antigen-antibody reaction was visualized by applying optimally diluted goat anti-swine IgG antibody conjugated with FITC (Kirkegaard and Perry Laboratories) and incubating for another 1 hr at 37°C. Each plate was observed for specific staining under a fluorescent microscope.

**Data analysis and Sequence comparison.** Statistical analyses on data were done using JMP (SAS Institute Inc., Cary, NC, USA). The repeated measurements of virus growth were analyzed with multivariate analysis of variance (MANOVA) to determine the growth difference among the structural gene-replaced mutant viruses. The VN test results on mutant
viruses were analyzed with Wilcoxon Rank Sum test (9) to assess the difference in the susceptibility to VN antibody among the mutants. Nucleic acid sequences of ORFs 2 to 6 were aligned and analyzed using Lasergene® MegAlign software (DNASTAR Inc., Madison, WI, USA) and Molecular Evolutionary Genetic Analysis (MEGA) software (version 2) (28).

Results

Antibody profile of tested antisera. Since each antiserum specific for VR-2332 or JA142 was generated by pooling sera from 10 challenged animals, there was little concern that the antisera used in the VN test would be deprived of antibody against any structural gene product. Nonetheless, the antisera were tested with each recombinant structural protein expressed by a baculovirus system to confirm the presence of antibodies for all structural viral proteins of PRRSV. On Western immunoblotting and immunofluorescence microscopy, antibodies specific for linear or conformational epitopes of all structure proteins were detected in the antisera (Figure 2). Linear epitope-dependent antibodies were apparently detected against ORF3 and ORF5 (Figure 2A and 2B) while conformational epitope-dependent antibodies were detected against all recombinant proteins (Figure 2C).

Role of the major envelope protein (GP5) of PRRSV in cross neutralization. As shown in Table 3, four ORF5-replaced mutants (JAP5, SDP5, 124P5, and 11715P5) whose ORF5 genes were donated from JA142, SDSU73, PRRS124 and 2M11715, respectively, were generated. In the VN test with anti-VR2332 antiserum, the susceptibility of these mutants to the VN activity of the VR-2332 antiserum was decreased by 2- to 4-fold
compared to that of VR2332E which was rescued from the original VR-2332 infectious clone. However, those mutants failed to recover the same level of resistance as their donor viruses to the VN activity of the VR-2332 antiserum. Their susceptibility to the VR-2332 antiserum was significantly higher than that of their donor viruses (p<0.01) (Figure 3A).

When the mutants were tested against the corresponding antiserum raised to each donor virus, their susceptibility was increased by 4- to 16-fold compared to that of VR2332E. However, they failed to recover the same level of susceptibility as their donor viruses to the VN activity of the corresponding antiserum. Their susceptibility to the antiserum generated against their donor viruses was significantly lower than that of their donor viruses (p<0.01) (Figure 3B, 3C, and 3D).

**Association of other envelope-associated proteins (GP2, GP3, GP4, M) of PRRSV with cross-neutralization.** Since the replacement of ORF5 failed to accomplish the full reversal of cross-neutralization between different viruses, single or multiple structural genes (ORFs 2 to 6) of the infectious clone was/were replaced with that/those of JA142 to generate a variety of chimeric viruses (Table 3). The growth ability of single ORF-replaced viruses (i.e., JAP2, JAP3, JAP4, JAP5, and JAP6) in MARC-145 was compatible (p=0.211) with that of VR-2332E as shown in Figure 4. However, the JAP3 mutant grew a bit better than the rest of the chimeric mutant viruses (p=0.0038).

**Effect of replacement of single envelope-associated structural gene on cross neutralization of PRRSV.** When the VN test was performed on single gene-replaced chimeric mutant viruses against the VR-2332 antiserum (Figure 5A), JAP3 and JAP5
demonstrated a significantly higher level of resistance \((p<0.004)\) to the VN activity of the antiserum as compared to VR2332E, whereas JAP4 showed a lower level of resistance compared to JAP3 \((p=0.004)\) even though JAP4 still represented a higher level of resistance \((p=0.007)\) to the VN activity of the VR-2332 antiserum as compared to VR2332E. When JAP3N containing the N-terminal region of ORF3 of JA142 (a.a.1-194) and JAP3C containing the C-terminal region of ORF3 of JA142 (a.a.183-255) were tested against the VR-2332 antiserum, JAP3N showed the level of resistance similar to that of JAP3 to the VN activity of the antiserum and the resistance of JAP3C to the VN activity of the antiserum was similar to that of JAP4 (Figure 5A). In contrast, replacement of ORF2 or ORF6 of the VR-2332 infectious clone with those of JA142 did not have any effect on the susceptibility of these chimeric mutant viruses to the VN activity of the VR-2332 antiserum (Figure 5A), suggesting that the antigenic similarity of GP2 and M proteins between the two viruses is less critical for cross neutralization.

When the chimeric viruses were tested against JA142 antiserum, similar observations, but in an opposite way, were made as with the VR-2332 antiserum. JAP3 and JAP5 showed the highest susceptibility to the JA142 antiserum among the single gene-replaced mutants (Figure 5B). As was the case with the VR-2332 antiserum, the N-terminal portion of GP3 played a significant role in cross neutralization as JAP3N showed the level of susceptibility similar to that of JAP3 to the VN activity of the JA142 antiserum while the susceptibility of JAP3C to the VN activity of the JA142 antiserum was similar to that of JAP4 (Figure 5B). JAP6 also showed significant increase in the susceptibility against the JA142 antiserum compared to VR2332E \((p=0.005)\). In contrast, JAP2 and JAP4 represented a lower level of susceptibility to the VN activity of the JA142 antiserum in comparison to JAP3, JAP5, and
JAP6, although the susceptibility of JAP2 was statistically significant as compared to that of VR2332E ($p=0.025$) (Figure 5B).

**Effect of replacement of multiple envelope-associated structural genes on cross neutralization of PRRSV.** When multiple genes-replaced mutants (i.e., JAP56, JAP456, JAP3456, and JAP23456) were tested against the VR-2332 antiserum (Figure 5A), JAP56 showed significantly lower resistance to the VN activity of the antiserum than did JA142 ($p=0.003$). The resistance of JAP456 to the VN activity of the VR-2332 antiserum was higher than that of JAP56 ($p=0.023$) and the level of resistance to the VN activity of the VR-2332 antiserum was increased by incorporating more ORFs of JA142 to the VR-2332 infectious clone (i.e., JAP3456) which showed a complete reversion to resistance to the VN activity of the VR-2332 antiserum like JA142. Addition of ORF2 to JAP3456 did not increase the resistance to the VN activity of the VR-2332 antiserum higher than that of JAP3456.

On the other hand, JAP56 showed substantial increase in the susceptibility to the VN activity of the JA142 antiserum, and the level of susceptibility of JAP56 and JAP456 was statistically similar to that of JA142 ($p>0.05$) (Figure 5B). The mean value of susceptibility of chimeric mutant viruses to the VN activity of the JA142 antiserum as determined by the VN titer of the antiserum was increased to a higher level by replacing more ORFs of the VR-2332 infectious clone with the corresponding ORFs of JA142. JAP3456 and JAP23456 showed complete reversion to susceptibility to the VN activity of the JA142 antiserum (Figure 5B).
Discussion

It has been reported that GP4, GP5 and M proteins contain neutralizing epitopes (13) and GP5 is believed to be most important in inducing the protective antibody since GP5-neutralizing antibody was demonstrated to be most effective in virus neutralization (17;40;49). Nonetheless, there has been no direct evidence proving that GP5 is a prerequisite for cross-neutralization between different PRRS viruses. Furthermore, even though a linear conserved neutralizing epitope (NE) has been identified in the N-terminal ectodomain (37-45 SHLQLIYNL) of GP5, the question was raised if other determinants associated with virus neutralization exist to explain the negative effect of genetic variation on cross protection among PRRS viruses (41) since the linear GP5 NE is highly conserved in most of the PRRS viruses (42).

In a previous study conducted in our laboratory (26), two hypervariable regions (32-34 and 57-59) and two residues, histidine (H38) and leucine (L39) at N-terminus of the NE were identified to be significantly associated with virus neutralization by GP5. In addition, that study suggested that GP5 was critical but might not be the only element involved in virus neutralization because the complete reversion to resistance or susceptibility against homologous or heterologous antiserum could not be achieved even after the entire ORF5 region was replaced with that of a heterologous donor virus (26). In the present study, four chimeric mutants (JAP5, SDP5, 124P5, and 11715P5) that received foreign ORF5 genes from four antigenically and genetically distinct PRRSV strains were constructed from the VR-2332 based infectious clone and subjected to the VN test. As expected, those ORF5-replaced mutants failed to accomplish the complete reversion of cross-neutralization, even though they showed higher susceptibility to antisera raised against the donor viruses or
higher resistance to the VR-2332 antiserum (Figure 3). These observations further indicated that other gene products of PRRSV should also be involved in inducing VN antibody and play a significant role in cross neutralization.

The substitution of ORF3 or ORF5 of the VR-2332 infectious clone with that of JA142 (JAP3 and JAP5, respectively) resulted in the most apparent reversal effect on the susceptibility or resistance of the virus to the VN activity of VR-2332 or JA142 antisera (Figure 5A and B). This was, to our knowledge, the first direct demonstration of the involvement of GP3 in virus neutralization. The GP3 protein has been presumed not to be related to virus neutralization due to its equivocal identity in the virion, although immunization with baculovirus-expressed GP3 of a type 1 PRRSV demonstrated to confer protection against reproductive loss by PRRSV infection (43), and a monoclonal antibody against GP3 of PRRSV type 2 showed an even higher degree of virus neutralization activity than a neutralizing monoclonal antibody against GP5 in vitro (6). It has been shown that GP3 is incorporated into a virus particle, thus a structural protein in the case of PRRSV type 1 (34;47). However, the association of GP3 with virion structure could not be demonstrated for PRRSV type 2 (16;31). Furthermore, GP3 was shown to lack a typical membrane anchor and to be secreted in a considerable amount from infected cells (52;55). While its presence in virion is controversial, GP3 is reported to be essential for PRRS virus infectivity, and the binding of GP3 to GP2a/GP4 is required to release minor envelope proteins from ER to Golgi complex; otherwise GP2a/GP4 dimers are retained in ER (55). The involvement of the secreted form of glycoproteins in virus neutralization is not uncommon. Classical swine fever virus (CSFV), a single-stranded positive-sense RNA virus like PRRSV, expresses Erns glycoprotein which lacks a typical membrane anchor and is secreted from infected cells like
GP3 of PRRSV. Nonetheless, the Erns protein is the major protein to induce virus neutralizing antibody against CSFV besides E2 protein which is the major envelope glycoprotein of CSFV (54;58). In addition, Ebola virus, a negative-sense RNA virus, produces two versions of glycoprotein. While naive glycoprotein forms peplomers on the viral surface, a truncated version of the same protein is secreted in a large quantity from infected cells. This secreted form of the protein was speculated to waste the anti-Ebola virus neutralizing antibody, hence inhibiting the antiviral activity by antibody (20).

The VN test on JAP3N and JAP3C mutants demonstrated: a) that the N-terminal portion of GP3 was responsible for most of the negative effect on virus neutralization which resulted from the replacement of ORF3 and b) that the C-terminal portion of GP3 was responsible for the effect which resulted from ORF4-substitution (Figure 5). The unique region of JAP3N is between 72 and 181, which does not overlap with any other mutants except JAP3. As illustrated in Figure 6, the genetic variability is concentrated in two regions (79-106 and 134-153). The first variable region (79-106) resides in B-cell epitopes identified by previous studies (10;59), and tyrosine (Y79) and glycine (G83) in this region were demonstrated to be critical for the affinity of the monoclonal antibodies to these epitopes (59); however, no antigenic information is available for the second region (134-153). In comparison, JAP3C contains most of the overlapped region with ORF4. A neutralizing epitope in ORF4 of type 1 PRRSV was identified in the region between 59 and 67, which is overlapped with the C-terminus of ORF3. However, this appears not to be applicable to type 2 PRRSV because there is a gap of 4 residues in the corresponding site and the ORF4-neutralizing monoclonal antibody raised against this particular epitope of type 1 PRRSV could not block the infectivity of type 2 PRRSV (35). Therefore, we conclude that
the antigenic similarity of GP4 may have a limited impact on cross neutralization and its
effect may vary among strains even though detailed mapping of neutralizing determinants in
the C-terminus of GP3 and GP4 remains to be further studied.

The involvement of M protein in virus neutralization seemed to be variable depending
on the virus strain since the substitution of ORF6 showed significantly enhanced
susceptibility of the chimeric mutant virus (JAP6) against JA142 antiserum but did not have
much affect on the resistance against VR-2332 antiserum. Similarly, the susceptibility of
JAP56 against JA142 antiserum was significantly increased compared to JAP5 but the
resistance against VR-2332 antiserum did not have much affect in comparison with that of
JAP5 (Figure 5). The importance of GP5/M heterodimerization in the induction of
neutralizing antibody and the stimulation of lymphocytes proliferation has been demonstrated
in previous studies by other investigators (21;22). Therefore, it can be assumed that variable
factors in GP5 and M proteins may affect the general capacity of virus neutralization induced
by GP5/M dimers. Recently, the negative effect of N-glycosylation in the GP5 ectodomain
on the induction of neutralizing antibody has been reported (4;15). When two non-essential
N-glycosylation sites of GP5 were removed by mutagenesis, the mutant virus induced a
higher titer of neutralizing antibody more quickly than did the wild type virus. Interestingly,
the VR-2332 strain possesses one more N-glycosylation site (at position 30) in the GP5
ectodomain than JA142. Moreover, in our previous study (25), JA142 induced a much
higher level of neutralizing antibody (SVN=1:16-128) compared to VR-2332 (SVN=1:<2-
32) even though the levels of total antibody detected by IFA and ELISA were similar
between the 2 viruses. Therefore, it is presumed that heavy glycosylation in the GP5
ectodomain might mask epitopes in GP5 and M protein or conformational epitopes of GP5/M
dimers, resulting in enhanced resistance to the VN antibody. The ectodomain of M protein, which was predicted as a 10-18 amino acids-long stretch at N-terminus, is believed to be responsible for the interaction with GP5 protein (13). Histidine at position 10, which is located very next to the cysteine residue responsible for the disulfide-linkage to the GP5 ectodomain, was substituted with tyrosine in this region of JA142. In addition, a variable region was observed between 63 and 66 in M protein. B-cell epitope was identified between 151 and 174 of M protein (10), but no substitution was found in this region for JA142 (Figure 7). The effect of those substitutions on virus neutralization remains to be further determined.

Our study demonstrated that overall reversion of cross neutralization (i.e., susceptibility vs resistance) among PRRS viruses was additively affected by the antigenic similarity of various membrane-associated proteins. Complete reversion of cross-neutralization could be achieved by replacing all envelope-associated proteins (ORFs 2-6) although ORF2 substitution had a very minimal affect (Figure 5). Among the membrane-associated proteins of PRRSV, the results of the study strongly indicate that the genetic similarity of ORFs 3 and 6 besides ORF5 must be taken into consideration to achieve the full capacity of virus neutralization between two different PRRS viruses. Fine mapping of neutralizing determinants in each viral protein should be carried out to improve antibody-mediated immune protection against PRRSV infection.

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transport of proteins encoded by ORFs 5 to 7 of porcine reproductive and respiratory syndrome virus. Virology 221:98-112.


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Characterization of two new structural glycoproteins, GP(3) and GP(4), of

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antigenic stability of PRRS virus in pigs. Field and experimental prospectives. 


### Tables

**Table 1.** Amino acid sequence identities (%) between VR-2332 and other virus strains OR F2 ORF3 ORF4 ORF5 ORF6

<table>
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<tr>
<th>Virus strain</th>
<th>ORF2</th>
<th>ORF3</th>
<th>ORF4</th>
<th>ORF5</th>
<th>ORF6</th>
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<td>VR-2332</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
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</tr>
<tr>
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<td>86.3</td>
<td>89.9</td>
<td>84.6</td>
<td>96.0</td>
</tr>
<tr>
<td>2M11715</td>
<td>94.2</td>
<td>89</td>
<td>89.4</td>
<td>88.6</td>
<td>98.3</td>
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Table 2. Oligonucleotide primers used to amplify each open-reading frame of PRRSV as part of construction of gene-swapped mutants from VR-2332-based infectious cDNA clone

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5’-3’)</th>
<th>Position*</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>JAP2F</td>
<td>TCCCCCGGGCCCTGTCATT</td>
<td>12031-12049</td>
<td>Forward primer for JAP2; 44-nucleotides upstream from start codon</td>
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<tr>
<td>JAP2R</td>
<td>CCTGCCTGGTGCAGTA</td>
<td>12894-12910</td>
<td>Reverse primer for JAP2; 49-nucleotides downstream from stop codon</td>
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<tr>
<td>JAP3F</td>
<td>^ATGGCTAATAGCTGTCAC</td>
<td>12698-12718</td>
<td>Forward primer for JAP3 and JAP3N</td>
</tr>
<tr>
<td>JAP3-557R</td>
<td>AAACCAACCAAGAGGAAAAGA</td>
<td>13254-13277</td>
<td>Reverse primer for JAP3N</td>
</tr>
<tr>
<td>JAP3-549F</td>
<td>GCTGCGCCCTCTTTTT</td>
<td>13246-13262</td>
<td>Forward primer for JAP3C</td>
</tr>
<tr>
<td>JAP3R</td>
<td>ATTCTCATCTGTGACATTGG</td>
<td>13489-13509</td>
<td>Forward primer for JAP3 and JAP3C; 27-nucleotides downstream from stop codon</td>
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<tr>
<td>JAP4F</td>
<td>GCTGCGCCCTTCTTTTC</td>
<td>13246-13263</td>
<td>Forward primer for JAP4; 3 nucleotides downstream from start codon</td>
</tr>
<tr>
<td>JAP4R</td>
<td>ATTCATATGGCCAGTAAGATG</td>
<td>13761-13781</td>
<td>Reverse primer for JAP4</td>
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<tr>
<td>P5F</td>
<td>CCTGAGACCATGAGGTGGG</td>
<td>13717-13735</td>
<td>Forward primer for all ORF5-swapped mutants; 73-nucleotides upstream from start codon</td>
</tr>
<tr>
<td>P5R</td>
<td>TTTAGGGCATTatatCATCACTGG</td>
<td>14458-14480</td>
<td>Reverse primer for all ORF5-swapped mutants; 66-nucleotides downstream from stop codon</td>
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<td>14875-14896</td>
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* Based on JA142 full-length sequence (AY424271).

# Start or stop codon was indicated with underline, otherwise refer to the note.
Table 3. List of gene-swapped mutants constructed from VR2332-based infectious clone

<table>
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<th>Category</th>
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<th>Replaced gene(s)</th>
<th>Mutation</th>
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<td>JAP2</td>
<td>ORF2</td>
<td>Replacement with ORF2 of JA142</td>
</tr>
<tr>
<td></td>
<td>JAP3</td>
<td>ORF3</td>
<td>Replacement with entire ORF3 of JA142</td>
</tr>
<tr>
<td></td>
<td>JAP3N</td>
<td>ORF3 (a.a.1-194)</td>
<td>Replacement with N-terminal portion of ORF3 of JA142</td>
</tr>
<tr>
<td></td>
<td>JAP3C</td>
<td>ORF3 (a.a.183-255)</td>
<td>Replacement with C-terminal portion of ORF3 of JA142</td>
</tr>
<tr>
<td></td>
<td>JAP4</td>
<td>ORF4</td>
<td>Replacement with ORF4 of JA142</td>
</tr>
<tr>
<td></td>
<td>JAP5</td>
<td>ORF5</td>
<td>Replacement with ORF5 of JA142</td>
</tr>
<tr>
<td></td>
<td>JAP6</td>
<td>ORF6</td>
<td>Replacement with ORF6 of JA142</td>
</tr>
<tr>
<td></td>
<td>SDP5</td>
<td>ORF5</td>
<td>Replacement with ORF5 of SDSU73</td>
</tr>
<tr>
<td></td>
<td>124P5</td>
<td>ORF5</td>
<td>Replacement with ORF5 of PRRS124</td>
</tr>
<tr>
<td></td>
<td>11715P5</td>
<td>ORF5</td>
<td>Replacement with ORF5 of 2M11715</td>
</tr>
<tr>
<td>Multiple genes-replaced</td>
<td>JAP56</td>
<td>ORFs 5-6</td>
<td>Replacement with ORF5-6 of JA142</td>
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<td></td>
<td>JAP456</td>
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<td>JAP3456</td>
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<td>Replacement with ORF3-6 of JA142</td>
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<td></td>
<td>JAP23456</td>
<td>ORFs 2-6</td>
<td>Replacement with ORF2-6 of JA142</td>
</tr>
</tbody>
</table>
Figure 1. Genetic diversity of PRRS viruses used in the study. The radial dendogram was created based on sequences of ORFs 2-6 and rooted with VR-2332. The bar represents one nucleic acid change in 100 sequences.
Figure 2. PRRS viral protein specificity profile of antisera using recombinant proteins as determined by Western immunoblotting against VR-2332 antiserum (A) and JA142 antiserum (B) or immunofluorescence microscopy against both antisera (C). Mock and WT indicate Sf-9 cells uninfected or infected with wild-type baculovirus, respectively. Specific bands for viral proteins (ORF3 and ORF5) are indicated with arrow heads.
Figure 3. Virus neutralizing antibody titer of VR-2332 antiserum (A), JA142 antiserum (B), SDSU73 antiserum (C) and PRRS124 antiserum (D) against wild type (JA142, SDSU73, PRRS124, 2M11715) or ORF5-replaced mutant (JAP5, SDP5, 124P5, 11715P5) viruses. Y axis indicates VN titer (log2). Each mutant was constructed from VR2332-based infectious cDNA clone by replacing its ORF5 with that of wild type viruses. Each box represents the 25 to 75% of the observations obtained from 6 separate attempts of VN test. Solid and dash lines in each box are median and mean, respectively. When both values were identical, only median value is shown. Where only mean value is indicated, median value is either of the box lines (bottom or top) closer to the mean value. The name of mutants is shown at the bottom of each plot. Asterisk represents the significant reduction or increase in the susceptibility to the antisera in comparison with VR2332E rescued from the VR2332 infectious clone ($p<0.05$).
Figure 4. Multiple-step growth curves of various mutant PRRS viruses constructed by replacing each structure gene of the VR2332-based infectious clone with the corresponding gene of the JA142 strain. Each replaced structure gene is indicated by the number following “JAP”. VR2332E is the virus rescued from the intact VR-2332 infectious clone.
Figure 5. Virus neutralizing activity of VR-2332 antiserum (A) and JA142 antiserum (B) against wild type viruses (JA142 and VR2332E) or chimeric mutant viruses constructed by replacing one or more open-reading frames (ORFs) of VR-2332 based infectious clone with the corresponding ORFs of the JA142 strain. Y axis indicates VN titer (log₂). Each swapped gene is identified by the number on each mutant. The name of mutants is shown at the bottom of each plot. Each box represents the 25 to 75% of the observations obtained from 6 separate attempts of VN test. Solid and dash lines in each box are median and mean, respectively. When both values were identical, only median value is shown. Where only mean value is indicated, median value is either of the box lines (bottom or top) closer to the mean value. Asterisk represents the significant reduction or increase in the susceptibility to the antisera in comparison with VR2332E rescued from the VR2332 infectious clone ($p<0.05$).
Figure 6. Alignments of ORF3 sequences of various field isolates of PRRSV. B-cell epitopes previously identified are underlined (10) or boxed (68).
Figure 7. Alignments of ORF6 sequences of various field isolates of PRRSV. Putative ectotomain is underlined. Identified B-cell epitope is boxed (10).
CHAPTER 5: GENERAL CONCLUSIONS

Cross neutralization is greatly affected by genetic variation among challenge PRRS viruses. The detrimental affect of antigenic diversity on the serodiagnosis of PRRS virus infection has been documented between two major genotypes of PRRS viruses (i.e., PRRSV type 1 and 2) (136). In addition, many studies have demonstrated a great deal of genetic and antigenic diversity among PRRS viruses within the genotypes (10;70;124;153). However, the effect of lesser degrees of genotypic heterology on the performance of PRRS serodiagnostics has not been investigated. Therefore, the first study was conducted to determine if serologic data and the performance of serologic assays are influenced by genotypic and/or biotypic differences of PRRS viruses and, if so, to assess the degree of effect.

The IFA and ELISA (IDEXX HerdChek® PRRS 2XR) tests showed a similar diagnostic performance on samples collected during the study period with respect to detecting animals seropositive for various antigenically distinct PRRS viruses. Observations in this study clearly indicate that the IFA test using a strain of PRRS virus can detect pigs exposed to antigenically distinct PRRS viruses, which was unexpected, and is able to detect the exposed animals earlier than ELISA. Therefore, the IFA test may be a good alternative test for confirmation when ELISA results are questionable. On the other hand, the cross serum virus neutralization (SVN) test was greatly affected by genetic variation among the challenge viruses. Therefore, SVN antibody was efficacious only for the homologous or its related virus.
Sequence variations in the N-terminal ectodomain of ORF5 play a critical role in cross neutralization among PRRS viruses. As the first study demonstrated that the degree of cross neutralization among PRRS viruses can be severely affected by genetic variation among the viruses, subsequent studies were conducted to identify the genetic determinants of PRRSV which are significantly associated with cross protection. Since GP5, the major envelope protein of PRRSV, has been postulated as the most important protein of PRRSV to induce neutralizing antibody against the virus (75;155;213), the second study focused on ORF5 which is the gene encoding for GP5.

Based on the classification determined by in vitro and in vivo neutralization assays, five candidate sites (sites I-V) as neutralizing determinants were determined in GP5. From in vitro neutralization assays on mutants constructed from a VR-2332 based infectious cDNA clone to contain deduced amino acid sequences identified in GP5 of resistant viruses, three sites designated as I, II and IV were determined to be the most important for virus neutralization. Many resistant viruses had amino acid sequence substitutions for H38L39 in site I of GP5 (K or N at 38 and F or I at 39). This site is located in the linear neutralizing epitope (NE) within the N-terminal ectodomain of GP5 (37-46) (149;161), which is conserved among PRRS viruses. Site IV (32-34) and site II (57-59) were located immediately before or after this linear NE, respectively, in VR-2332 and replaced with various sequences in resistant viruses. These sites are located in two hypervariable regions (32-39 and 57-61) of the N-terminal ectodomain of PRRSV GP5 (92;108).
**ORFs 3 and 6 besides ORF5 are critically involved in cross neutralization between two different PRRS viruses.** Even though GP5 played a significant role in the induction of anti-PRRSV neutralizing antibody, JAP5 and SDP5 mutants whose entire ORF5 was replaced with that of JA142 or SDSU73, PRRS viruses antigenically very distinct from VR-2332, failed to obtain the complete resistance against VR-2332 antiserum as did JA142 or SDSU73 and couldn’t restore the complete susceptibility against JA142 or SDSU73 antiserum as compared to JA142 or SDSU73, suggesting that ORF5 is not the only element involved in cross neutralization. *In vitro* virus neutralization assays on chimeric mutants constructed by swapping one or more ORFs of the VR-2332 infectious clone with the corresponding ORF(s) of JA142 or other antigenically distinct PRRS viruses revealed envelope-associated proteins other than GP5 also contributed in an additive manner and at varying degrees to cross neutralization of the viruses since complete reversion of the susceptibility to the neutralizing activity of the antisera was obtained when all envelope-associated protein genes were substituted. In particular, the N terminal portion of GP3 and M protein played a significant role in cross neutralization. Therefore, the genetic similarity of ORFs 3 and 6 in addition to ORF5 should be taken into consideration to achieve complete cross neutralization between two different PRRS viruses.

**Future direction and application of the current results in vaccine development and virus classification.** The ORF5 of PRRSV is the gene most commonly sequenced for differential diagnosis and molecular epidemiology of PRRSV. However, overall sequence homology of ORF5 and phylogenetic relatedness of ORF5 are not always correlated with immunological relatedness (i.e. cross protection) among PRRS viruses. Even though a linear
neutralizing epitope was identified in ORF5, the prediction for cross protection between two different PRRS viruses was still vague since this epitope is conserved among the viruses and no other immunobiological properties are available for ORF5 and its product (i.e., GP5). The genetic determinants of ORF5 identified in the present study will be useful for this specific purpose because those determinants were demonstrated to be critically associated with cross neutralization between two different viruses. Thus, comparison of deduced amino acid sequence with the 32-59 short region in GP5 would favor a better prediction for cross protection between two PRRS viruses although fine mapping for ORFs 3 and 6 remains to be conducted and the role of those determinants in cross protection needs to be confirmed in an animal study.

The VR-2332 strain of PRRSV, the prototype of North American PRRSV (i.e., type 2) shows the elevated resistance against VN antibody generated against other heterologous PRRS viruses. The enhanced resistance of the virus might be attributed to its tolerance to the VN activity of antibody directed to GP5 and M proteins because of the high glycosylation in its GP5. In this study, GP3 appeared to be as important as GP5 in cross neutralization among PRRS viruses whereas the M protein did not convey a critical role in virus neutralization of the VR-2332 strain. In this regard, GP3 would be a better target protein to control certain PRRSV strains, such as VR-2332, which continue to be implicated in PRRS outbreaks as a major virus group. It is then an option to consider the development of a chimeric virus of two different PRRS viruses which can confer a broader protection against those viruses. For example, a chimeric virus that has the sequence of ORFs 2-4 of VR-2332 and that of ORFs 5-6 of JA142 can be used to prevent the infection of PRRS viruses antigenically related to VR-2332 and JA142.
Since some of the VN-related genetic determinants identified in ORF5 were located in the hypervariable region of ORF5, which are variable for different PRRS viruses, the development of a polyvalent vaccine that comprises a cocktail of antigens should also be considered to confer a broader range of cross protection against various PRRS viruses. Results of the current study should provide the basis for classification of PRRS viruses based on their immunological similarity; hence, assisting in the selection of candidate viruses to maximize the efficacy of the vaccine.

In addition to considering B-cell epitope based vaccines, elucidation of T-cell epitopes of PRRSV will be important because T-cell epitopes are generally more conserved than B-cell epitopes and the balanced stimulation of both humoral and cellular immunity is the necessity of a successful vaccine. Once T-cell epitopes are identified, a different virus-specific vaccine platform can be considered, such as vectored vaccine simultaneously displaying B-cell epitope(s) on the surface and endogenously expressing a gene of T-cell epitopes. This type of vaccine platform has been explored for a few viral pathogens of humans. It would provide not only the balanced humoral and cell-mediate immunity for better protection but also a way to develop a marker vaccine which will be required for the eradication of PRRS.
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