

Reverse Genetics-based Approaches to Attenuate Porcine Reproductive and Respiratory
Syndrome Virus (PRRSV)

Yanyan Ni

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Xiang-Jin Meng, Chair
Zachary N Adelman
Tanya LeRoith
P. Christopher Roberts
Lijuan Yuan

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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) is arguably the most economically-important swine pathogen. As the emergences of novel virulent strains of PRRSV continue to occur worldwide, rapid vaccine development is the key for effective control of ongoing PRRSV outbreaks. With the availability of the PRRSV reverse genetics systems, rapid vaccine development against PRRSV through the manipulation of the reverse genetics becomes feasible.

To facilitate the vaccine development effort and study of PRRSV genes, we first established a DNA-launched infectious clone of the passage 14 PRRSV strain VR2385, pIR-VR2385-CA, and identified a spontaneous 435-bp deletion in the *nsp2* gene. To characterize the biological and pathological significance of this *nsp2* deletion, we restored deleted *nsp2* sequence back to pIR-VR2385-CA and constructed another clone pIR-VR2385-R. VR2385-CA and VR2385-R were successfully rescued *in vitro*. The results from this study indicates that the spontaneous *nsp2* deletion plays a role for enhanced PRRSV replication *in vitro* but has no significant effect on the pathogenicity of the virus.

With the availability of the DNA-launched infectious clone of PRRSV, we successfully applied the molecular breeding approach to rapidly attenuate PRRSV. The GP5 envelope genes of 7 genetically divergent PRRSV strains and the GP5-M genes of 6 different PRRSV strains were molecularly bred. DS722 with shuffled GP5 genes and DS5M3 with shuffled GP5-M genes, were successfully rescued *in vitro* and shown to be attenuated both *in vitro* and *in vivo*.

Furthermore, DS722, but not DS5M3, still elicit similar protection against PRRSV challenge as its parental virus. This study reveals a unique approach through DNA shuffling of viral envelope genes to attenuate a positive-strand RNA virus.

We subsequently utilized the novel synthetic attenuated virus engineering (SAVE) approach to attenuate PRRSV. The GP5 and nsp9 genes of PRRSV were codon-pair deoptimized with the aid of a computer algorithm. SAVE5 and SAVE9 with deoptimized GP5 gene and SAVE9 with deoptimized nsp9 gene, were successfully rescued and shown to be attenuated *in vitro*. An *in vivo* pathogenicity study indicated the attenuation of SAVE5 virus *in vivo*. The results have important implications for rapid vaccine development against PRRSV and other important viruses.

DEDICATION

I would like to dedicate this work to my husband, mother, grandma and grandpa for their support during my graduate training at Virginia Tech.

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ATTRIBUTION

Several colleagues aided in the research behind one or two of my chapters presented as part of this dissertation. Other colleagues aided with the research or *in vivo* study for Chapter 2, 3 and 4. A brief description of their contribution is included here.

Chapter2: Establishment of a DNA-launched infectious clone for a highly pneumovirulent strain of type 2 porcine reproductive and respiratory syndrome virus: Identification and *in vitro* and *in vivo* characterization of a large spontaneous deletion in the nsp2 region.

Yao-Wei Huang, PhD (department of biomedical sciences and pathobiology) is currently a professor in Zhejiang University in China. Dr. Huang was a co-author in this paper and helped with the molecular techniques used.

Chapter3: Attenuation of porcine reproductive and respiratory syndrome virus by molecular breeding of the virus envelope genes from genetically divergent strains.

Yao-Wei Huang, PhD (department of biomedical sciences and pathobiology) is currently a professor in Zhejiang University in China. Dr. Huang was a co-author in this paper and helped with the molecular techniques used.

Lei Zhou, PhD (department of biomedical sciences and pathobiology) is currently an assistant professor in China Agricultural University in China. Dr. Zhou was a co-author in this paper and contributed DNA shuffling research.

Patrick Halbur, PhD (Veterinary diagnostic laboratory) is currently a professor at Iowa State University. Dr. Halbur was a co-author in this paper and helped with *in vivo* study.

Chapter4: Computer-aided codon-pairs deoptimization of viral genes attenuates porcine reproductive and respiratory syndrome virus.

Zhao Zhao, PhD candidate (department of computer science), is currently a student at Virginia Tech. Zhao was a co-author in this paper and contributed for providing algorithm to deoptimize the codon-pairs of viral genes.

Sakthivel Subramaniam, PhD (department of biomedical sciences and pathobiology), is currently a post doctorate at Virginia Tech. Dr. Subramaniam was a co-author in this paper who provided PK-15 cells expressing CD163 and helped with the study of protein analysis.

Lei Zhou, PhD (department of biomedical sciences and pathobiology) is currently an assistant professor in China Agricultural University in China. Dr. Zhou was a co-author in this paper and helped with providing monoclonal antibody against Nsp9 protein of PRRSV.

Qian Cao, graduate student (department of biomedical sciences and pathobiology) is currently a PhD student at Virginia Tech. Qian was a co-author in this paper and helped with cell culture experiment.

Hanchun Yang, PhD (Key laboratory of animal epidemiology and zoonosis of ministry of agriculture) is currently a professor in China Agricultural University in China. Dr. Yang was a co-author in this paper and provided monoclonal antibody against Nsp9 protein of PRRSV.

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Tanja Opriessnig, PhD (Veterinary diagnostic laboratory) is currently a professor at Iowa State University. Dr. Opriessnig was a co-author in these papers and helped with *in vivo* studies.

Dianjun Cao, PhD (department of biomedical sciences and pathobiology) is currently a research scientist at Virginia Tech. Dr. Cao was a co-author in these papers and helped with the molecular techniques used.

Xiang-Jin Meng, PhD (department of biomedical sciences and pathobiology) is currently a distinguish professor in department of biomedical sciences and pathobiology at Virginia Tech. Dr. Meng was a co-author in these papers, principal investigator for grants supporting the research, wrote and contributed editorial comments .

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GENERAL INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is currently a world-wide economically important disease in the global swine industry. It was first reported in the United States in 1987 (Keffaber, 1989) and later in Europe (Wensvoort et al., 1991). The causative pathogen of PRRS is porcine reproductive and respiratory syndrome virus (PRRSV), which is an enveloped, single-strand RNA virus belonging to the family *Arteriviridae* of the order *Nidovirales*. According to their genetic difference, PRRSV has been classified into two genotypes: European (Type 1) and North American (Type 2) types (Benfield et al., 1992, Wensvoort et al., 1991).

The cell tropism of PRRSV is very restricted. *In vivo*, PRRSV preferentially replicates in macrophage-lineage cells (Wensvoort et al., 1992). *In vitro*, MARC-145 and CL2621 cells (subclones of MA104 monkey kidney cell line) are non-porcine permissive cells for PRRSV replication (Benfield et al., 1992; Bautista et al., 1993; Kim et al., 1993). In addition, PRRSV is able to replicate in other cells such as PK-15 and BHK-21 expressing the CD163 molecule (Calvert et al., 2007).

PRRSV possesses a high degree of genetic and antigenic variations (Andreyev et al., 1997; Kapur et al., 1996; Meng et al., 1995). The lack of immunological cross-protection leads to the failure of current vaccines against heterologous strains of PRRSV. The applications of new technologies such as reverse genetics in study of PRRSV opens a new avenue for researchers to search for the solution to these problems in control of PRRSV.

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Chapter I

Literature Review

Porcine reproductive and respiratory syndrome virus (PRRSV)

PRRSV was first isolated in the Netherlands in 1991 and designated as “Lelystad” virus (LV). Subsequently, the North American type of PRRSV was isolated in 1992 and designated as ATCC VR-2332. The Lelystad virus sequence was first determined in 1993 (Meulenbergh et al., 1993), and the sequences of the North American PRRSV were first determined in 1994 (Meng et al., 1994; Mardassi et al., 1994). The PRRS viruses isolated in Europe and North America were genetically distinct with less than 70% sequence identity, thus they are categorized into two genotypes: European (type 1) and North American (type 2) types (Meng et al., 1995; Benfield et al., 1992; Wensvoort et al., 1991). PRRSV is an enveloped, single-strand, positive RNA virus which belongs to the family *Arteriviridae* of the order *Nidovirales* which include members of the *Coronaviridae* and *Roniviridae* families (Gorbalenya et al., 2006).

Porcine reproductive and respiratory syndrome (PRRS)

The disease caused by PRRSV, PRRS, has two major clinical manifestations: reproductive failure in sows including late-term abortions, increased chances of stillborns, mummified and weak-born pigs, and respiratory disease in all ages of pigs such as interstitial pneumonia (Albina, 1997a; Benfield et al., 1992). PRRS is a devastating global swine disease which costs approximately \$664 million dollars to the U.S. swine industry every year

(<http://www.pork.org/News/1265/PRRSCostsIndustry664Million.aspx>).

PRRSV genome and structure

The genome of PRRSV is approximately 15kb in size and is capped at the 5' end and polyadenylated at the 3' terminus (Meulenberg et al., 1993). It contains at least 9 overlapping open reading frames (ORFs) (Dea et al., 2000). ORF2-ORF5 encodes glycosylated membrane proteins GP2-GP5, ORF6 encodes non-glycosylated matrix (M) protein and ORF7 encodes nucleocapsid (N) protein (Meulenberg et al., 1993). ORF2b within ORF2 encodes non-glycosylated protein E (Wu et al., 2001) and a novel structural protein encoded by ORF5a was recently identified (Johnson et al., 2011). The viral particles are pleomorphic and they have round to egg-like shapes with an average diameter of 58 nm. The envelope is smooth with a few protruding features, and the nucleocapsid, separated from the envelope by a 2-3 nm is double layered with an average diameter of 39 nm (Spilman et al., 2009).

Structural proteins

GP5 and M

GP5 and M encoded by ORF5 and ORF6, respectively, are the major PRRSV structural proteins which are critical to produce viral particles (Wissink et al., 2005). These two proteins form disulfide linked heterodimers in the virus envelope which bind the heparin sulfate for viral attachment (Delputte et al., 2002). The GP5 protein contains a signal peptide with approximately 30 amino acids (aa) at the N terminus which is assumed to be cleaved, followed by a glycosylated ectodomain which contains a decoy non-neutralizing epitope and a neutralizing

epitope (Plagemann et al., 2002). The presence of N-glycans around the neutralizing epitope of GP5 is important for the virus to escape the function of neutralizing antibody (Ansari et al., 2006). A hydrophobic region including one or three transmembrane domains is located between residues 60 and 125 followed by a large C-terminal endodomain (Plana Duran et al., 1997).

M protein is the most conserved protein among the structural proteins of PRRSV (Meng et al., 1995). An ectodomain of 10-18 aa was located at its N-terminus and followed by three membrane-spanning regions (Snijder and Meulenberg, 1998). Like GP5, there is an endodomain of 81-87 aa at the C-terminus of M. However, the significance of the large endodomains of M is still unclear since there is no detectable interaction between M endodomains and N proteins (Music and Gagnon, 2010).

Nucleocapsid (N) protein

The N protein encoded by ORF7 is a small basic protein with a molecular mass of 15kDa. Similar as other arteriviruses and coronaviruses, the N protein of PRRSV is a serine phosphoprotein. It is predominantly expressed during infection and induces abundant non-neutralizing anti-N antibodies (Murtaugh et al., 2002). The N-terminus of N protein contains RNA-binding domain. The first half of N gene sequence has significant sequence difference between PRRSV strains (Meng et al., 1995). There is a hydrophobic sequence from 21-33 residues involved in assembly by forming α -helix. The nuclear and nucleolar localization sequences have been identified residing in the RNA-binding domain (Yoo et al., 2003). The C-terminus of N protein contains dimerization domains which fold similarly to coronavirus. The Cys 23 in the N protein is involved in the formation of disulfide bond-linked dimers of N

proteins (Wootton et al., 2002) and later is incorporated into virions and plays a critical role in PRRSV infectivity (Lee and Yoo, 2005).

Minor structural proteins

In addition to the major envelope proteins GP5, M and N which comprise 90-95% of the structural content of PRRSV particles (Snijder et al., 1998), minor proteins GP2, GP3 and GP4 encoded by ORF2a, ORF3 and ORF4 are also incorporated to virions as disulfide linked heterotrimers (Wissink et al., 2005).

GP2 belongs to the Class I integral membrane protein which has been shown to be indispensable in equine arteritis virus (EAV), another member in the *Arteriviridae* Family (van Dinten et al., 1997). GP2 contains a N-terminal signal peptide followed by an ectodomain, transmembrane (TM) helix and an endodomain (Music and Gagnon, 2010). Two conserved putative N-link glycosylated sites are embedded in the GP2 at position Asn 171 and Asn 178 in type 2 PRRSV, and Asn173 and Asn179 in type 1 PRRSV. However, the glycosylation of GP2 is dispensable for the infectivity of PRRSV (Wissink et al., 2004).

ORF2b embedded within ORF2a encodes a non-glycosylated small protein E. The E protein containing a putative single TM helix is proven to be necessary for infectivity of PRRSV but is dispensable for the formation of viral particles (Lee and Yoo, 2006). It was found in EAV that the E protein covalently interacts with the GP2-GP3-GP4 heterotrimer (Wissink et al., 2005), indicating a critical role of GP3 for the heteromultimeric complex. There is a conserved myristoylation site “1MGXXS6” in the E protein which is dispensable for PRRSV infectivity but affects the viral growth (Du et al., 2010).

GP3 is the most heavily glycosylated protein which contains 6 glycosylation sites in the ectodomain. The topology of this protein remains unclear (Wieringa et al., 2003). It is predicted that the GP3 contains a N-terminal TM domain located in the most variable region of GP3 that has less than 30% sequence identity shared between type 1 and type 2 PRRSV (Murtaugh et al., 1995). It is also predicted that the TM region comprises a signal peptide in both PRRSV and EAV, however, this prediction has not been verified experimentally by studies in EAV since the peptide is not cleaved.

GP4 is a class I integral membrane protein which contains a putative N-terminal signal peptide region and an additional hydrophobic region in the C-terminal region at residues 156-177 in type 2 PRRSV and 161-181 in type 1 PRRSV. Like GP3, GP4 is highly glycosylated with 4 putative glycosylation sites.

PRRSV Replication

Entry

Attachment and internalization

At the early stage of attachment, PRRSV predominantly binds to one of the entry mediators, heparan sulfate, although the binding capacity to this receptor differed among different PRRSV isolate. Later, the virus gradually increases its binding to another entry mediator, porcine sialoadhesin (pSn), and this binding requires the presence of sialic acid on the GP5/M complex. The binding efficiency is related to the sialic acid binding capacity of receptor pSn. Mutations on pSn which affects its binding to sialic acid lead to reduction of PRRSV binding to pSn (Delputte

et al., 2007). Another study showed that a truncated pSn with the N-terminal V-set domain is sufficient for PRRSV binding to pSn (An et al., 2010a). The ligand of PRRSV for interaction with these two receptors was proved to be GP5/M complex.

Internalization and uncoating

The internalization of PRRSV is via a clathrin-mediated endocytosis process and the binding of PRRSV to pSn facilitates this process (Vanderheijden et al., 2003).

After PRRSV enters the endocytic pathway, acidification in early endosomes enables the uncoating of PRRSV (Calvert et al., 2007). Several proteinases are involved in this process including cathepsin E and trypsin-like serine protease (Misinzo et al., 2008). CD163 has been shown to be a receptor for PRRSV (Calvert et al., 2007) and it was shown that transfection of CD163 gene to permissive but not susceptible cell lines such as BHK-21 enables the infection of these cells by PRRSV (Van Gorp et al., 2008). In addition, CD163 was co-localized with the structural proteins GP2a and GP4 of PRRSV in early endosomes but not under the plasma membrane (Van Gorp et al., 2009; Das et al., 2010). The viral proteins GP2 and GP4 were found to bind the CD163 molecule through co-immunoprecipitation experiments (Das et al., 2010). Therefore, the interaction with CD163 is related to viral release and critical for the infection of PRRSV. CD163 is a group B SRCR protein containing 8 cysteine residues per domain except domain 8 which contains only 6 cysteine residues (Sarrias et al., 2004). Domain 5 (SRCR 5), which is an important functional part of CD163, is critical for PRRSV infection. The N-terminal domains 1 and 2, as well as the C-terminal tails of CD163 molecule are dispensable for viral

infection, and deletion of C-terminal tails of CD163 facilitated viral replication in cells (Yoo et al., 2010).

Replication

Replication of full-length genomic RNA

The uncoated genomic RNA of PRRSV is first translated to polypeptides. The polypeptides (pp)1a and pp1ab are encoded by ORF1a and 1b, respectively, which consist of two-third of the PRRSV genome. The translation of ORF1b is initiated by a frameshift mechanism with a slippery structure “G(C)UUUAAAC ” and a downstream RNA pseudoknot. The polypeptides are subsequently processed into 14 functional non-structural proteins (nsp) by four proteases (PCP α and PCP β , PL2, SP) derived from ORF1a. The PCP1 α and PCP1 β , which reside in nsp1 α and nsp1 β respectively, are papain-like protease. PCP α directs the release of nsp1 α and PCP β cleaves nsp1 β /nsp2 site to release nsp1 β (Chen et al., 2010; den Boon et al., 1995; Kroese et al., 2008; Sun et al., 2009). The PL2 located in the nsp2 is a papain-like cysteine proteinase. PL2 self-cleaves nsp2/nsp3 site and releases nsp2 from the precursor (Han et al., 2009). A downstream protein nsp4 contains a 3C-like serine proteinase SP which executes the maturation of the remaining ppa1 and pp1ab (Tian et al., 2009; van Dinten et al., 1999).

The matured nsp's are then assembled into the replication/transcription complex associated with an ER-derived double membrane-like structure called double membrane vesicle (DMV) (Fang and Snijder, 2010). The formation of DMV is mediated by nsp2 and nsp3 during the infection of EAV (Posthuma et al., 2008; Snijder et al., 2001). A recent study revealed that the outer membranes of DMVs formed by EAV infection are interconnected with each other and with the

ER membrane which forms a reticulovesicular network (RVN) similar to SARS-coronavirus (Knoops et al., 2008, Knoops et al., 2012). The nsp2 is the largest nonstructural protein, which spans amino acids (aa) 383-1579 in type 2 PRRSV (Han, et al., 2009), and consists of an N-terminal papain-like cysteine proteinase domain, a hypervariable middle region that contributes to the genomic differentiation between the type 1 and the type 2 PRRSV, and a trans-membrane (TM) domain near the C-terminus (Music and Gagnon, 2010). Nsp2 is the most variable region between types 1 and 2 strains of PRRSV with <40% sequence identity at the amino acid level (Allende et al., 1999; Darwich et al., 2011). Natural deletions in the nsp2 region have been reported in field strains of PRRSV (Fang et al., 2004; Gao et al., 2004; Han et al., 2006; Zhou et al., 2009). Furthermore, the nonessential regions in nsp2 for viral replication in cell culture were identified, and the nsp2 region shows flexibility with respect to virus infectivity, especially in the middle region (Faaberg et al., 2010; Fang et al., 2008; Han et al., 2007; Kim et al., 2009). It was demonstrated that a 30-aa deletion in the hypervariable region of the nsp2 was not related to the virulence of the high pathogenic PRRSV strains that had recently emerged in China (Zhou et al., 2009). It was also shown that a conserved nsp2-coding region decreased the level of ubiquitin and ISG15 conjugates in transfected 293T cells (Frias-Staheli et al., 2007).

Negative strand RNA synthesis is initiated at the 3' end of the viral genome. Nsp9, a replicase subunit, contains a putative RNA dependent RNA polymerase (RdRp) domain which initiates RNA synthesis by a *de novo* mechanism according to a study of nsp9 of EAV (Beeren et al., 2007). A putative NTPase/RNA helicase motif is identified in nsp10 which contains an N-terminal putative zinc-binding domain critical for helicase activity *in vitro* (Seybert et al., 2005). A stretch of 34 nucleotides (14653-14686) of ORF7 in type 1 PRRSV is required for negative strand RNA synthesis and a 7-base sequence in this region forms a kissing interaction with a

loop structure in 3' noncoding region (Verheije et al., 2002). The nsp1 of EAV modulates the accumulation of negative strand RNA for mRNA synthesis (Nedialkova et al., 2010). The synthesized full-length negative strand RNA is then utilized as a template for the replication of full-length genomic RNA. The 3' end conserved sequence of the negative strand RNA forming a stem loop structure is involved in the initiation of viral genomic RNA replication and this sequence can be replaced with a novel AU-rich sequence which does not alter the RNA structure (Choi et al., 2006).

Subgenomic mRNA synthesis

The subgenomic (sg) mRNA synthesis starts with the synthesis of a nested set of negative strand sg RNAs. The synthesis of negative strand sg RNA utilizes a mechanism of discontinuous negative-strand RNA synthesis which requires a common leader sequence at the 5' end of genomic RNA and body junction sites of different segments at 3' proximate region. In EAV, negative strand RNA synthesis is initiated at the 3' end of viral genomic RNA, and elongates to the body junction site. This nascent strand is then translocated to the 5' end of the viral genomic RNA and base-pairs with the leader sequence. The negative strand subsequently resumes the synthesis until the most 5' end of genomic RNA (Van Den Born et al., 2004). The synthesized negative strand sg RNAs then serve as templates for sg mRNA synthesis.

Assembly and release

Assembly of structural proteins

The glycoproteins are retained in the ER when they are expressed individually but they can be collectively transported through Golgi apparatus to the plasma membrane when expressed together (Wissink et al., 2005). The GP5 and M are required for the production of viral particles (Wissink et al., 2005). In addition, studies on type 1 PRRSV indicated that oligosaccharides linked to N46 but not N53 of GP5 protein is involved in viral particle formation (Wissink et al., 2004). However, minor proteins like GP2a, GP3 and GP4 are dispensable for the viral particle production. These minor proteins interact with each other, and are incorporated to the viral particles as multimeric complexes (Wissink et al., 2005). It has been shown that the dimerizations of N protein mediated by disulfide linkages are not required for viral assembly (Zhang et al., 2012).

Packaging of viral RNA genome

A basic amino acid-rich domain (aa 37-57) within the N protein is believed to be related to the viral RNA packaging (Wootton et al., 2002; Yoo et al., 2003). The packaging of heteroclite sgRNAs which contain the 5' end of ORF1a indicates that a putative packaging signal may be included in this region (Yuan et al., 2004). One of these heteroclite sgRNAs with a minimal binding region of 35 nt was capable of packaging non-viral RNA sequence into viral particles and the 35 nt sequence is essential for the binding activities (Baig and Zakhartchouk, 2011).

PRRSV pathogenesis and virulence

PRRSV specifically targets a small subset of monocyte/macrophage lineages *in vivo* (Duan et al., 1997). The viral tropism *in vitro* is determined by the minor envelope proteins of PRRSV (Tian et al., 2012). The major envelope proteins GP5 and M may not contribute to viral tropism according to the study on EAV (Lu et al., 2012). After virus enters the cells by receptor-mediated endocytosis, viral replication is initiated. The replication of PRRSV peaks within 2 weeks after infection in pigs and could persist for up to 5-7 weeks (Duan et al., 1997, Labarque et al., 2000). PRRSV first drives the infected cells to an anti-apoptotic state during replication and initiates apoptosis of these cells and bystander cells at the end of the replication cycle (Costers et al., 2008). Besides destroying first-line host defense by directly targeting macrophages, PRRSV manipulates and modulates the host immune system by hampering the type I interferon production (Sun et al., 2010) which leads to the delay and inefficiency of immune responses against viral infection. Two weeks after infection, certain subtypes of macrophages infected with PRRSV spread all over the body, including transplacentally which can lead to late-stage reproductive problem (Karniychuk and Nauwynck, 2009).

The virulence of PRRSV has an important impact on pathogenesis. High virus replication may be partly responsible for the virulence of PRRSV. The highly virulent PRRSV strains exhibited a higher replication rate than classical PRRSV (Halbur and Bush, 1997; Lu et al., 2008). The high replication of PRRSV results in a high level of viremia in both serum and tissues which are directly related to the pathogenic consequences and immunological responses of pigs to PRRSV infection (Johnson et al. 2004; Cho et al. 2006). In addition, the high replication also results in increased mutations of PRRSV genome (Hanada et al, 2005). The rapid generation of genetic variants provides more opportunity for the virus to evade the host immune response. The

change of mutated viral proteins may also increase the ability of the virus for attachment and invasion, as well as the capacity to impair the host immunity.

The genome-wide scanning of virulence-related genes of PRRSV was conducted by constructing a series of chimeric viruses of a highly virulent PRRSV strain with the replacement of corresponding regions from an attenuated vaccine PRRSV strain. The virulence and attenuation of these chimeric viruses were determined in a sow reproductive failure model. The results indicate the multi-genic character of PRRSV virulence. Among the tested viral proteins, Nsp3 – nsp8 and ORF5 contain the major virulence determinants (Kwon et al., 2008).

Genetic variation

Although type 1 and type 2 PRRSV were discovered at about the same time, the genetic differences between the two types of viruses are significant. The type 1 and type 2 PRRSV only share about 67% nucleotide sequence identity (Benfield et al., 1992, Wensvoort et al., 1991).

There is an approximately 30% genetic difference between type 1 PRRSV strains which is more diversified when compared to type 2 PRRSV with only 21% maximal difference (Murtaugh et al., 2010).

PRRSV nsp2 is the most variable gene which accounts for the major genetic differences between type 1 and type 2 PRRSV. There is less than 40% amino acid sequence similarity between the nsp2 of the two PRRSV types. GP5 gene is the most variable structural genes of PRRSV with a 60% nucleotide sequence identity between the two types of PRRSV (Fosberg, 2005; Hanada et al., 2005). In addition, there is only about 90% nucleotide sequence identity in the GP5 genes among the type 2 PRRSV (Suarez et al., 1996). The high variability of GP5 genes was used for

phylogenetic analysis of PRRSV. A recent phylogentic study (Shi et al., 2010) identified 9 distinct lineages of type 2 PRRSV based on available GP5 gene sequences. Among these 9 lineages, 7 lineages are predominant in North America.

The higher mutation rate of PRRSV (10^{-2} /amino acid site/year) compared to other RNA viruses (10^{-3} to 10^{-5} /amino acid site/year) might partly explain its genetic diversity (Hanada et al., 2005). In addition, PRRSV recombination has been demonstrated by computations and experiments to play an essential role in the genetic diversity of PRRSV isolates (Forsberg et al., 2002; Yuan et al., 1999).

Genetic diversity among PRRSV strains limited the efficient cross-protection of the current vaccines which result in the vaccine failures against PRRSV infection in the field. Therefore, developing a universal vaccine against genetically diversified field strains of PRRSV is a major challenge to the current vaccine development efforts.

Vaccines

Inactivated PRRSV vaccines

The current PRRS inactivated vaccines do not provide efficient protection in vaccinated animals against PRRSV infection although they are safe and have no adverse reactions (Meng, 2000; Papatsiros et al., 2006). Currently, there is no licensed PRRS inactivated vaccine in the United States. However, the PRRS inactivated vaccines such as Suvaxyn –PRRS (Fort Dodge), Ingelvac PRRS KV (Boehringer Ingelheim) and Suipravac –PRRS (Hipra) derived from either type 1 or type 2 PRRSV strains are licensed in Europe and other parts of the world.

The PRRS inactivated vaccines induce no measurable protective immunity and fail to prevent clinic signs such as reproductive losses, viremia and viral shedding in semen within both homologous and heterologous PRRSV infections (Nielsen, 1997, Piras, F., 2005, Scotti, M., 2007, Zuckermann, F. A. 2007). The inactivated vaccines are currently used in breeding herd to improve their reproductive performance. Overall the inactivated vaccine is ineffective against PRRSV.

Modified live-attenuated PRRSV vaccine (MLV)

Among all the types of conventional vaccines, MLVs provide the most efficient protection against PRRSV. Current PRRSV MLVs derived from both type 1 and type 2 PRRSV are licensed in many countries including the United States such as Ingelvac PRRS MLV (Boehringer Ingelheim), Ingelvac PRRS ATP, FosterPRRS (Zoetis), AMERVAC PRRS (Hipra) and PORCILIS PRRS (Merck).

The current MLVs against PRRSV are effective against homologous field strains but are of little use against heterologous field strains (Meng, 2000; Huang and Meng, 2010). Unfortunately the majority of the PRRSV strains circulating in swine herds worldwide today are genetically different from the MLV vaccine strains, making the current MLVs ineffective in many herds. The fact that the current MLVs do provide solid protection against homologous strains indicates that MLV is still a viable control strategy if the MLV vaccine viruses can be engineered to be broadly-protective against heterologous strains. The effectiveness of the MLV against heterologous strains will largely depend on the genetic relatedness of the PRRSV strain to which the pigs are exposed. Therefore, the design of an effective PRRSV vaccine must take the genetic

diversity of PRRSV into consideration or PRRS will remain difficult to control (Meng, 2000; Huang and Meng, 2010).

A major concern for MLV is the risk of reversion of vaccine virus strain to a pathogenic phenotype. It has been reported that PRRSV MLV could revert to virulent strain and cause reproductive problems in immunized sows and resulted in developing respiratory diseases in vaccine piglets (Rowland, 2010; Opriessnig et al., 2002). In addition, the shedding of vaccine viruses, which persists a long time (4 weeks) and causes the transmission of these viruses to other naïve animals, is another concern for the safety of MLVs (Thanawongnuwech and Suradhat, 2010).

PRRSV subunit vaccines and vector vaccines

Currently, there is no commercial PRRSV subunit vaccine or vector vaccine available in the market as the efficiency of these vaccines against PRRSV is not fully satisfactory.

Among the PRRSV structural proteins, GP3, GP5 and M proteins have been selected as target for subunit vaccine or vector vaccine development in the previous studies. Several vectors have been used for expressing PRRSV viral proteins including baculovirus, adenovirus, and *Mycobacterium bovis* (BCG) (Plana Duran et al., 1997; Jiang et al., 2007; Bastos et al., 2002). Plant tissues were also used for expressing PRRSV GP5 or M proteins as potential subunit vaccine (Chia et al., 2010; Hu et al., 2012). Expression of viral proteins as a fusion protein or co-expression of several viral proteins were proved to enhance the immune response including higher titers of neutralizing antibodies to PRRSV and increased lymphocyte proliferation (Jiang et al., 2006).

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Chapter 2

Establishment of a DNA-launched infectious clone for a highly pneumovirulent strain of type 2 porcine reproductive and respiratory syndrome virus: Identification and *in vitro* and *in vivo* characterization of a large spontaneous deletion in the nsp2 region

Yan-Yan Ni¹, Yao-Wei Huang¹, Dianjun Cao¹, Tanja Opriessnig², and Xiang-Jin Meng^{1*}

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ABSTRACT

A highly pneumovirulent strain of porcine reproductive and respiratory syndrome virus (PRRSV), ATCC VR2385, was isolated from a pig exhibiting typical PRRS in the early 90's. While passaging the virus in monkey kidney cells, we identified a large spontaneous deletion of a 435-bp in the nsp2 gene. To assess the biological significance of this spontaneous deletion, we first determined the full-length genomic sequence of this virus and established a DNA-launched infectious clone of the passage 14 virus containing the 435-bp nsp2 deletion (designated as pIR-VR2385-CA). The full-length viral genome engineered with two ribozyme elements at both ends was placed under the control of the eukaryotic CMV promoter. The infectious virus was successfully rescued from pIR-VR2385-CA DNA-transfected BHK-21 cells. To characterize the biological and pathological significance of this large nsp2 deletion, we subsequently constructed another DNA-launched infectious clone, pIR-VR2385-R, in which we restored the deleted 435-bp nsp2 sequence back to the pIR-VR2385-CA backbone. The growth characteristics of the two rescued viruses (VR2385-CA and VR2385-R) were compared, and the results showed that the VR2385-CA virus with the nsp2 deletion replicated more efficiently *in vitro* (1.0-1.5 log titer higher) than the VR2385-R virus with the restored nsp2 sequence but the VR2385-CA virus exhibited a significantly reduced serum viral RNA load *in vivo*. A comparative pathogenicity

study in pigs (n=10) revealed that the nsp2 deletion had no effect on virus virulence, and the restored nsp2 sequence in the VR2385-R virus remains stable during virus replication in pigs. The results from this study indicates that the spontaneous nsp2 deletion plays a role for enhanced PRRSV replication *in vitro* but has no effect on the pathogenicity of the virus.

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS), characterized by respiratory diseases in nursery pigs and reproductive failure in sows (Albina, 1997; Done and Paton, 1995), is one of the most economically important infectious diseases in the global swine industry (Neumann et al., 2005). The causative agent of PRRS is porcine reproductive and respiratory syndrome virus (PRRSV), which is a single-strand, positive-sense, enveloped RNA virus in the family *Arteriviridae* of the order *Nidovirales* (Benfield et al., 1992; Cavanagh, 1997; Faaberg and Plagemann, 1996). Two distinct genotypes of PRRSV were identified: the European genotype (type 1) and North American genotype (type 2) (Benfield et al., 1992; Meng, 2000; Meng et al., 1995; Murtaugh et al., 2010; Wensvoort et al., 1991).

The genome of PRRSV is approximately 15 kb in size (Meulenberg et al., 1993) and is capped at the 5' end and polyadenylated at the 3' terminus. It contains at least 9 overlapping open reading frames (ORFs) (Dea S, 2000). At least six structural proteins, including GP2 (or GP2a), GP3, GP4, GP5, M, and N, which are encoded by the ORFs 2 to 7 respectively, were identified (Lunney et al., 2010). M and GP5 form a complex that is involved in the binding of the sialoadhesin receptor (Van Breedam et al., 2010). More recently, a novel structural protein encoded by ORF5a was identified to be important for PRRSV replication (Johnson et al., 2011). The ORF1a and ORF1b, which constitute approximately two-third of the viral genome, encode a single polyprotein and subsequently process to 14 nonstructural proteins (NSPs) (Allende et al., 1999; Fang and Snijder, 2010; Nelsen et al., 1999; Wootton et al., 2000) that are cleaved by viral proteases NSP 1 α , NSP1 β , NSP2 and NSP4 (Han et al., 2010). Both Nsp1 and Nsp2 are involved in interacting with the host innate immune response by down-regulating interferons and other

cytokines (Beura et al., 2010; Chen et al., 2010; Darwich et al., 2010; Subramaniam et al., 2010; Sun et al., 2010). Nsp9 encodes a viral RNA-dependent RNA polymerase while nsp10 encodes a helicase (Bautista et al., 2002; Snijder and Meulenberg, 1998).

Nsp2 is the largest nonstructural protein, which spans amino acids (aa) 383-1579 in type 2 North American PRRSV (Han et al., 2009), and consists of an N-terminal papain-like cysteine proteinase domain, a hypervariable middle region that contributes to the genomic differentiation between the North American type and the European type, and a trans-membrane (TM) domain near the C-terminus (Music and Gagnon, 2010). Nsp2 is the most variable region between North American type and European type strains of PRRSV with <40% sequence identity at the amino acid level (Allende et al., 1999; Darwich et al., 2011). Natural deletions in the Nsp2 region have been reported in field strains of PRRSV (Fang et al., 2004; Gao et al., 2004; Han et al., 2006; Zhou et al., 2009). Furthermore, the nonessential regions in nsp2 for viral replication in cell culture were identified, and nsp2 region shows flexibility with respect to virus infectivity, especially in the middle region of nsp2 (de Lima et al., 2006; Faaberg et al., 2010; Fang et al., 2008; Han et al., 2007; Kim et al., 2009; Ran et al., 2008; Rowland, 2007). It was demonstrated that a 30-aa deletion in the hypervariable region of nsp2 was not related to the virulence of the high pathogenic PRRSV strains that had recently emerged in China (Zhou et al., 2009). It was also shown that a conserved nsp2-coding region decreased the level of ubiquitin and ISG15 conjugates in transfected 293T cells (Frias-Staheli et al., 2007). However, the impact of the spontaneous nsp2 deletions on virus replication and pathogenicity under controlled conditions remains largely unknown.

In 1994, we isolated a highly pneumovirulent strain of type 2 PRRSV (strain ATCC VR2385) (Meng et al., 1994; Meng et al., 1996). The full-length genomic sequence of this unique strain of

type 2 PRRSV had not yet been reported. In this present study, while passaging the VR2385 virus in cell cultures, we identified a large spontaneous deletion of 435-bp in the nsp2 region. To determine the potential biological role of this large nsp2 deletion in PRRSV replication and pathogenicity, we constructed and biologically and pathologically characterized two DNA-launched infectious cDNA clones of the VR2385 viruses with and without the nsp2 deletion. The VR2385-CA virus with the large spontaneous nsp2 deletion replicated more efficiently *in vitro* than the VR2385-R virus with an intact nsp2 but the VR2385-CA virus exhibited reduced replication ability *in vivo* with significantly lower serum viral RNA loads in experimentally-infected pigs, indicating that the nsp2 deletion is related to cell culture adaption of the virus.

MATERIALS AND METHODS

Virus and cells: The highly pneumovirulent strain of PRRSV, ATCC VR2385, was isolated from a pig exhibiting typical PRRS disease in the early 90's (Meng et al., 1996). A comparative pathogenicity study using 9 different strains of type 2 PRRSV revealed that the VR2385 strain is highly pneumovirulent in cesarean-derived colostrum-deprived pigs (Halbur et al., 1996). The virus stocks at passages 4 and 14 were used in this study. BHK-21 and MARC-145 cells were grown at 37°C in Dulbecco's minimum essential medium (DMEM) supplemented with 10% FBS and antibiotics.

Determination of the full-length genomic sequence of VR2385 and sequence analyses: The sequences of the ORFs 2 to 7 genes, but not ORF1, of the VR2385 isolate have been published (Meng et al., 1994). To determine the complete genomic sequence of VR2385, total RNAs were isolated from the passage 14 virus using TRI Reagent (MRC). Reverse transcription

and cDNA synthesis were performed at 42 °C for 60 min in a 20-µl reaction mixture containing 100 U of Superscript II reverse transcriptase (Invitrogen), 10mM deoxyribonucleoside triphosphate, 100 mM of DTT, 1 U of RNasin (Promega), and 0.5 µg of oligo dT primers (Invitrogen). The overlapping PCR fragments with approximately 2 kb in size for each fragment covering the entire genome of the VR2385 virus were amplified from the cDNA, and subsequently cloned into a pCR-2.1 vector (Invitrogen). Six individual clones of each fragment were selected for sequencing. The consensus sequences were assembled and used for sequence analysis utilizing the lasergene software (Version 8, DNA STAR, Inc.).

Construction of an infectious cDNA clone of the passage 14 VR2385 virus containing the nsp2 deletion: After determining the sequence at the extreme 5'-end of the viral genome using the GeneRACER kit (Invitrogen), a total of 7 overlapping fragments (AB, BF, FS, SP, PS, SA, AN) with unique restriction enzyme sites that cover the entire viral genome were amplified from the cDNA of the passage 14 VR2385 virus stock. A T7 RNA polymerase core sequence was engineered immediately upstream of the 5'-end of the VR2385 genome in the fragment AB during the RT-PCR using primers T7ABf and ABr (Fig. 1A; Table 1). A 20-poly(A) nucleotides were introduced immediately downstream of the 3'-end of the viral genome in the fragment AN. A total of 6 individual clones for each fragment were selected for sequencing, and the clone containing the consensus sequence was used for the assembly of the full-length cDNA clone. Primers mFSr and mSPf (Table 1) were used to mutate a single nucleotide to generate a SphI restriction site in the fragments FS and SP for assembly. A low-copy number plasmid, pACYC177 (New England BioLab), was modified by replacing the fragment between the BamHI and BglI sites with a stuffer fragment to facilitate the cloning and assembly process

(Fang et al., 2006). Each of the RT-PCR fragments was used to sequentially replace the stuffer with the same restriction enzyme sites on the modified vector pACYC-177, resulting in the assembly of a full-length cDNA clone of the VR2385 virus. The single nucleotide mutation introduced in the SP fragment was then changed back to the consensus sequence by using a site-directed mutagenesis kit (Invitrogen) with primers mutSphIf and mutSphIr (Table 1) after assembly of the fragments FS and SP to the backbone. This full-length cDNA clone of the passage 14 virus, which contains the 435-bp nsp2 deletion, was designated as pACYC-VR2385-CA.

Construction of a DNA-launched infectious clone of pACYC-VR2385-CA containing the nsp2 deletion: To facilitate efficient rescue of the cloned virus, we converted the RNA-launched infectious cDNA clone of pACYC-VR2385-CA into a DNA-launched infectious clone essentially as described previously (Huang et al., 2009b; Huang and Meng, 2010). A three-step subcloning procedure was performed to introduce the ribozyme elements and construct the final infectious clone (Fig. 1C). Briefly, A PCR fragment with the fusion of a hammerhead ribozyme (HHRz) to the 5'-end of the viral genome was cloned into pACYC-VR2385-CA by using the single restriction site BamHI. Next, a hepatitis delta virus ribozyme (HDVRz) was engineered to the 3'-end using the same fragment-replacing strategy with the restriction sites AclI and XbaI. Subsequently, the fragment IR-XA from the vector pIRES-EGFP2 containing the CMV promoter (1987→5308, 0→619) was amplified using primers pIR-XbaIf and pIR-AscIr (Table 1), incorporating the unique restriction sites AscI and XbaI, which were then used to ligate this fragment upstream to the viral genome. The full-length viral genome engineered with HHRz and HDRz at both termini was released from the backbone vector pACYC-177 by double digestion

with AscI and XbaI, and subsequently ligated to the PCR product IR-XA digested with the same restriction enzymes AscI and XbaI (Fig 1c) to produce the DNA-launched infectious clone pIR-VR2385-CA.

Construction of a DNA-launched infectious clone with the restored original nsp2 sequence: A fragment containing the 435-bp nsp2 sequence was amplified by RT-PCR with primers BFdelf and BFdelr (Table 1) from the passage 4 VR2385 virus stock which does not have the nsp2 deletion. A three-fragment fusion PCR was used to incorporate this fragment containing the 435-bp nsp2 sequence into two flanking fragments containing restriction sites AscI and FseI with primers AscInsp2f and FseInsp2r (Table 1). This fusion PCR product was then used to replace the corresponding region of the DNA-launched infectious clone pIR-VR2385-CA with the restriction sites AscI and FseI, and the resulting DNA-launched infectious clone was designated as pIR-VR2385-R.

***In vitro* transcription, transfection and recovery of infectious viruses from the infectious clones:** The clone pACYC-VR2385-CA was linearized with the restriction enzyme NdeI, and capped RNA transcripts were transcribed *in vitro* with T7 RNA polymerase using the mMessage Machine kit (Ambion) as described previously (Huang and Meng, 2010). To test the infectivity of the clone pACYC-VR2385-CA, BHK-21 cells were seeded in 6-well plates (approximately 2×10^5 cells per well) one day before transfection. Approximately 10 µg of the RNA transcripts of pACYC-VR2385-CA were transfected into BHK-21 cells at a 60% confluency using the DMRIE-C reagent (Invitrogen). The cells were incubated at 37 °C for 6 h, after which the transfection mixture was replaced with fresh DMEM containing 10% FBS. The

supernatant of transfected cells was harvested at 48 h post-transfection and subsequently passaged onto MARC-145 cells. The rescue of virus was confirmed by an immunofluorescence assay (IFA) with an anti-PRRSV N-specific antibody (SDOW17).

For rescue of infectious PRRSV from the two DNA-launched infectious clones, BHK-21 cells at a 60% confluency in 6-well plates were transfected with 3 µg of pIR-VR2385-CA or pIR-VR2385-R DNA with 8 µl of Lipofectamine LTX (Invitrogen) according to the manufacturer's instruction. At 48 h post-transfection, the cell culture supernatant was harvested and passaged onto MARC-145 cells. The rescue of virus was confirmed by IFA with the anti-PRRSV N monoclonal antibody (SDOW17), and the rescued viruses were designated as VR2385-CA and VR2385-R, respectively.

Immunofluorescence assay (IFA): To determine the viability of the cloned virus, MARC-145 cells were infected with supernatant of transfected BHK-21 cells as described previously. Three days later, cells were washed with 0.05% PBS-Tween, fixed with 80% acetone (Sigma). The fixed cells were incubated with anti-PRRSV N monoclonal antibody SDOW17 (Rural Technologies, Inc) (Fang et al., 2006; Meng et al., 1996; Meulenberg et al., 1998) at 37 °C for 2 h. After washing three times, the cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin at 37 °C for 2 h. The stained cells were visualized with a Nikon Eclipse TE300 fluorescence microscope fitted with a camera (Nikon).

Plaque assay: Confluent monolayers of MARC-145 cells cultured in a 6-well plate were infected with serially-diluted viruses (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , and 10^{-5}). After 1 h incubation, the inoculum was removed and an agar overlay was applied to the monolayer. Plaques were stained

with neutral red solution (Sigma) 4 days post-infection at 37 °C. The plaques were counted and the titer of a virus stock was calculated and expressed in plaque-forming unit (PFU) per milliliter. Plaque morphology between the VR2385-CA virus with the nsp2 deletion and the VR2385-R virus with the restored original nsp2 sequence was compared in MARC-145 cells.

Characterization of virus growth *in vitro*: To compare the growth characteristics of the VR2385-CA and VR2385-R viruses, growth curve analysis was performed. To generate the virus stocks for the growth curve study, approximately 18 µg of the pIR-VR2385-R plasmid DNA was transfected into the BHK-21 cells at a 60% confluency in a T-75 flask, and approximately 6 µg of the pIR-VR2385-CA plasmid DNA was transfected to BHK-21 cells at a 60% confluency in a T-25 flask. At 48 hr post-transfection, the transfected cells were lysed by three repeated freeze-thaw cycles, and clarified at 3,000 x g for 10 min to produce virus-containing supernatant. The infectious titers of the VR2385-R and VR2385-CA virus stocks were determined by plaque assay as described above and quantified as PFU. For the growth curve analysis, confluent monolayers of MARC-145 cells seeded in 24-well plates were infected with VR2385-R and VR2385-CA viruses at the same multiplicity of infection (M.O.I.) of 0.1, respectively. As a control, the passage 14 virus stock (VR2385-14) was also included in parallel in the growth curve analysis (Fig. 3A). The infected cells were harvested at 6, 12, 24, 36, 48, 60 and 72 hours post-infection (hpi). The titers of virus harvested at different time points were measured by IFA in MARC-145 cells and quantified as focus forming unit (FFU) per ml as described previously (Huang et al., 2009a). All experiments in this study were assayed in triplicate.

Comparative pathogenicity and replication of VR2385-R and VR2385-CA viruses in pigs: To determine the effect of the nsp2 deletion on virus pathogenicity and replication *in vivo*, thirty pigs at 3-weeks of age were randomly assigned to three groups of 10 pigs each, and inoculated with 8 ml of VR2385-CA virus ($8 \times 10^{6.33}$ TCID₅₀/pig), 8 ml of VR2385-R virus ($8 \times 10^{6.33}$ TCID₅₀/pig), and DMEM containing 10% FBS (8ml/pig), respectively, via a combination of the intra-nasal (4 ml) and intra-muscular (4 ml) routes. Serum samples were collected prior to inoculation and weekly thereafter, and sections of lung tissues were collected from each pig during necropsy and stored at -80 °C until usage. Eight pigs from each group were necropsied at 14 days post-inoculation (dpi) to assess the gross and histological pathological lesions. The remaining two pigs from each group were euthanized at 28 dpi to determine the genetic stability of the nsp2 sequence during *in vivo* infection.

Gross lung lesions: The total amount of macroscopic lung lesions (0–100% of the lung affected by grossly visible pneumonia) was estimated and recorded for each pig (Halbur et al., 1995). The scoring system was based on the approximate volume that each lung lobe contributes to the entire lung: the right cranial lobe, right middle lobe, cranial part of the left cranial lobe, and the caudal part of the left cranial lobe contribute 10% each to the total lung volume, the accessory lobe contributes 5%, and the right and left caudal lobes contribute 27.5% each.

Histological lesions: Five sections of the lung representative of all lobes from each pig were collected during necropsy at 14 dpi and submerged in 10% neutral buffered formalin. Sections of lymph nodes (superficial inguinal, mediastinal, tracheobronchial, mesenteric), tonsil, thymus, ileum, kidney, colon, spleen, and liver were also collected at necropsy and fixed in 10% neutral

buffered formalin and routinely processed for histological examination. Microscopic lesions were evaluated in a blinded fashion to treatment status. Microscopic lung sections were scored for the presence and severity of interstitial pneumonia characterized by type 2 pneumocyte hypertrophy and hyperplasia and alveolar septal infiltration with inflammatory cells ranging from 0 to 6 (0, normal; 1, mild multifocal; 2, mild diffuse; 3, moderate multi-focal; 4, moderate diffuse; 5, severe multifocal; 6, severe diffuse) (Halbur et al., 1995). Sections of lymphoid tissues, heart, liver, kidney, ileum, and colon were evaluated for the presence of lymphohistiocytic inflammation and scored from 0 (none) to 3 (severe).

Real-time PCR to quantify the amounts of viral genome in serum: Total RNAs were isolated from serum using TRI Reagen (MRC), and the amount of PRRSV RNA genome copy numbers were quantified by a SYBR Green-based real-time qPCR. A pair of primers (forward primer: 5'-GGCAACTCAGACGACCGAAC-3'; reverse primer: 5'-AGTAGTAATTGGACAGCGAGAAGG-3') was designed based on the nsp2 region by the Beacon software. The qPCR assay was conducted in a MyiQ real-time PCR machine (Bio-Rad Laboratories, Hercules, CA). The reactions were performed in 25- μ l PCR volumes containing 12.5 μ l semi-quantitative master mix (Quantum), 0.5 μ l of each primer (10 μ M), 5 μ l of template cDNA, and 6.5 μ l of nuclease-free water. The cycling parameter included 45 cycles of denaturation at 95 $^{\circ}$ C for 5 s, annealing at 58 $^{\circ}$ C for 15 s, and extension at 72 $^{\circ}$ C for 15 s, with an initial denaturation at 95 $^{\circ}$ C for 10 min. Dissociation curve analysis were performed using the instrument's default setting immediately after each PCR run. Each reaction was measured in triplicates.

Virus isolation from serum samples of infected pigs and infectivity titer determination:

To isolate virus from serum samples of infected pigs and to quantify the infectivity titer, serum samples from pigs were centrifuged at 3,000 x g for 15 min and filtered through a 0.2 µm membrane (Corning). The sterile serum samples were then serially diluted, and used to infect MARC-145 cells to determine the infectivity titer by IFA as described above.

Enzyme-linked immunosorbent assay (ELISA) to detect the kinetics of PRRSV-specific antibody responses in infected pigs: Serum samples collected at different days post-inoculation from all pigs were tested by ELISA for anti-PRRSV antibody responses using the HerdChek PRRS virus antibody test kit X3 (IDEXX Laboratories Inc.), following the manufacturers' instruction.

Statistical analyses: A two-tail Student's *t* test was used to evaluate the differences ($P < 0.05$) between the samples for the two groups both *in vitro* and *in vivo*.

RESULTS

Identification of a large spontaneous deletion in the nsp2 region of the passage 14 VR2385 virus but not in passage 4 virus: After we obtained the full-length genomic sequence of the VR2385, a sequence comparison of the nsp2 region between the passage 14 virus and passage 4 virus was performed. To our surprise, a 435-bp deletion in the nsp2 region was identified in the passage 14 virus (Fig 1b) but not in the passage 4 virus. The deletion was in frame and spanned from 3080 nt to 3514 nt which is predicted to be within the hypervariable region 2 of the nsp2. The passage 4 VR2385 virus, when compared to the sequence of the

prototype type 2 VR2332 strain of PRRSV, contains no deletion in the nsp2. However, after only 10 passages, a spontaneous deletion of 435-bp occurred in the nsp2 region of the VR2385 virus.

Generation of a RNA-launched (pACYC-VR2385-CA) and a DNA-launched (pIR-VR2385-CA) infectious clones of the passage 14 PRRSV VR2385: The full-length genomic sequence of the VR2385 was first determined in this study. Subsequently, we developed an RNA-launched infectious cDNA clone, pACYC-VR2385-CA, for the passage 14 VR2385 virus (Fig 1A). The full-length cDNA clone of passage 14 virus containing the nsp2 deletion was assembled in the vector pACYC177. To facilitate the biological characterization of this infectious clone, we subsequently converted this RNA-launched infectious clone into a DNA-launched infectious clone, pIR-VR2385-CA, by introducing a CMV promoter and two ribozyme sequences (Fig 1C). Transfection of BHK-21 cells with the RNA transcripts from the clone pACYC-VR2385-CA or with the plasmid DNA of clone pIR-VR2385-CA, followed by infection of MARC-145 cells with the supernatant from the transfected cells, resulted in successful rescue of the virus VR2385-CA containing the 435-bp nsp2 deletion. PRRSV VR2385-specific antigen was detected by IFA with an anti-PRRSV N monoclonal antibody both in the transfected BHK-21 cells and the infected MARC-145 cells (Fig 2A, B).

Rescue of a PRRSV with the restored original nsp2 sequence from the DNA-launched infectious clone: The original nsp2 sequence of PRRSV VR2385 was amplified from the total RNAs extracted from the passage 4 VR2385 virus stock. No deletion was found in the nsp2 region from the passage 4 virus. A modified DNA-launched clone, pIR-VR2385-R, was constructed by restoring the 435-bp nsp2 deletion back to the corresponding region of the clone

pIR-VR2385-CA. The virus VR2385-R, which contains the restored original 435-bp nsp2 sequence, was successfully rescued from the modified DNA-launched infectious clone and confirmed by IFA with an anti-PRRSV N monoclonal antibody in the transfected BHK-21 cells and the infected MARC-145 cells (Fig. 2C, D). Compared to the VR2385-CA virus which produced an infectious titer of approximately $10^{6.3}$ TCID₅₀/ml after two passages in MARC-145 cells, the VR2385-R virus replicated much slower during the first two passages and produced an infectious titer of only approximately 10^4 TCID₅₀/ml after two passages in MARC-145 cells.

The 435-bp nsp2 deletion in the VR2385-CA virus enhanced virus replication *in vitro*: To compare the growth characteristics between the two rescued viruses (VR2385-CA and VR2385-R) with and without the 435-bp nsp2 deletion, the growth kinetics of the two viruses were analyzed by infection of MARC-145 cells with the respective virus at the same M.O.I. of 0.1 for each virus. The infectious virus titers were determined at different time points post-infection by FFA. The results showed that both viruses reached the peak infectivity titer at approximately 48 hpi. However, the infectious titer of the VR2385-R virus with the restored original nsp2 sequence was consistently lower than that of the VR2385-CA virus during the first 48 hrs of infection, especially at 24 hpi when there was a significant difference in the virus titers between the two viruses (Fig 3A).

The VR2385-CA virus with a 435-bp nsp2 deletion forms larger plaques: The results from the plaque assay showed that both VR2385-CA and VR2385-R viruses developed plaques within 4 days post-infection. However, the size of plaques developed by the VR2385-R virus

was smaller than that of VR2385-CA (Fig 3B), further indicating that the VR2385-CA virus with the nsp2 deletion replicated more efficiency *in vitro* than the VR2385-R virus.

Serum viral loads and serum infectious titers of VR2385-CA and VR2385-R viruses in experimentally infected pigs. The viral RNA loads (copy numbers) in serum samples of infected pigs were quantified by a SYBR Green-based real-time PCR. The results showed that pigs infected with the VR2385-R virus had a significantly higher serum viral RNA load (1.30×10^9 copies/ml) than pigs infected with the VR2385-CA virus (3.07×10^8 copies/ml) at 14 dpi (Fig. 4A) ($p = 0.013$). We also determined the infectious virus titers in serum samples by virus isolation followed by infectivity titration in MARC-145 cells. However, the serum virus infectious titers were not statistically different between the two groups (Fig. 4B).

Serum anti-PRRSV antibody levels in pigs experimentally-infected with VR2385-CA and VR2385-R viruses. The levels of PRRSV-specific antibody response in serum samples of all pigs were tested by ELISA. The results showed that both groups of pigs infected with VR2385-CA and VR2385-R developed the PRRSV-specific antibody responses at 14 dpi. The pigs infected with VR2385-CA virus had a higher level of PRRSV-specific antibody response compared to the pigs infected with the VR2385-R virus, although the difference between the two groups is not significant (Fig. 5). The pigs in the negative control group are tested negative for PRRSV antibody (data not shown).

Gross lung lesions: Pigs infected with the VR2385-CA virus had 44-53% of their lung surface affected by visible pneumonia lesions. The means were 16.1 ± 5.8 for group 1 pigs

inoculated with the VR2385-CA virus, 17.6 ± 7.1 for group 2 pigs inoculated with the VR2385-R virus, and 1.9 ± 1.3 for the negative control pigs at 14 dpi, and the difference was not statistically significant between the two PRRSV-infected groups ($p=0.87$) (Table 2A). Similarly, macroscopic lung lesions were not different ($p=1$) between the two PRRSV-infected groups at 28 dpi (2.0 ± 0.0 for group 1 pigs, 2.0 ± 2.0 for group 2 pigs, and 0.0 ± 0.0 for the negative control pigs).

Microscopic lesions: Microscopic lung lesions were characterized by mild-to-moderate focal-to-diffuse thickening of alveolar septa mainly by macrophages and lymphocytes and pneumocytes type 2 hypertrophy and hyperplasia. The group mean scores were 2.5 ± 0.5 for the group 1 pigs inoculated with the VR2385-CA virus, 2.5 ± 0.5 for the group 2 pigs inoculated with the VR2385-R virus, and 1.1 ± 0.2 for the negative control pigs at 14 dpi, and there was no significant difference ($p=1$) between the two PRRSV-infected groups (Table 2B). At 28 dpi, the group mean interstitial pneumonia scores were 3.0 ± 0.0 for group 1 pigs, 3.0 ± 1.0 for group 2 pigs ($p=0.464$), and 2.0 ± 0.0 for the negative control pigs. Individual pigs in all groups had mild lymphohistiocytic hepatitis or myocarditis.

The restored 435-bp nsp2 sequence in the VR2385-R virus is stable in pigs: To determine if the VR2385-R virus with the restored 435-bp original nsp2 sequence is stable during *in vivo* replication of the virus in pigs, we amplified and sequenced the nsp2 region from the viruses recovered from the two infected pigs that were necropsied at 28 dpi. Sequence analyses revealed that the 435-bp nsp2 restored sequence in VR2385-R virus is genetically stable *in vivo* during the 4-week infection period, as reversion or change in the size of the nsp2 was not observed in the viruses recovered from pigs at 28 dpi.

DISCUSSION

The PRRSV nsp2 region is a variable and flexible domain, and deletions in the nsp2 region have been reported (Fang et al., 2004; Gao et al., 2004; Han et al., 2006; Shen et al., 2000; Tian et al., 2007). However, a study on cross-reactivity of the humoral responses to both nsp1 and nsp2 indicates that, despite extensive genetic diversity in nsp2, many antigenic determinants in this region are conserved (Johnson et al., 2007). Under experimental conditions using an artificial construct, the maximal size of nsp2 deletion that can be tolerated for PRRSV is 403-aa (Han et al., 2007). However, a spontaneous large nsp2 deletion of 145-aa observed in the VR2385 virus from this study rarely occurs, and the mechanism of the occurrence for such a large spontaneous deletion during virus passage in cells and its effect on virus replication and pathogenicity remains largely unknown.

In this study, we identified a large 435-bp deletion in nsp2 region spanning aa 581-725 during continuous passaging of the VR2385 virus in cell culture. To characterize the biological implication of this nsp2 deletion, we first determined the complete genomic sequence of the VR2385 virus and constructed both DNA-launched and RNA-launched infectious clones of the VR2385 virus. By using the DNA-launched infectious clone, we were able to restore the 435-bp original nsp2 sequence back into the virus genome and rescued the restored virus *in vitro*. Comparison of the growth kinetics and characteristics of the rescued viruses revealed that the VR2385-CA virus with the nsp2 deletion replicated more efficiently with approximately 1.0-1.5 log higher titer in MARC-145 cells than the VR2385-R virus with the restored original nsp2 sequence. This observation is similar to a report showing that the replacement of the nsp2 deletion region of a Chinese high-pathogenic PRRSV with the corresponding region from a low virulence strain of PRRSV resulted in a slower growth *in vitro* (Zhou et al., 2009), although the

nsp2 deletion of the Chinese high-pathogenic PRRSV was much further upstream in the nsp2 compared to the large nsp2 deletion observed in VR2385 virus from this study. Furthermore, in this study, we demonstrated that the plaques formed by the VR2385-CA virus with the nsp2 deletion are larger than those formed by the VR2385-R virus with the restored original nsp2 sequence, further confirming the enhanced replication ability of the VR2385-CA virus. However, the enhanced *in vitro* replication of the VR2385-CA virus did not correlate with higher serum viral RNA load or infectious viral titer *in vivo*. In fact, pigs infected with the VR2385-CA virus had a significantly lower serum viral RNA load compared to pigs infected with the VR2385-R virus at 14 dpi, although the serum virus infectivity titers in pigs infected with either of the two viruses were not different. The PRRSV-specific antibody responses are not significantly different in pigs experimentally infected with the two recombinant viruses. Therefore, it appears that this large spontaneous nsp2 deletion is directly related to the cell culture adaption, as the deletion improved the virus replication efficiency *in vitro* but there was no enhancement of virus replication *in vivo*. Consequently, our results suggested that, under the selection pressure of cell culture, this spontaneously occurring large nsp2 deletion is advantageous to the virus replication *in vitro*.

To determine whether the nsp2 deletion mutant altered virus virulence in pigs, a comparative pathogenicity study with sufficient numbers of pigs in each group (n=10) was conducted. The results showed that there was no significance difference in the gross or histological lesion scores in the lungs between pigs infected with the two viruses, indicating that the nsp2 deletion had no effect on the pathogenicity of the virus *in vivo*. This large nsp2 deletion in the VR2385-CA virus was genetically stable in pigs during the 4 weeks of infection period, as the viruses amplified from the infected pigs at 28 dpi had no reversion or change in the nsp2 size. Similarly, the nsp2-

restored VR2385-R virus is also genetically stable, as the restored nsp2 sequence in the virus was not lost during the 4-week virus replication in pigs.

In summary, we identified a spontaneous large 435-bp deletion in the hypervariable region of nsp2 from a highly pneumovirulent strain of PRRSV. Biological and pathological characterization using reverse genetics system revealed that the nsp2 deletion enhanced virus replication efficiency *in vitro*. However, this nsp2 deletion reduced the serum viral RNA loads in experimentally infected pigs compared to the virus without the nsp2 deletion, although the deletion had no effect on the infectious virus titers in the sera of infected pigs and did not affect the virulence of the virus either. Furthermore, the 435-bp nsp2 sequence in the restored virus is genetically stable during the 4-week infection period in pigs. The results from this study further underscore the importance and yet flexibility of the intriguing multifunctional nsp2 region of PRRSV in virus replication.

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Fig. 1. Constructions of RNA-launched and DNA-launched infectious clones of a highly pneumovirulent strain of PRRSV: **(A)** Strategy for the construction of a full-length infectious clone of pACYC-VR2385-CA containing the 435-bp nsp2 deletion. A total of 7 fragments amplified from total RNAs extracted from the passage 14 VR2385-CA virus were assembled into a modified low-copy number vector pACYC177 including T7 promoter and poly (A) tail. Unique restriction enzyme sites used for the assembly of the full-length clone were indicated for each fragment. **(B)** Alignment of amino acid sequences in the nsp2 region of the passage 14 virus (VR2385-P14) and the passage 4 virus (VR2385-P4). A 145-aa (435-bp) deletion in the nsp2 of the VR2385-P14 virus was identified when compared to the VR2385-P4 virus. Dash (-) indicates a deletion. **(C)** Strategy for the construction of a DNA-launched infectious clone (pIR-VR2385-CA) and restoration of the 145-aa (435-bp) nsp2 sequence to the pIR-VR2385-CA infectious clone backbone. The VR2385 virus genome with ribozyme introduced at both ends was placed downstream the CMV promoter in pIRES-EGFP2 vector to construct the DNA-launched infectious clone, pIR-VR2385-CA. The 435-bp (145-aa) nsp2 sequence amplified from the passage 4 VR2385 virus was inserted into the DNA-launched clone pIR-VR2385-CA between the aa 580 and 581 of the nsp2.

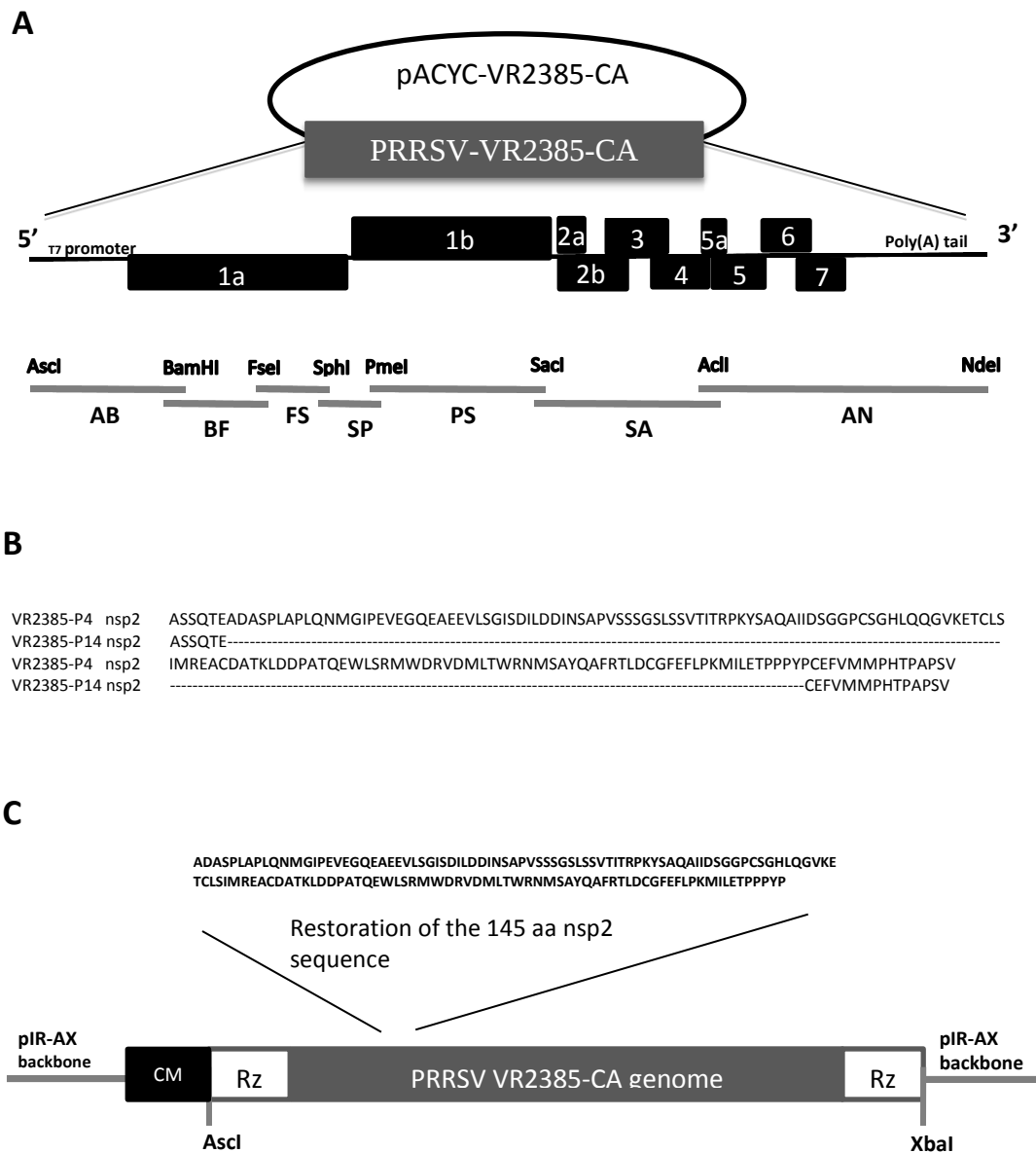


Fig. 1

Fig. 2. Rescue and passage of cloned viruses VR2385-CA and VR2385-R. (A) BHK-21 cells were transfected with DNA-launched clone pIR-VR2385-CA, and stained with an anti-PRRSV N antibody by IFA at 48 h post-transfection. **(B)** Immunofluorescence assay (IFA) confirmation of the rescue of the VR2385-CA virus from MARC-145 cells infected with the supernatant of transfected cells from (A). **(C)** BHK-21 cells were transfected with DNA-launched clone pIR-VR2385-R, and stained with an anti-PRRSV N antibody by IFA at 48 h post-transfection. **(D)** IFA confirmation of the rescue of the nsp2 sequence-restored virus from MARC-145 cells infected with the supernatant of transfected cells from (C).

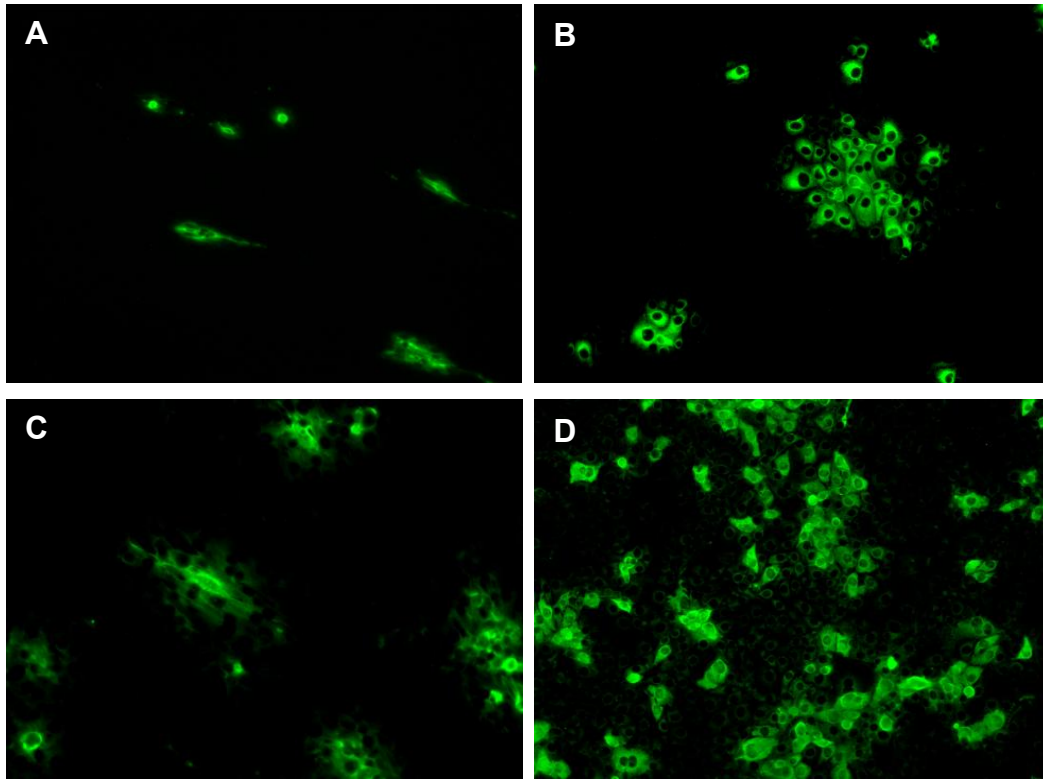


Fig. 2

Fig. 3. *In vitro* growth kinetics and plaque morphology of VR2385-CA virus with the 435-bp nsp2 deletion and VR2385-R virus with the restored nsp2 sequence. (A) Growth kinetics of the VR2385-CA and VR2385-R viruses derived from infectious clones. MARC-145 cells were infected with each of the VR2385-CA and VR2385-R viruses at the same M.O.I. of 0.1, and as a control the passage 14 virus (VR2385-14) was also included in the experiment. The virus titers (FFU/ml) were determined at the indicated time points post-infection. The asterisk (*) indicates a statistical significant difference between the two groups at that time point. The standard error bars are indicated. **(B)** Plaque morphology of the VR2385-CA virus and VR2385-R virus. Confluent MARC-145 cells in 6-well plate were infected with serial dilutions of each of the two viruses, respectively. After 1h incubation, the virus inoculum was removed and an agar overlay was applied. The plaques were stained with neutral red at 4 days post-infection. Note the smaller plaque size for the VR2385-R virus.

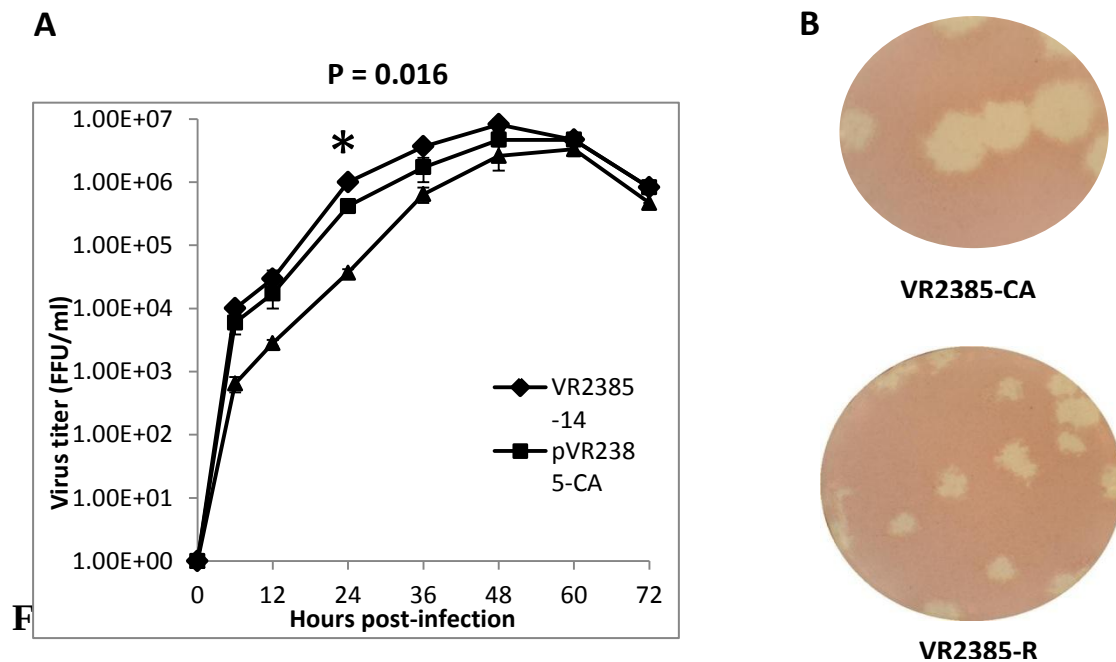


Fig. 4. Serum viral RNA loads and serum infectious titer of PRRSV in pigs experimentally infected with VR2385-CA with the nsp2 deletion and VR2385-R with restored nsp2 sequence. (A) PRRSV viral RNA copy numbers in serum samples at 14 DPI from the 8 pigs infected with VR2385-CA and VR2385-R viruses, respectively. There was a significant difference of serum viral RNA loads between the two groups (indicated with *). The virus with nsp2 deletion had a significantly lower serum viral RNA loads compared to the virus without nsp2 deletion. **(B)** Comparison of serum virus infectivity titers at 14 DPI from the 8 pigs infected with VR2385-CA and VR2385-R viruses. There was no significant difference in the infectivity titers between the two groups. The standard error bars are indicated.

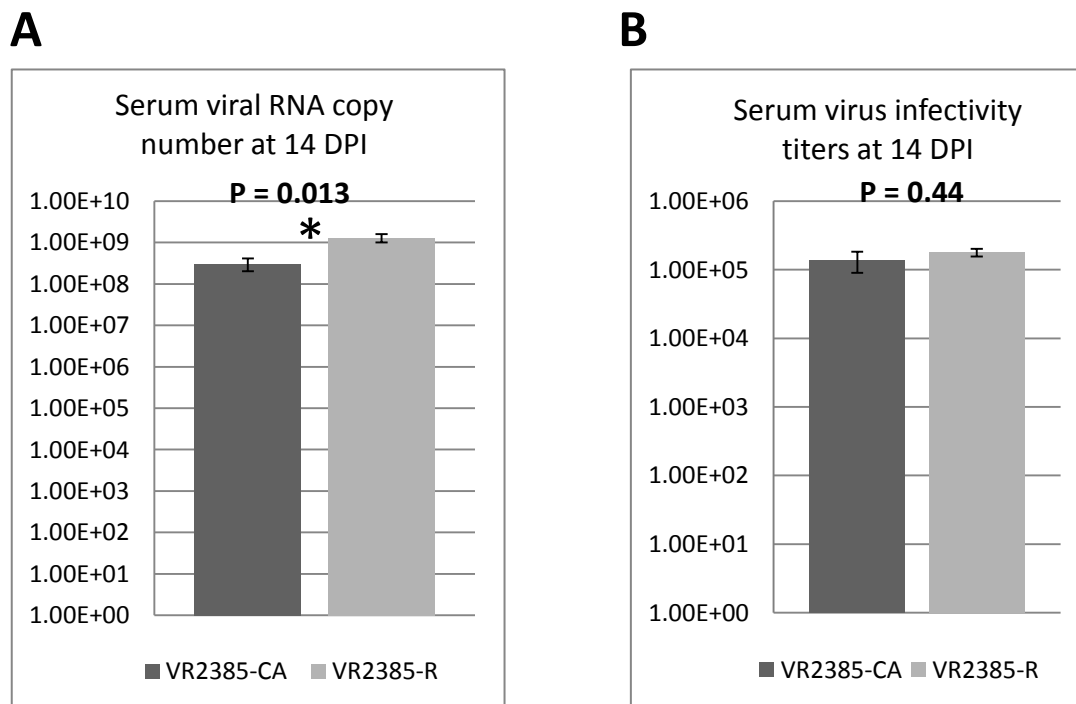


Fig. 4

Fig. 5. Serum anti-PRRSV antibody responses in pigs experimentally infected with VR2385-CA virus with the nsp2 deletion and the VR2385-R virus with restored nsp2 sequence. Comparison of serum PRRSV-specific antibody levels at 0, 7, and 14 days post-inoculation (dpi) from the 8 pigs infected with VR2385-CA and VR2385-R viruses and necropsied at 14 dpi. The pigs in the negative control group are all tested negative for PRRSV antibody (data not shown). The standard error bars are indicated.

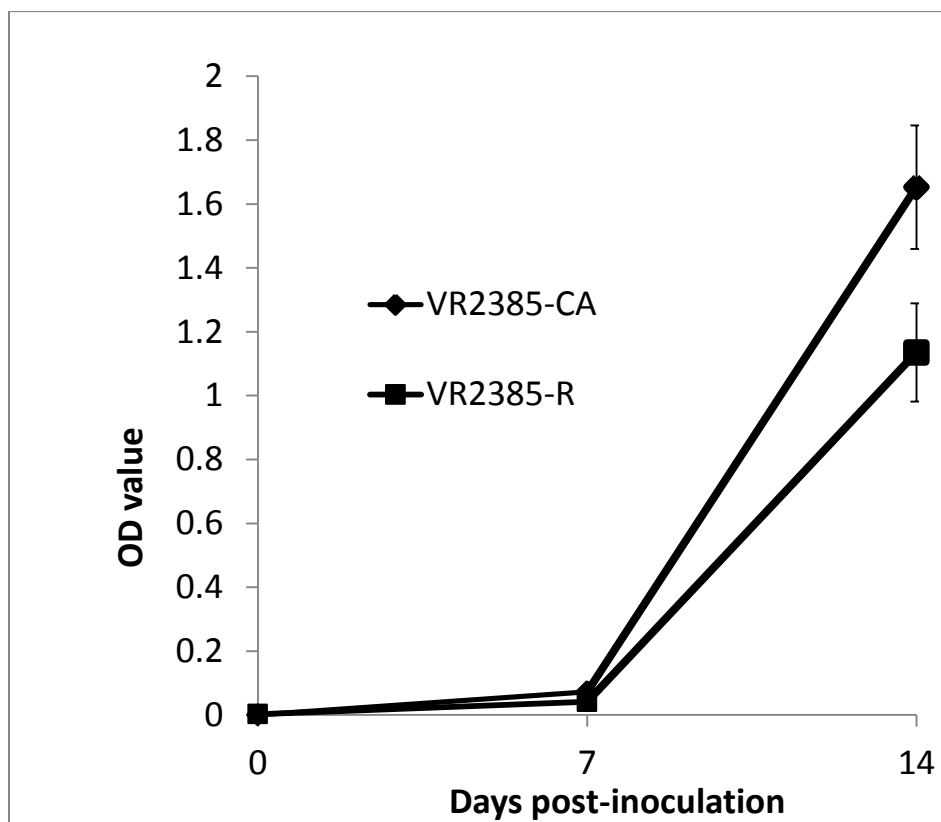


Fig. 5

Table 1. Oligonucleotide primers used in this study

Primer ID	Sequence (5'>3')	nt position in VR2385-CA virus genome
Fragment AB		
ABf	ATGACGTATAGGTGTTGGCTCT	1-22
ABr	GGGCTCAACCCTTATTCTA	1255-1273
T7ABf	TTGGATCCGGCGCGCCTAATACGACTCACTATAGGAT GACGTATAGGTGTT	1-16
Fragment BF		
BFf	CCCAAATGGACCTATCGT	1150-1167
BFr	TCAGAGGGTTGCTCAATGGG	4118-4137
Fragment FS		
FSf	GACCTGTGTGATCGGTTTTTGCG	4031-4052
mFSr	GTCAAGGGCAGGGTAAGGGCAT	4680-4701
Fragment SP		
SPr	TGGCGGCTAGCAGTTTAAACAC	7252-7273
mSPf	AGCATGCCCTTACCCTGCCCTT	4677-4698
Fragment PS		
PSf	GCCTGACTAAGGAGCAGTGTTT	7236-7257
PSr	TTGGGGAGCGGAGAGCTCGA	10510-10529
Fragment SA		
SAf	CTGATCTAGAAGGGTCGAGCTC	10496-10517
SAr	TGGTCAACTACCAAGGAACGTT	13234-13255
Fragment AN		
ANf	GTCAAGGAATTTACCCAACGTT	13218-13239
ANrpolyA	TACATATGTTTTTTTTTTTTTTTTTTTTTTTAAATTTCG GCC	14968-14977
For site-directed point mutation		
mutSph1-f	CATTTCCCAGCATGGCCTTACCCTGCCCTTG	4669-4699
mutSph1-r	CAAGGGCAGGGTAAGGCCATGCTGGGAAATG	4669-4699
For the construction of DNA-launched infectious clones		
pIR-XbaI	GCTCTAGAGCATAATCAGCCATACCACATTTGTAGAGG	
pIR-AscI	GAAGGCGCGCCTCGAGATCTGAGTCCGGTAG	
For restoring the original nsp2 sequence		
BFdelf	AGCCCCTAGATTTGTCTGCAT	3045-3065
BFdelr	CGACTGAGCCAATGGTAAGG	3136-3155
AscInsp2f	TGCTGAAACTAGCCAACGCCAGACAACCT	2523-2552
FseInsp2r	TAGGAAAAAAGGGGGCTCGGAATGGAAC	4301-4328

Table 2. Gross and histological lung lesions in pigs experimentally inoculated with the VR2385-CA virus with the nsp2 deletion and the VR2385-R virus with the restored original nsp2 sequence

Group	Average % of gross lung lesions at DPI 14*	Std Err
Negative	1.9	1.3
VR2385-CA	16.1	5.8
VR2385-R	17.6	7.1
Average scores of histological lung lesions at DPI 14*		
Negative	1.1	0.2
VR2385-CA	2.5	0.5
VR2385-R	2.5	0.5

*P > 0.05

Chapter 3

Attenuation of porcine reproductive and respiratory syndrome virus by molecular breeding of the virus envelope genes from genetically divergent strains

Yan-Yan Ni, Tanja Opriessnig, Lei Zhou, Dianjun Cao, Yao-Wei Huang, Patrick G. Halbur,
and Xiang-Jin Meng

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ABSTRACT

Molecular breeding via DNA shuffling can direct the evolution of viruses with desired traits. By using a positive-strand RNA virus, porcine reproductive and respiratory syndrome virus (PRRSV), as a model, rapid attenuation of the virus is achieved in this study by DNA shuffling of the viral envelope genes from multiple strains. The GP5 envelope genes of 7 genetically divergent PRRSV strains and the GP5-M genes of 6 different PRRSV strains were molecularly bred by DNA shuffling and iteration of the process, and the shuffled genes were cloned into the backbone of a DNA-launched PRRSV infectious clone. Two representative chimeric viruses, DS722 with shuffled GP5 genes and DS5M3 with shuffled GP5-M genes, were rescued and shown to replicate at a lower level and formed smaller plaques *in vitro* when compared to its parental virus. An *in vivo* pathogenicity study revealed that pigs infected with the two chimeric viruses have significant reductions in viral RNA loads in sera and lungs, and in gross and microscopic lung lesions, indicating attenuation of the chimeric viruses. Furthermore, pigs vaccinated with the chimeric virus DS722, but not with DS5M3, still induced protection against PRRSV challenge at a level similar to that of its parental virus. Therefore, this study reveals a

unique approach through DNA shuffling of viral envelope genes to rapidly attenuate a positive-strand RNA virus. The results have important implications for future vaccine development and will generate broad general interest in the scientific community for rapidly attenuating other important human and veterinary viruses.

INTRODUCTION

Molecular breeding through DNA shuffling mimics natural recombination at an accelerated rate and can direct the evolution of viruses with desired traits (21). In the traditional DNA shuffling approach, a set of related parental viral genomes is first selected, digested with DNase I to create a pool of short DNA fragments, which is then reassembled by repeated thermocycling and amplification (6, 37, 46). The shuffled chimeric viruses can then be selected for desired properties (3). Thus far, DNA shuffling has been mainly used to generate chimeric viruses with novel tissue tropism or with broader antigenic representation (3, 36, 44). To our knowledge, attenuation of a virus by DNA shuffling has never been reported.

In this study we hypothesize that DNA shuffling of viral genes that are important virulence determinants could lead to rapid attenuation of viruses. To test our hypothesis, a single-strand positive-sense RNA virus, porcine reproductive and respiratory syndrome virus (PRRSV), was utilized as a model virus system for DNA shuffling in this study. PRRSV caused a devastating global swine disease with immense economic losses (22, 30). It is estimated that the losses associated with PRRSV infection are approximately \$560.32 million per year in the United States alone (28). In 2006, “swine high fever disease” outbreaks with a mortality of 20-100% caused by a variant strain of PRRSV have devastated the swine industry in China and its neighboring countries (1, 38). Rapid development of vaccines is critical for the control of such devastating future outbreaks.

PRRSV, a member of family *Arteriviridae*, consists of two distinct genotypes: type 1 (European type) and type 2 (North American type) (4, 27). Within the type 2, there exist at least 9 distinct genetic lineages (34). The PRRSV genome consists of at least 9 overlapping open reading frames (ORFs) (12, 23). The GP5 and M encoded by ORF5 and ORF6, respectively, are

the major structural proteins of PRRSV (8, 24, 43). These two proteins form disulfide-linked heterodimers that bind to the heparin sulfate for virus attachment (7, 9, 35). The GP5 protein contains a signal peptide at its N-terminus (15) and a glycosylated ectodomain which contains a decoy non-neutralizing epitope and a neutralizing epitope (2, 10, 32, 33, 40). A hydrophobic region is located between amino acid (aa) residues 60 and 125 followed by a large C-terminal endodomain (39, 42). More recently, a novel structural protein encoded by ORF5a was identified and may play a role in virus replication (17). The GP5 is the most variable structural protein with 89-94% aa sequence identity among type 2 PRRSV (26) but only 51-55% identity between types 1 and 2 PRRSV (13, 19, 27). Importantly for this study, it has been shown that one of the major virulence determinants of PRRSV is located in the GP5 (20). Therefore, we selected the GP5 as the main target for DNA shuffling in our attempts to rapidly attenuate PRRSV. The M is a non-glycosylated membrane protein that likely plays a key role in virus assembly and budding (43). Since the M is closely associated with the function of the GP5, therefore we also included the M as the target for the DNA shuffling to attenuate PRRSV.

The main objective of this study is to explore if virus attenuation can be achieved by molecular breeding of the virus envelope genes using PRRSV as a model virus. We molecularly bred PRRSV by DNA shuffling of the GP5 genes of 7 and the GP5-M genes of 6 genetically divergent strains of PRRSV. The shuffled chimeric viruses were infectious *in vitro* and, most importantly, attenuated in pigs. This represents the first report of successful virus attenuation by DNA shuffling approach. Furthermore, one shuffled chimeric virus elicited protection against PRRSV challenge at a level similar to its parental virus in pigs.

MATERIALS AND METHODS

Cells and viruses. BHK-21 and MARC-145 cells were grown at 37°C in Dulbecco's minimum essential medium (DMEM) supplemented with 10% FBS and antibiotics. PAM cells were obtained by lung lavage of 8-week-old PRRSV-naïve piglets as described previously (45). PAM cells were grown at 37 °C in RPMI 1640 medium supplemented with 10% FBS and 2X antibiotics. The North American type 2 PRRSV was systematically classified into 9 genetically distinct lineages based on the ORF5 gene sequences of 8,624 PRRSV strains (34). To produce a chimeric virus by molecular breeding, a total of 7 genetically different strains of PRRSV each representing a distinct genetic lineage or sublineage in the phylogenetic tree (34) including MN184B (lineage 1), VR2385 (lineage 5.1), VR2430 (lineage 5.2), S132 (lineage 6), Chinese highly pathogenic strain JXA1 (lineage 8.7), FL-12 (lineage 8.9), and NADC20 (lineage 9) were selected for the DNA shuffling in the study. The genetic relationship of these selected strains of PRRSV used in the DNA shuffling was shown in the phylogenetic tree (Fig. 1). The GP5 gene sequences of VR2385 and FL-12 were amplified from the infectious clones pIR-VR2385-CA (1) and pFL-12 (3), respectively. The GP5 gene sequence of the strain VR2430 was amplified from a viral stock. The GP5 gene sequences of the other 4 PRRSV strains [MN184B, S132, JXA1, and NADC20] were commercially synthesized (Genscript) based on the sequences in the GenBank database.

DNA shuffling of the GP5 and GP5-M genes. For the GP5 gene shuffling, the GP5 genes from seven strains of PRRSV were mixed in equimolar amounts with a total 5 µg DNA and diluted in a 50 µl of 50 mM Tris•HCl (pH 7.4) and 10 mM MgCl₂. The mixture was incubated at 15°C for 2 min with 0.15 U of DNase I (Sigma). DNA fragments of 50-150 bp in size were purified from 2% agarose gels. The purified DNA fragments were subsequently added

to the *Pfu* PCR mixture consisting of 1X *Pfu* buffer, 0.4 mM of each dNTP, and 0.06 U *Pfu* polymerase. A PCR program without primers (95°C for 4 min; 35 cycles of 95°C for 30 s, 60°C for 30 s, 57°C for 30 s, 54°C for 30 s, 51°C for 30 s, 48°C for 30 s, 45°C for 30 s, 42°C for 30 s, 72°C for 2 min; and final 72°C for 7 min) was performed to reassemble the digested DNA fragments. Subsequently, specific primers flanking the shuffled GP5 gene region, GP5trunc-F (5'-GGGAACAGCAGCTCAAATTTACAG-3') and GP5trunc-R (5'-AGGGGTAGCCGCGGAACCAT-3'), were used to amplify the assembled shuffling products. Similar approaches were used to shuffle the GP5-M genes from 6 different strains of PRRSV. Unlike the GP5 shuffling, the strain S132 was not included in the GP5-M gene shuffling since the M gene sequence for S132 strain was not available. GP5F (5'-ATGTTGGGGAAATGCTTGACCG-3') and mfu3'R (5'-GCCGCAATCGGATGAAAGCCTG-3'), were used to amplify the assembled shuffling products.

Construction of chimeric PRRSV libraries. The shuffled product libraries were cloned into a blunt-end vector pCR-BLUNT to assess the quality of the DNA shuffling. The recombination efficiency was analyzed by sequencing the shuffled genes from 30 randomly selected clones to delineate crossovers. The nucleotide changes among the parental strains served as the markers to delineate the origin of each fragment between two proximate crossover sites incorporated in the shuffled product. The fragment between the crossover sites with the same nucleotide pattern as a particular parental strain was considered to be derived from that particular parental strain (Fig. 2, and Fig. 3). The shuffled products contained segments derived from all parental viruses and have a good number of crossovers were selected for the study. The GP5 clone DS722 and the GP5-M clone DS5M3 were ultimately selected from respective libraries for

the construction of chimeric viruses in the backbone of a DNA-launched PRRSV infectious clone pIR-VR2385-CA (16, 29). For cloning purpose, two flanking fragments amplified from pIR-VR2385-CA containing naturally-occurring unique restriction sites *Acl*I and *Xba*I, respectively, were fused to the corresponding shuffled products and the fusion fragments were cloned into the DNA-launched infectious clone to produce chimeric viruses containing the shuffled GP5 or GP5-M genes. The amino acid differences in GP5 among the 7 parental virus strains and the two chimeric viruses (DS722 and DS5M3) were presented in Figure 3.

In vitro transfection and immunofluorescence assay (IFA). To rescue the infectious chimeric PRRSV from the recombinant DNA-launched infectious clones, BHK-21 cells at a 60% confluency in 6-well plates were transfected with 3 μ g of chimeric PRRSV DNA using 8 μ l of Lipofectamine LTX (Invitrogen) according to the manufacturer's instruction. At 48 hr post-transfection, the cell culture supernatant was harvested and passaged onto MARC-145 cells. Two days later, cells were washed with 0.05% PBS-Tween, and fixed with 80% acetone (Sigma). The fixed cells were incubated with anti-PRRSV N monoclonal antibody SDOW17 (Rural Technologies, Inc) at 37°C for 1 hr. After washing three times, the cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG at 37°C for 1 hr. The stained cells were visualized with a Nikon Eclipse TE300 fluorescence microscope fitted with a digital camera (Nikon).

Plaque assay. Confluent monolayers of MARC-145 cells cultured in a 6-well plate were infected with 10-fold serially-diluted viruses (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , and 10^{-5}). After 1 hr incubation, the inoculum was removed and an agar overlay was applied to the cell monolayer.

Plaques were stained with neutral red solution (Sigma) 4 days post-infection at 37°C. The wells containing 10-100 plaques in each plate were selected to measure the diameter for each plaque. Plaque morphology and size were compared between the parental virus VR2385 and the two chimeric viruses.

Growth characterization of the chimeric viruses *in vitro*. To analyze the growth characteristics of the chimeric viruses *in vitro*, a growth curve was performed in MARC-145 cells and porcine alveolar macrophages (PAM). Briefly, confluent monolayers of MARC-145 cells or PAM seeded in 96-well plates were infected with the parental virus VR2385 and two rescued chimeric viruses (DS722, DS5M3) at the same multiplicity of infection (M.O.I.) of 0.1, respectively. Both infected MARC-145 cells and PAM cells were harvested at 6, 12, 24, 36, 48, 60, 72 and infected MARC-145 cells were harvested with an additional time point at 84 hours post-infection (hpi). The titers of virus harvested at different time points were determined by IFA in MARC-145 cells and quantified as TCID₅₀/ml. All *in vitro* experiments were assayed in triplicate.

Pathogenicity study of the two chimeric viruses in specific-pathogen-free (SPF) pigs. To determine and compare the virulence in pigs between the two chimeric viruses and the parental virus, we used a nursery pig respiratory disease model to assess the pathogenicity of PRRSV since this nursery pig model has been widely used worldwide for evaluating PRRSV virulence and vaccine efficacy (5, 11, 14, 18, 25). A total of 24 SPF pigs at 3 weeks of age were divided into 4 groups of 6 each and intramuscularly inoculated with respective virus as shown in Table 1. All six pigs from each group were necropsied at 14 days post-inoculation (dpi). At

necropsy, the lung tissues were collected from each pig for histological examination and for quantification of viral RNA loads.

Challenge and protection study in SPF pigs vaccinated with the chimeric viruses. A total of 72 SPF pigs at 3 weeks of age were divided into 8 groups of 9 pigs per group and vaccinated as shown in Table 2. At 35 days post-vaccination (dpv), pigs in each group were challenged with either the parental VR2385 virus or a heterologous NADC20 virus (Table 2). At 14 days post-challenge (dpc), all pigs were necropsied, and gross pathological lung lesions were recorded and scored (13). Lung tissues were also collected for histological examination and viral RNA load quantification.

Real-time PCR to quantify viral RNA loads in sera and lung tissues of pigs. To quantify viral RNA loads in the lung tissues, samples of lung tissues (500 mg) collected at each necropsy were homogenized in 10% (w/v) sterile PBS. Homogenates were centrifuged at 3,000 rpm at 4°C for 15 min, and the supernatant were used for the quantification of PRRSV RNA. Total RNAs were extracted from weekly serum samples and homogenates of lung tissues using TRI Reagent (MRC), and used to synthesize cDNA using Superscript II kit (Invitrogen). PRRSV genomes were quantified using a SYBR Green-based qPCR. A pair of primers (forward primer: 5'-TTAAATATGCCAAATAACAACGG-3'; reverse primer: 5'-TGCCTCTGGACTGGTT-3') was designed based on the conserved region in the nucleocapsid gene by the Beacon software. The qPCR assay was conducted in a CFX96 real-time PCR system (Bio-Rad). The reactions were performed in a 20 µl PCR volume containing 10 µl SsoFast EvaGreen Supermix (Bio-Rad), 0.5 µl of each primer (10 µM), 5 µl of the template cDNA, and 4 µl of nuclease-free water. The

cycling parameter included an initial denaturation at 95°C for 10 min followed by 39 cycles of denaturation at 95°C for 10 s, annealing and extension at 58°C for 20 s. Dissociation curve analysis was performed using the instrument's default setting immediately after each PCR run. Each reaction was measured in triplicates.

Necropsy and gross pathology evaluation. All pigs were humanely euthanized by intravenous pentobarbital overdose (Fatal-Plus® Vortech Pharmaceutical, LTD; Dearborn, MI, USA). Veterinary pathologists were blinded to treatment status of the pigs for evaluation of gross lung lesions. The total amount of lung affected by pneumonia (0-100% of the lung affected by grossly visible pneumonia) was recorded for each pig at necropsy in a blinded fashion to the treatment status as described previously (13). Briefly, the scoring system is based on the approximate volume that each lung lobe contributes to the entire lung: the right cranial lobe, right middle lobe, cranial part of the left cranial lobe, and the caudal part of the left cranial lobe contribute 10% each to the total lung volume, the accessory lobe contributes 5%, and the right and left caudal lobes contribute 27.5% each (13). Five defined sections of lungs (13) were collected, immediately immersed in 10% neutral-buffered formalin, and routinely processed for histological examination. In addition, fresh lung tissues were collected separately and immediately stored at -80°C for virological testing.

Histopathology evaluation. Microscopic lung lesions were evaluated independently by two veterinary pathologists (TO, PGH) blinded to the treatment status. Lung sections were scored for presence and severity of interstitial pneumonia ranging from 0 to 6 (0=normal; 1=mild multifocal; 2=mild diffuse; 3=moderate multifocal; 4=moderate diffuse; 5=severe multifocal;

6=severe diffuse) (13). If the results obtained by the two pathologists on a certain tissue differed, the mean of the two scores was used.

Statistical analyses. A two-tail Student's *t* test was used to evaluate the differences ($p < 0.05$) between the samples in the two groups for both *in vitro* and *in vivo* studies. Data were analyzed using software GraphPad Prism (version 5.0).

RESULTS

Generation of infectious chimeric viruses containing well-shuffled GP5 or GP5-M genes from 7 and 6 genetically distinct strains of PRRSV, respectively. To generate GP5-shuffled chimeric viruses with good growth fitness, we excluded the 96 nt from the 5' end of the GP5 gene including the signal peptide sequence and the 16 nt overlapping region with M gene as well as the junction site sequence from 23 to 18 nt upstream of the M gene for DNA shuffling of the GP5 genes. The resulting 468 nt GP5 genes from seven PRRSV strains each representing a distinct genetic lineage or sublineage (34) (Fig. 1) was shuffled with DNase I digestion followed by PCR without primers for the reassembly. A PCR product consisting of reassembled shuffled products with an expected size of 468 bp was obtained after a second PCR with specific primers flanking the shuffled region. To generate chimeras containing segments derived from all seven parental viral strains, the shuffling process was iterated by using the shuffled DNA pool from the first round shuffling as the parents (6, 46).

Sequence analyses of 30 representative clones that were randomly selected from the shuffled library revealed that all contained chimeric GP5 sequences but only two clones contained sequences from all 7 parental viruses. The numbers of crossovers ranged from 8 to 12 in the

shuffled GP5 gene products. The GP5 clone DS722 that contains segments derived from all seven parental viral sequences with 12 crossovers was selected from the shuffled library (Fig. 2) and cloned into the backbone of a DNA-launched PRRSV infectious clone (29). The resulting chimeric virus DS722 containing the shuffled GP5 gene from 7 different strains of PRRSV was successfully rescued from transfected cells (Fig. 4B).

Similar approaches were used to shuffle the region spanning the GP5-M genes of 6 distinct strains of PRRSV. Sequence analyses of 10 representative clones that were randomly selected from the shuffled library revealed that all contained the chimeric GP5 sequences, and 6 of the 10 clones contained sequences derived from all the 6 parental viruses. The numbers of crossovers ranged from 12 to 22 in the shuffled GP5 gene products. The chimeric GP5-M clone DS5M3 containing segments derived from all 6 parental viral sequences with 18 crossovers (Fig. 2) was selected and cloned into the backbone of the DNA-launched PRRSV infectious clone. The GP5-M chimeric virus DS5M3 was successfully rescued from transfected cells (Fig. 4C). The amino acid differences in GP5 among the parental and shuffled viruses are indicated in Figure 3.

The two chimeric viruses replicated at a lower level both in MARC-145 cells and PAM cells and formed smaller plaques in MARC-145 cells compared to the parental virus. To characterize and compare the growth characteristics between the two chimeric viruses (DS722 and DS5M3) and the parental virus (VR2385), the growth kinetics of these three viruses were analyzed by infection of MARC-145 cells or PAM cells with the respective virus at the same M.O.I. of 0.1. The infectious virus titers were determined at different hpi. The results showed that in MARC-145 cells, the chimeric virus DS722 replicated significantly lower than the parental virus VR2385 at all the time points except 60 and 84 hpi whereas the chimeric virus

DS5M3 also displayed a significantly lower level of replication compared to VR2385 (Fig. 5A). In PAM cells, the chimeric virus DS722 replicated significantly lower than the parental virus VR2385 at all the time point except 6 and 12 hpi whereas the chimeric virus DS5M3 displayed a significant lower level of replication at all the time point compared to VR2385 (Fig. 6).

Both chimeric viruses and the parental virus developed plaques within 4 dpi. However, the diameters of the plaques formed by the two chimeric viruses were significantly smaller than that by the parental virus ($p < 0.001$) (Fig. 5B), further indicating that the chimeric viruses had a reduced growth capacity *in vitro* than the parental virus.

Both chimeric viruses (DS722 and DS5M3) were attenuated in pigs. The pathogenicity study revealed that pigs infected with the chimera DS5M3 had significantly lower serum viral RNA loads than pigs infected with the parental virus VR2385 at both 7 ($p = 0.02$) (Fig. 7A) and 14 dpi ($p = 0.0009$) (Fig. 7B). Similarly, the serum samples from pigs infected with chimera DS722 also had lower viral RNA loads than pigs infected with the parental virus at both 7 and 14 dpi, and the difference was significant at 7 dpi ($p = 0.03$) (Fig. 7A) but not at 14 dpi, although most sera from the DS722 group displayed lower viral RNA loads than those from the VR2385 group at 14 dpi (Fig. 7B).

Similarly, pigs infected with the chimera DS5M3 had significantly lower viral RNA loads ($p < 0.0001$) in the lung tissues than pigs infected with the VR2385. The pigs infected with the chimera DS722 also had a lower viral load in the lung than pigs infected with the parental virus, although the difference was not significant (Fig. 7C).

Macroscopic lung lesions were generally absent or mild in pigs inoculated with DMEM and with the two chimeras DS5M3 and DS722 (Fig. 8A). In pigs infected with the parental VR2385

virus, visible gross lung lesions were more pronounced and affected an average of 42% of the lung surfaces (Fig. 8A). The mean scores of the gross lung lesions in pigs inoculated with the two chimeric viruses DS722 ($p = 0.005$) and DS5M3 ($p < 0.0001$) were significantly lower than that of the parental virus VR2385 pigs (Fig. 8A). The mean scores of the histological lung lesions in pigs infected with the chimeric viruses DS722 ($p = 0.0002$) and DS5M3 ($p < 0.0001$) were significantly lower than that in pigs infected by the parental VR2385 virus (Fig. 8B).

Chimera DS722, but not chimera DS5M3, elicited protection in pigs against PRRSV challenge at a level similar to its parental virus. To investigate whether the two attenuated chimeras can still elicit protection against PRRSV, pigs were first vaccinated with the parental virus VR2385, chimeras DS722, DS5M3 or DMEM, respectively (Table 2). Eight to nine vaccinated pigs each in groups 1, 3, 5 and 7 were then challenged at 35 dpv with the parental VR2385 virus (lineage 5). Nine vaccinated pigs each in groups 2, 4, 6 and 8 were challenged at 35 dpv with a heterologous NADC20 virus (lineage 9) (Table 2). At the time of challenge at 35 dpv, viremia was not detected in any of the pigs by RT-PCR. All pigs were necropsied at 14 dpc.

For the pigs challenged with the parental strain VR2385, at 7 dpc, the serum viral RNA loads significantly decreased in pigs vaccinated with the VR2385 ($p=0.005$) or with the chimera DS722 ($p=0.003$) but not with the chimera DS5M3 when compared to group 7 control pigs (Fig. 9A). Similarly, at 14 dpc, the serum viral RNA loads significantly decreased in pigs that were vaccinated with the VR2385 ($p=0.01$) or with the chimera DS722 ($p=0.01$) but not with the chimera DS5M3 when compared to group 7 control pigs (Fig. 9B). The reduction of the serum viral RNA loads against challenges with parental VR2385 and heterologous NADC20 in pigs vaccinated with the chimera DS722 was similar to that in pigs vaccinated with the parental virus

VR2385 both at 7 and 14 dpc (Fig. 9A, 9B). The viral RNA loads in the lung tissues at 14 dpc were significantly decreased in pigs vaccinated with the parental virus VR2385 ($p=0.002$) or with the chimera DS722 ($p<0.0001$) but not with the chimera DS5M3 when compared to group 7 control pigs (Fig. 8C). The pigs vaccinated with the chimera DS722 displayed a significant lower viral RNA loads in the lung tissues compared to the pigs vaccinated with VR2385 (Fig. 9C).

For pigs challenged with a heterologous NADC20 strain, at 7 dpc, the serum viral RNA loads were significantly lower in pigs that vaccinated with the parental virus ($p = 0.0002$) or with the two chimeras DS722 ($p=0.0002$) and DS5M3 ($p=0.02$) when compared to group 8 controls (Fig. 9A). Similarly, at 14 dpc, there were significant reductions in the serum viral RNA loads in pigs vaccinated with the parental virus VR2385 ($p=0.01$) or with two chimeras DS722 ($p=0.03$) and DS5M3 ($p=0.02$) when compared to group 8 control pigs (Fig. 9B). Also, the viral RNA loads in the lung tissues at 14 dpc were significantly reduced in pigs vaccinated with the parental virus VR2385 ($p=0.002$) and with the chimera DS722 ($p=0.01$) but not with the chimera DS5M3 when compared to group 8 control pigs (Fig. 9C). The reduction of viral RNA loads in the lung tissues of pigs vaccinated with the chimera DS722 was similar to that in pigs vaccinated with the parental virus VR2385 both at 7 and 14 dpc (Fig. 9C).

At necropsy, the average scores of both macroscopic and microscopic lung lesions in pigs vaccinated with two chimeras (groups 3, 4, 5, 6) were significantly lower than those in the control pigs in groups 7 and 8 (Fig. 10A, 10B). The protection based on the macroscopic and microscopic lung lesions was much more effective in the DS722-vaccinated pigs than in DS5M3-vaccinated pigs. The average scores of gross and microscopic lung lesions in DS722-vaccinated pigs were mostly similar to those in VR2385-vaccinated pigs, although the scores in DS5M3-vaccinated pigs were significantly higher than those in VR2385-vaccinated pigs (Fig.

10).

Both chimeras DS722 and DS5M3 were stable *in vivo*. The shuffled genes of chimeric viruses DS722 and DS5M3 recovered from the sera of pigs in the respective groups at 14 dpi were amplified by RT-PCR and sequenced. Sequence analyses revealed that the sequences of recovered viruses were the same as the original virus inocula, thus indicating the genetic stability of these two chimeric viruses in animals.

DISCUSSION

Molecular breeding through DNA shuffling can direct the evolution of viruses *in vitro* and select new strains with desired traits. To determine if molecular breeding of virus envelope genes that are important virulence determinants can produce an attenuated virus that retain its protective ability against challenge, we bred the GP5 gene of 7 and the GP5-M genes of 6 genetically distinct strains of PRRSV by DNA shuffling and iteration of the shuffling process. The application of iteration of DNA shuffling process increased the chance to incorporate all parental viral genes into the small GP5 region (6, 46). Two representative chimeric viruses, a GP5 chimera DS722 and a GP5-M chimera DS5M3, were rescued and selected for further studies. Although both chimeras were infectious *in vitro*, they both displayed a lower level of virus replication in both MARC-145 cells and PAM cells. In addition, on MARC-145 cells, both chimeric viruses formed smaller plaques when compared to the parental virus, indicating that the two shuffled chimeric viruses exhibited an attenuated phenotype *in vitro*.

To further determine whether DNA shuffling of the GP5 or GP5-M gene altered the virus virulence *in vivo*, we conducted a pathogenicity study (Table 1) and showed that there was a

significant reduction in both the macroscopic and microscopic lung lesion scores in pigs infected with the two chimeras when compared to those infected with the parental virus. Significant reductions in viral RNA loads in sera and lung tissues were also found in pigs infected with the chimera DS5M3. The *in vitro* growth and the *in vivo* pathogenicity studies indicated that both chimeras were attenuated. Therefore, rapid attenuation of PRRSV was achieved in this study by shuffling of the virulence-determinant GP5 genes from multiple genetically divergent virus strains. This unique DNA shuffling approach to attenuate a virus is advantageous than many other traditional reverse genetics system approaches in that DNA shuffling mimics the natural evolution of viruses and that it does not require an understanding of the functionality of the shuffling regions, rather the approach relies on a functional screening for the desired traits of the shuffled viruses such as attenuation phenotype in this study.

Since the two chimeric viruses displayed an attenuated phenotype *in vivo*, we next evaluated whether the chimeric viruses can still induce protection against PRRSV challenge. Eight groups of pigs were first vaccinated with the parental virus VR2385, the two chimeras or DMEM medium, respectively (Table 2). At 35 dpc, pigs in each group were challenged with a homologous (lineage 5) or a heterologous PRRSV (lineage 9). The results revealed that the chimera DS722 still elicited a solid protection against challenges by both homologous and heterologous PRRSV strains. However, the GP5-M shuffled chimeric virus DS5M3 did not induce sufficient level of protection, even though there was a significant reduction in macroscopic and microscopic lung lesion scores. Thus, the GP5-shuffled chimeric virus DS722 still retains its ability to elicit protection against PRRSV, suggesting that the DNA shuffling of the virulence-determinant gene attenuated the virus but did not impair the ability of this shuffled virus for eliciting protection.

We had initially thought that the GP5-M chimera DS5M3 would also retain its ability to elicit protection since GP5 and M form heterodimers (31). The observed poor protection of chimera DS5M3 was likely due to the low replication fitness of this chimera *in vivo* since only low levels of chimera DS5M3 viral RNA were detected in both sera and lung tissues in the pathogenicity study. The GP5-M DNA shuffling included some critical regions for virus replication such as the GP5 signal peptide sequence and the overlapping region, and thus shuffling of these critical regions may have likely affected the viral replication efficiency *in vivo*, leading to over-attenuation of the chimera DS5M3 and thus poor protection compared to the GP5 chimera DS722. In addition, a missing of a glycosylation site at 34 aa of DS5M3 compared to its parental virus VR2385 may contribute to the attenuation of viral virulence according to the recent study (41). Although the exact mechanism of attenuation by DNA shuffling remains unknown, the attenuation phenotype of the shuffled viruses may be attributed to the altered growth efficiency of the chimeric viruses. In addition, potential conformational changes of the shuffled GP5 in the chimeras may alter its interactions with other viral proteins or host cells leading to attenuation (36).

In conclusion, attenuation of a virus was demonstrated, for the first time, by DNA shuffling of its envelope genes. We successfully produced two chimeric viruses that displayed attenuated phenotypes both *in vitro* and *in vivo* by shuffling the GP5 gene containing major virulence determinants and the GP5-M genes. The attenuated shuffled virus DS722 still induced protection similar to its parental virus against PRRSV infection. Although development of an improved PRRSV vaccine with better protection is not the scope of the present study, it is logical to speculate that further shuffling of other structural genes such as GP3 and GP4 that are relevant for neutralizing activities in the future may lead to the generation of a more broadly-protective

PRRSV MLV vaccine. Therefore, attenuation of a positive-strand RNA virus by DNA shuffling as demonstrated in this study has important implications for future vaccine development and thus has broad general interests to the scientific community as this approach of rapid virus attenuation can be easily applied to other important human and veterinary viruses.

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Fig. 1. A phylogenetic tree based on the GP5 genes of selected PRRSV strains from different genetic lineages of type 2 PRRSV as reported by Shi et al (110). The phylogenetic tree was constructed by using the neighbor-joining method with bootstraps in 1000 replicates. The numbers above each major branch indicate the bootstrap values. The GP5 sequences of the 7 parental strains selected for the shuffling in the study were indicated with boldface letters and *italic* font in the tree. Each lineage, corresponding to those reported by Shi et al (110), was labeled as “L” with a number besides a vertical line.

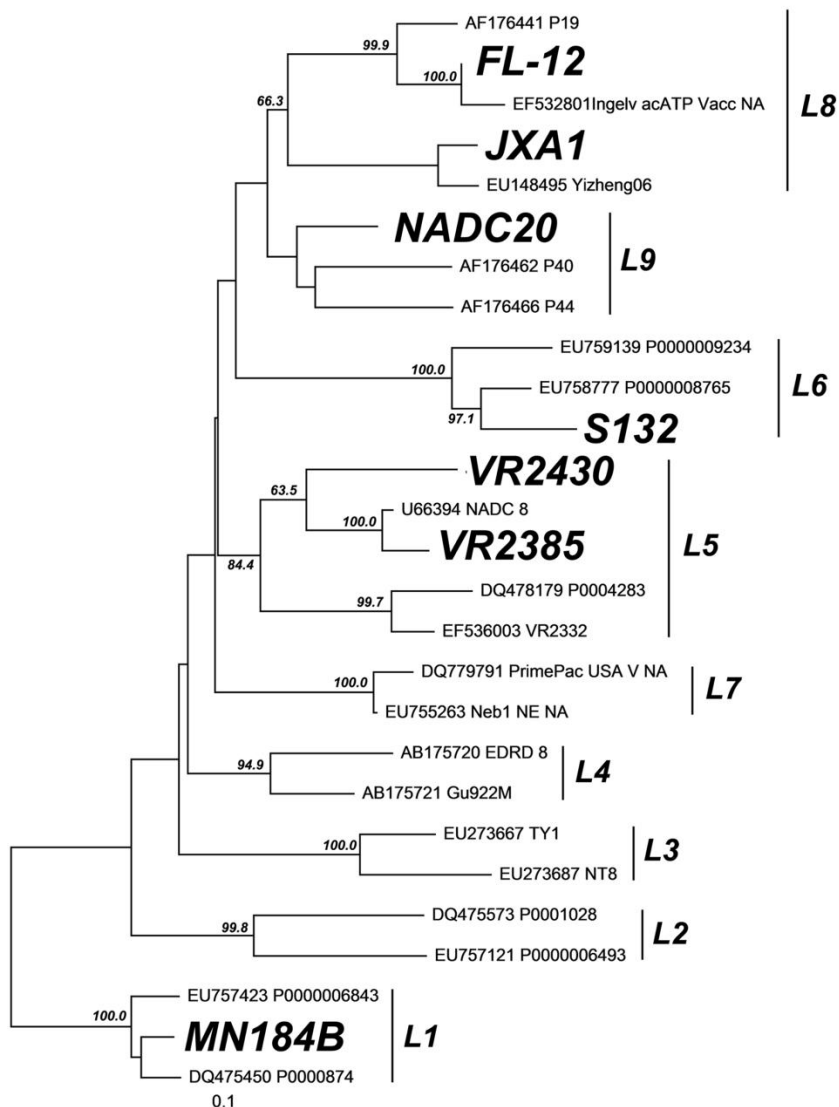


Fig. 1

Fig. 2. Schematic diagram of the shuffled chimeric GP5 gene sequences in two representative chimeras (DS722 and DS5M3). The parental virus sequences of the GP5 gene for the two chimeras are depicted schematically. The exact boundaries of crossovers were indicated with the nucleotide position numbers relative to the GP5 gene. Each pattern represents the sequence derived from an individual parental virus strain. If two patterns are displayed in same region, it indicates that this particular region contains sequences shared by two different parental strains. The GP5 sequences of the parental virus strains and the two shuffled chimeras were deposited in GenBank database and the GenBank accession numbers are JX044140 (VR2385), JX050225 (VR2430), AY545985 (FL-12), DQ176020 (MN184B), EF112445 (JXA1), JX069953 (NADC20), JX069952 (S132), JX044138 (DS722), and JX044139 (DS5M3).

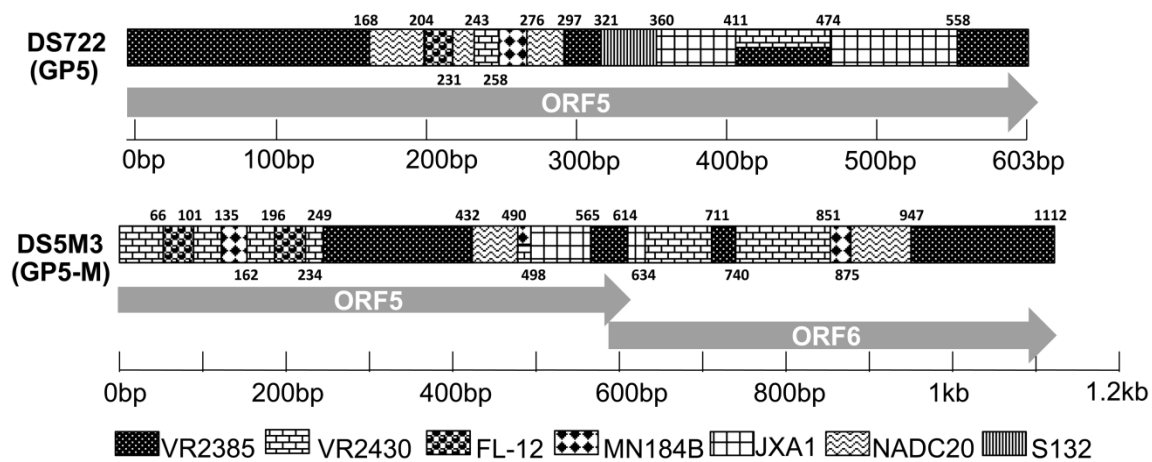


Fig. 2

Fig. 3. An alignment of the GP5 amino acid sequences among the seven parental virus strains and the two chimeras (DS722 and DS5M3). The GP5 sequence of the backbone virus VR2385 was shown on top. Only differences were indicated for other strains.

	10	20	30	40	50	60	70	80	90	100
VR2385	MLGKCLTAGCCSQLLFLWCIVPSCFVALVSANGNSSNLQLIYNLTLCELNGTDWLANKFDWAVECFVIFPVLTHIVSYGALTTSHEFLDTVGLVTVSTAG									
VR2430	Y..S.....W....A...SIN.PH.....GE.....									
FL12	...R.....R..S.....F..A...N..S...H.....KD...T.....									
MN184	Y...P.....F..LA...N..SS...H.....I.....NDH.S...T.....I.....									
JXA1	C...R.....FYLA.V..N..SN.N..HI.....Q.....T.....A.....									
NADC20	R..P.....F..AVI.N..SS...HF.S.....KR.....T.....									
S132	..VN.....R.....CFP.V.CN.SNANG.H.....T.....A.....									
DS722	Y..S.....F..A...N..SIN.PH.....I.....GE.....T.....									
DS5M3	Y..S.....F..A...N..SIN.PH.....I.....GE.....T.....									
	110	120	130	140	150	160	170	180	190	200
VR2385	FVHGRYVLSSIIYAVCALAALICFVIRLAKNCMSWRYSCTRYTNFLDDTKGRLYRWRSPIIEKRGKVEVEGHLIDLKRVVLDGSAATFVTRVSAEQWGRP									
VR2430	.S.....FT.....I..KI.....									
FL12	.Y.....G.....V..L.....L									
MN184	YY.A.....T.....I..G.D.....K.....									
JXA1	YY.....V..G.....L.....L..L									
NADC20	.Y.....F.....G.....V..L.....L									
S132	.L.....FA..G..F.....Y.....G.....V..L.....L									
DS722	V..G.....V..L.....L									
DS5M3	V..G.....V..L.....L									

Fig. 3

Fig. 4. Rescue and *in vitro* passages of the shuffled chimeric viruses DS722 and DS5M3.

(A): Immunofluorescence assay (IFA) confirmation using anti-PRRSV N monoclonal antibody for the rescue of the parental virus VR2385 in MARC-145 cells infected with the supernatant of BHK-21 cells transfected with a DNA-launched PRRSV infectious clone pIR-VR2385-CA. **(B) and (C):** IFA confirmation for the rescues of the chimeric viruses DS722 **(B)** and DS5M3 **(C)** in MARC-145 cells infected with the supernatant of BHK-21 cells transfected cells with shuffled clones pIR-DS722 and pIR-DS5M3, respectively. **(D):** There is no fluorescent signal in the MARC-145 cells infected with the supernatant of BHK-21 cells transfected with a plasmid containing a defective PRRSV VR2385 backbone.

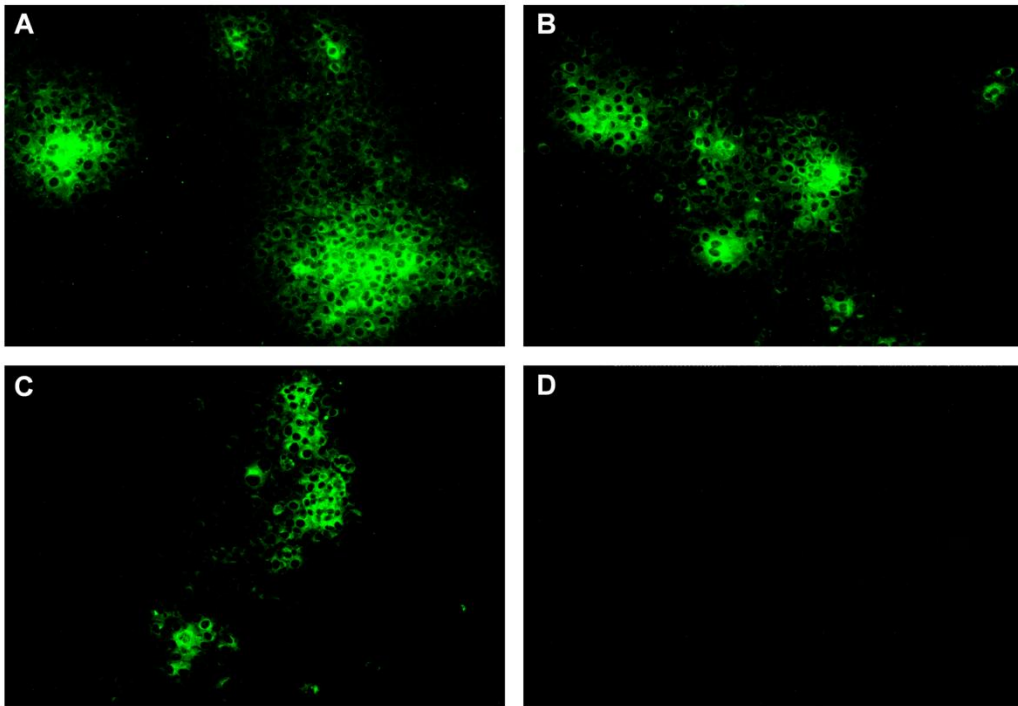


Fig. 4

Fig. 5. *In vitro* growth kinetics and plaque morphology of the shuffled chimeric viruses DS722 and DS5M3 on MARC-145 cells. (A): Growth kinetics of the parental virus VR2385 and the two chimeric viruses (DS722, DS5M3). The virus infectious titers (TCID₅₀/ml) were determined at the indicated time points post-infection. The asterisk (*) and pound (#) signs indicate a statistically significant difference between the chimeric viruses and its parental virus at that time point. The experiments were performed in triplicates and the bars indicate standard errors. **(B):** Plaque morphology and diameter of the parental virus VR2385 and chimeric viruses DS722 and DS5M3.

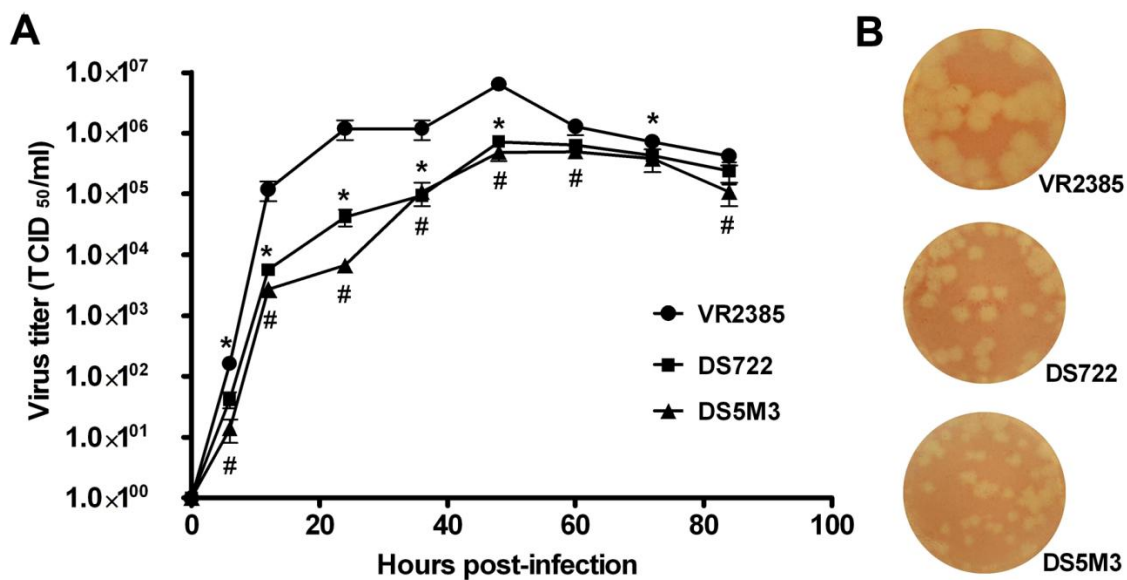


Fig. 5

Fig. 6. *In vitro* growth kinetics of the shuffled chimeric viruses DS722 and DS5M3 on PAM cells. The virus infectious titers (TCID₅₀/ml) were determined at the indicated time points post-infection. The asterisk (*) and pound (#) signs indicate a statistically significant difference between the chimeric viruses and its parental virus at that time point. The experiments were performed in triplicates and the bars indicate standard errors.

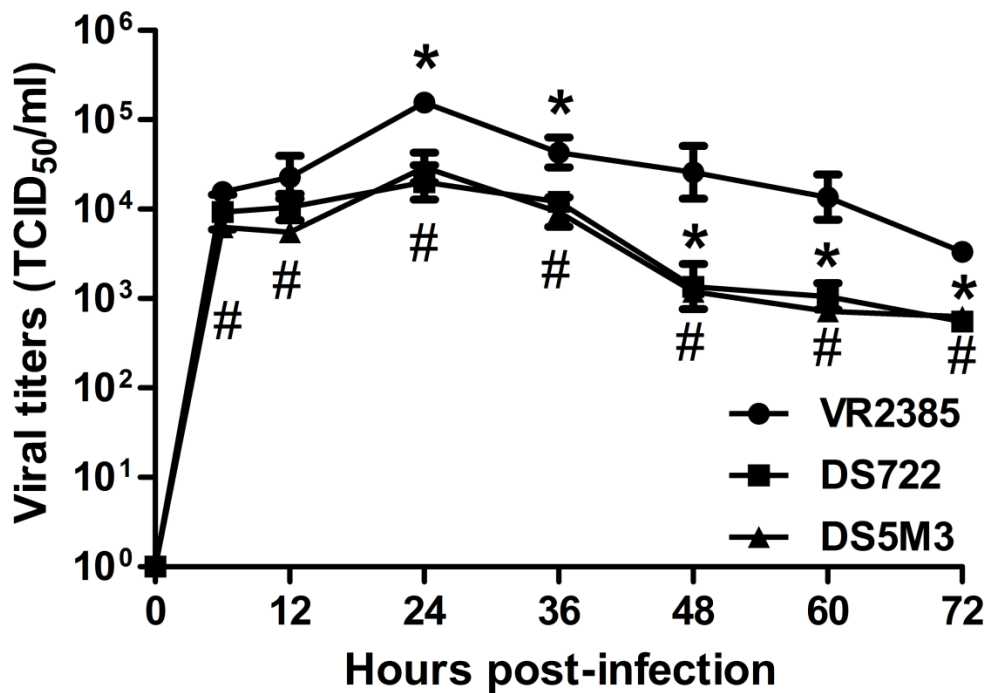


Fig. 6

Fig. 7. Viral RNA loads in serum samples and lung tissues in the comparative pathogenicity study from pigs infected with parental virus VR2385 and chimeric viruses DS722 or DS5M3 or inoculated with DMEM (negative control) at 7 and 14 dpi, respectively. (A): PRRSV viral RNA loads in serum samples at 7 dpi. (B): PRRSV viral RNA loads in serum samples at 14 dpi. (C): Viral RNA loads in the lung collected during necropsy. Significant differences are indicated with asterisk signs (* $p<0.05$, ** $p<0.01$, * $p<0.001$). In all three panels, the numbers within a circle along the x axis indicate the numbers of animals in each group that were tested negative for viral RNA.**

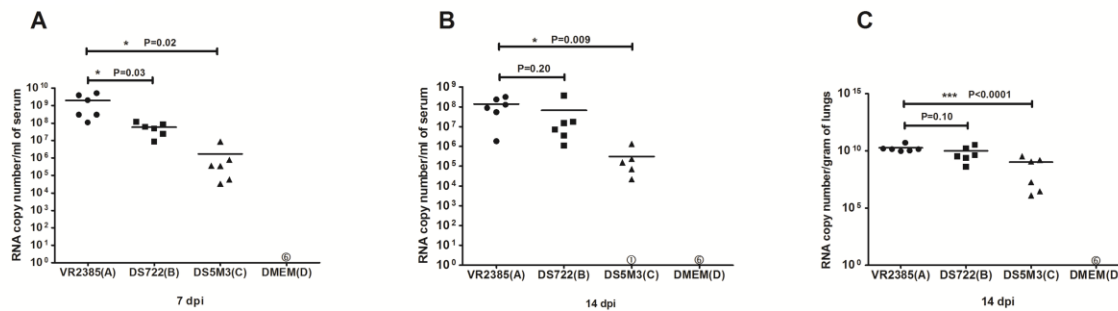


Fig. 7

Fig. 8. Macroscopic and microscopic lesions in the lung tissues from pigs experimentally infected with the parental virus VR2385 and the two chimeric viruses DS722 or DS5M3 during necropsy at 14 dpi. (A): Macroscopic lesion scores of the lung tissues at 14 dpi from the 6 pigs infected with the parental virus VR2385, chimeric viruses DS722 and DS5M3, or inoculated with DMEM (negative control), respectively. **(B):** Microscopic lesion scores of the lung tissues at 14 dpi. Significant differences are indicated with asterisk signs (** $p<0.01$, *** $p<0.001$, and **** $p<0.0001$).

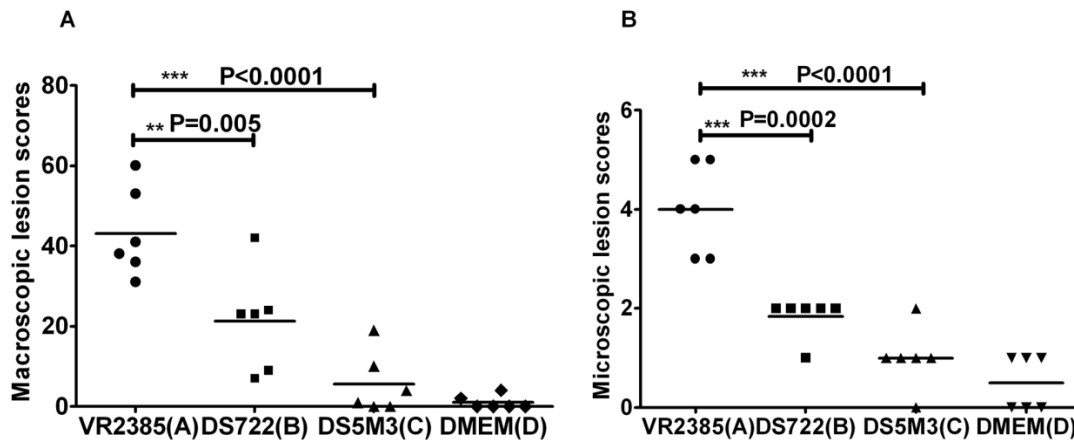


Fig. 8

Fig. 9. Viral RNA loads in serum samples at 7 and 14 days and in lung tissues at 14 days post-challenge (dpc) from the challenge/protection study in pigs vaccinated with the chimeric viruses (DS722 or DS5M3) followed by challenge. (A): PRRSV viral RNA loads in serum samples from pigs at 7 dpc with a homologous PRRSV VR2385 or a heterologous PRRSV NADC20. (B): PRRSV viral RNA loads in serum samples from pigs at 14 dpc with PRRSV VR2385 or NADC20. (C): PRRSV viral RNA loads in the lung tissues from pigs at 14 dpc. Significant differences are indicated with asterisk signs (* p <0.05, ** p <0.01, * p <0.001). In all three panels, the numbers within a circle along the x axis indicate the numbers of animals in each group that were tested negative for serum viral RNA.**

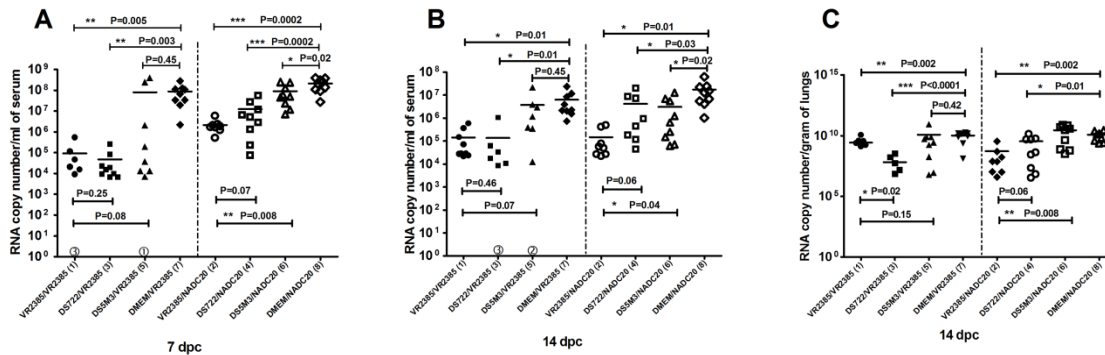


Fig. 9

Fig. 10. Macroscopic and microscopic lesion scores of lung tissues at necropsy at 14 dpc following challenge in pigs vaccinated with the chimeric viruses DS722 and DS5M3. (A): Macroscopic lung lesion scores from pigs at necropsy 14 dpc. **(B):** Microscopic lesion scores of the lung tissues from pigs at necropsy 14 dpc. Significant differences are indicated with asterisk signs (* $p<0.05$, *** $p<0.001$).

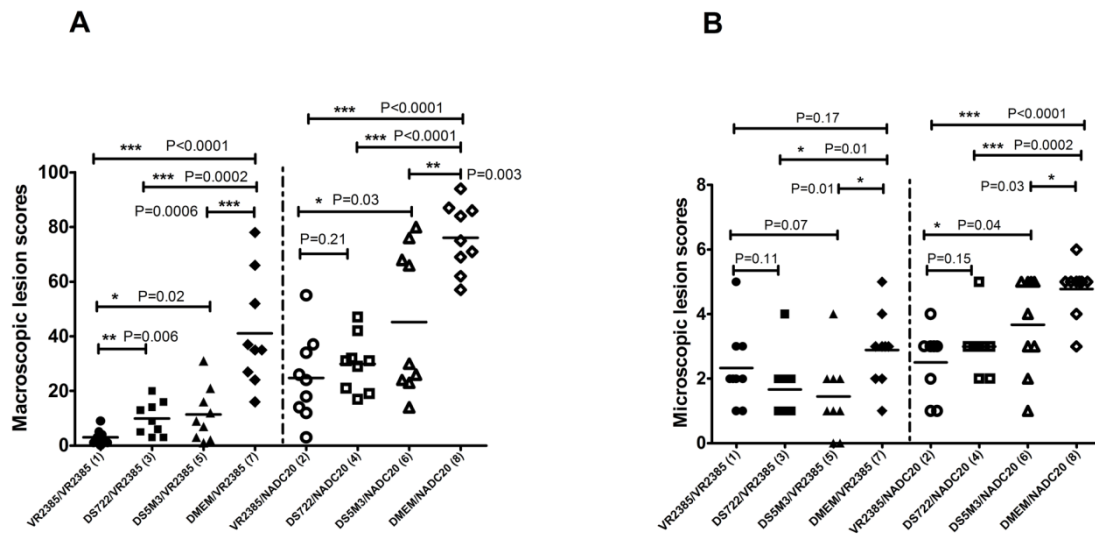


Fig. 10

Table 1 Experimental design for the comparative pathogenicity study to determine the virulence of the shuffled chimeric viruses in pigs

Group	Inocula (2×10^5 TCID ₅₀ /pig)	No of pigs	Necropsy at 14 days post-inoculation
A	Parental VR2385	6	6
B	Chimera DS722	6	6
C	Chimera DS5M3	6	6
D	DMEM control	6	6

Table 2 PRRSV challenge and protection study in pigs vaccinated with the attenuated chimeric viruses or with the parental virus

Group	No of pigs	Vaccination at 0 day (2×10^5 TCID ₅₀ /pig)	Challenge virus at 35 days post-vaccination ^a	Necropsy at 14 days post-challenge
1	9	Parental VR2385	VR2385	8 ^b
2	9	Parental VR2385	NADC20	9
3	9	Chimera DS722	VR2385	9
4	9	Chimera DS722	NADC20	9
5	9	Chimera DS5M3	VR2385	9
6	9	Chimera DS5M3	NADC20	9
7	9	DMEM control	VR2385	9
8	9	DMEM control	NADC20	9

^aThe challenge virus dose for all viruses is 2×10^5 TCID₅₀/pig.

^b1 pig died of unrelated cause before the challenge.

Chapter 4

Computer-aided codon-pairs deoptimization of viral genes attenuates porcine reproductive and respiratory syndrome virus

Yan-Yan Ni, Zhao Zhao, Tanja Opriessnig, Sakthivel Subramaniam, Lei Zhou, Dianjun Cao,

Qian Cao, Hanchun Yang, and Xiang-Jin Meng

Submitted to Virology

ABSTRACT

Synthetic attenuated virus engineering (SAVE) is an emerging technology that enables to rapidly attenuate viruses. In this study, by using SAVE we demonstrated rapid attenuation of an arterivirus, porcine reproductive and respiratory syndrome virus (PRRSV). The gp5 and nsp9 genes of PRRSV were each codon-pair deoptimized aided by a computer algorithm. Two codon-pair deoptizmiend viruses, SAVE5 with deoptimized gp5 gene and SAVE9 with deoptimized nsp9 gene, were successfully rescued *in vitro*. The SAVE5 and SAVE9 viruses both replicated at a lower level *in vitro* with a significant decrease of corresponding protein expression compared to the wild-type VR2385 virus. Pigs experimentally infected with the SAVE5 virus had significantly lower viremia level up to 14 days post-infection as well as significantly reduced gross and histological lung lesions when compared to wild-type VR2385 virus-infected pigs, indicating attenuation of the SAVE5 virus. This study proved the feasibility of rapidly attenuating PRRSV by SAVE.

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is arguably the most economically-important swine disease facing the global swine industry today causing devastating economic losses of approximately \$664 million per year in the United States alone (Lunney et al., 2010; Neumann et al., 2005; Nieuwenhuis et al., 2012). PRRS is caused by an enveloped, single-stranded, positive-sense RNA virus, PRRS virus (PRRSV) (Benfield et al., 1992; Cavanagh, 1997; de Groot RJ, 2012). The genome of PRRSV is approximately 15 kb in size and consists of structural genes (ORF2-ORF7) and non-structural genes (ORF1a and ORF1b) (Allende et al., 1999; Meulenberg et al., 1993; Nelsen et al., 1999). PRRSV is extremely heterogenic with at least 9 distinct subtypes among the type 2 PRRSV and at least 4 distinct subtypes of type 1 PRRSV (Shi et al., 2010; Stadejek et al., 2013). The current available vaccines, although effective against homologous or closely-related PRRSV strains, are not effective against heterologous PRRSV strains circulating in the swine population.

Extensive genetic variation and recombination led to frequent emergence of new and more virulent strains of PRRSV worldwide (Murtaugh et al., 2010; Shi et al., 2010; Tian et al., 2007). Therefore, rapid attenuation of emerging variant field strains of PRRSV for further development of effective vaccines is urgently needed. Traditional methods for attenuating PRRSV and other viruses are usually time-consuming, and have the additional disadvantage that the traditionally attenuated PRRSVs have an inherent risk of reversion to a pathogenic phenotype (Meng, 2000). To overcome these problems associated with traditional virus attenuation, in this study we utilized a computer-based

codon-pairs deoptimization technology, synthetic attenuated virus engineering (SAVE), for rapid attenuation of PRRSV.

It is known that, among the synonymous codon pairs encoding the same amino acid residues, some codon pairs occur more frequently than others in certain species (Fedorov et al., 2002; Gutman and Hatfield, 1989; Moura et al., 2007), which is known as codon-pair bias. Different species have different codon-pair biases, and it has been demonstrated that the codon-pair bias is related to the efficiency of the protein synthesis thereby affects the accumulation of synthesized proteins inside cell (Coleman et al., 2008; Mueller et al., 2010). Therefore, the principle of the SAVE technology is that codon-pair deoptimization of key viral gene(s) decreases the protein expression levels of the corresponding gene(s) that are related to viral virulence. The codon-pair deoptimization is based on computer-aided modification of the naturally optimized pairs of codons in a viral gene sequence without altering the codon bias and the amino acid sequence (Mueller et al., 2010). The SAVE technology was first applied to attenuate poliovirus (Coleman et al., 2008) by using a computer algorithm to rearrange codons existing in a poliovirus gene fragment to change the pattern of codon pairs but preserve codon bias or amino acids. It was shown that the efficiency of polio viral protein translation was decreased by codon-pair deoptimization which led to poliovirus attenuation. The SAVE approach was also applied to successfully attenuate influenza virus (Mueller et al., 2010). Unfortunately, the promising potential of this novel approach to rapidly attenuate other important viruses especially veterinary viruses has not been explored.

The ORF5 gene of PRRSV encodes the major envelope glycoprotein GP5 which is critical to form viral particles with the M protein encoded by the ORF6 (Wissink et al., 2005). The GP5 protein forms a heterodimer with the M protein which is important for viral assembly (Van Breedam et al., 2010). This GP5-M dimer is involved in virus entry particularly in the alveolar macrophages, the host cells in pigs (Delputte et al., 2007). In addition, the GP5 is associated with apoptosis induced by PRRSV in MARC-145 cells (Gagnon et al., 2003; Miller and Fox, 2004). It has also been demonstrated that the GP5 contains the major virulence determinants of PRRSV (Kwon et al., 2008). The GP5 contains a N-terminal signal peptide which overlaps with a small structural protein encoded by ORF5a and may play a role in viral replication (Johnson et al., 2011).

The ORF1a and ORF1b encode non-structural polyproteins, which are further processed to at least 15 non-structural proteins (Nsp) of PRRSV. The translation of ORF1b is initiated by a frame-shift mechanism with a slippery structure “G(C)UUUAAAC” and a downstream RNA pseudoknot (Fang and Snijder, 2010; Snijder and Meulenberg, 1998). The Nsp9 is a critical non-structural protein responsible for viral replication and contains a putative RNA-dependent RNA polymerase (RdRp) domain (Fang and Snijder, 2010).

In this study, we utilized the computer-aided codon-pair deoptimization approach to successfully attenuate PRRSV. We demonstrated that, by deoptimization of the codon-pairs of the GP5 structural gene and the nsp9 non-structural gene, two modified PRRSVs each with a codon-pair deoptimized gene were successfully rescued. The growth capacity

and virulence of these two modified viruses were compared with the wt PRRSV both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Application of computer-based algorithm to deoptimize codon pairs of PRRSV GP5 and nsp9 genes. We utilized the SAVE approach (Coleman et al., 2008) to deoptimize the PRRSV GP5 and nsp9 genes by minimizing their codon pair bias. The GP5 structural gene was chosen because it is one of the major virulence determinants of PRRSV, and the rationale to produce another modified virus by deoptimizing nsp9 gene is that the nsp9 is essential for PRRSV replication. In the GP5 gene, a region of 146 nucleotides (nt) overlapping with ORF5a and a 16-nt region overlapping with ORF6 were excluded for codon-pair deoptimization to avoid the alteration of unrelated proteins. The 5' end region of 81-nt in the nsp9 gene was also excluded for codon-pair deoptimization since this region is involved in the frame-shifting mechanism of PRRSV protein translation.

The representation of all amino acids (excluding stop codon) in terms of nucleotide triplets is 61, which produces the numbers of possible codon pairs as $61^2=3721$. The Codon Pair Score (CPS) is defined as the natural log of the ratio of the observed over the expected number of occurrences of each codon pair over all coding regions. Given two codons A and B, and the corresponding amino acid X and Y, respectively, the CPS is calculated with the equation below:

$$CPS = \ln\left(\frac{F(AB)}{\frac{F(A)F(B)}{F(X)F(Y)} \cdot F(XY)}\right)$$

Subsequently, the “CPB” (Codon Pair Bias) is computed with the equation below:

$$CPB = \sum_{i=1}^k \frac{CPS_i}{k-1}$$

This denotes the average CPS over an entire gene.

For a given gene, and two amino acid pairs within which, if they are the same in terms of amino acids, but utilize different codons, we can exchange the codon within one amino acid pair with the other, to manipulate the CPB. We used an approximation algorithm – simulated annealing – to minimize the CPB.

To preserve the functionality of the attenuated viral gene, we maintained the secondary structure of the gene while attempting the attenuation, and this was accomplished by restricting the difference of the free energy of the folding of the newly generated gene sequence with the original one. We used the RNAfold software in the Vienna RNA package (Schuster et al., 1994) to compute the minimum free energy (MFE).

DNA synthesis and cloning. The codon-pair deoptimized GP5 and nsp9 gene sequences were commercially synthesized *de novo* (Genscript). The codon-pair deoptimized GP5 and nsp9 gene fragments were each amplified with a pair of primers CpD5F/CpD5R and CpD9F/CpD9R, respectively (Table 1). For cloning purposes, the flanking fragments derived from the wt viral sequence containing naturally-occurring unique restriction sites were fused to the corresponding codon-pair deoptimized gene fragment (Table 1) and the fusion gene fragments were subsequently cloned into the backbone of a DNA-launched infectious clone of PRRSV, pIR-VR2385-CA, to replace the original GP5 or nsp9 gene sequence, respectively. These recombinant clones were designated as pIR-VR2385-SAVE5 and pIR-VR2385-SAVE9, respectively.

Cells and viruses. The only susceptible cell line to propagate PRRSV is the monkey kidney cell line MARC-145. However, in order to accurately assess the translation efficiency and replication of the codon-pair deoptimized viruses, a cell line of porcine origin that possesses the same translation system as the natural host animal of PRRSV is needed. Therefore, in this study, we utilized a susceptible porcine kidney PK15 cell line stably expressing PRRSV receptor molecule CD163, PK15-CD163, which was recently established in our lab for PRRSV infection followed the similar protocols described previously (Calvert et al., 2007). Cells were maintained in modified Eagle medium (MEM) (Invitrogen) supplemented with 2 to 10% fetal bovine serum (FBS) (Invitrogen), 1% antibiotic/antimycotic (Fisher), 1% sodium pyruvate (Invitrogen), and 1X MEM non-essential amino acids solution (Invitrogen). Hygromycin at 100µg /ml was added to growth medium for selection and maintenance of the PK15-CD163 cells. BHK-21 cells and MARC-145 cells were used for PRRSV rescue and propagation. Both cells were cultured in Dulbecco's modified Eagle medium (DMEM) (ATCC) supplemented with 2 to 10% FBS and 1% penicillin/streptomycin (Fisher).

Rescue of codon-pair deoptimized PRRSV. To rescue the codon-pair deoptimized viruses, recombinant clones pIR-VR2385-SAVE5 and pIR-VR2385-SAVE9 were each transfected into BHK-21 cells as described previously (Ni et al., 2011). The transfected cells were harvested at 24 hr post-transfection by freeze-thaw 3 times, and the cell lysates were passaged onto MARC-145 cells. To confirm the rescue of the codon-pair

deoptimized viruses, an immunofluorescence assay (IFA) with an anti-PRRSV N antibody was performed at 48 hours post-infection (hpi) as previously described (Ni et al., 2011). After three passages of the viruses on MARC-145 cells, the genomic region spanning the codon-pair deoptimized genes from the rescued viruses were amplified and sequenced to confirm the successful rescue of these viruses and the genetic stability of the codon-pair deoptimized viral genes *in vitro*. The viruses rescued from pIR-VR2385-SAVE5 and pIR-VR2385-SAVE9 were designated as SAVE5 and SAVE9, respectively.

Growth characterization of codon pair deoptimized viruses *in vitro*. To analyze the impact of codon-pair deoptimization of GP5 or nsp9 gene on the growth capacity of the rescued viruses *in vitro*, a PRRSV-susceptible porcine kidney cell line PK15-CD163 was used for viral infection and growth characterization. Confluent monolayers of PK15-CD163 cells seeded in 96-well plates were infected with the wt PRRSV VR2385 as well as with the codon-pair deoptimized SAVE5 and SAVE9 viruses at multiplicity of infection (m.o.i.) of 0.1, respectively. The infected cells were harvested at 6, 12, 24, 36, 48, 60 and 72 hpi, and the infectious titers of the viruses were determined as described previously (Ni et al., 2013). Triplicate experiments were performed in this study.

Western blotting analysis. To analyze the effect of codon-pair deoptimization of viral genes on viral protein translation, we performed western blot analyses. Briefly, at 24 hpi, the infected PK15-CD163 cells were rinsed twice with 4 °C phosphate-buffered saline (PBS) and lysed with RIPA buffer. The BCA Protein Assay Kit (Pierce) was used,

according to the manufacturer's instruction, to quantify the total protein concentration of the lysed samples. The same amount of the total proteins (50 µg) from each sample was loaded and separated by SDS-polyacrylamide gel electrophoresis (PAGE) and subsequently transferred to a nitrocellulose membrane. For Western blotting analysis, the membrane was first blocked with Odyssey blocking buffer (Licor Biosciences) for 1 hr, and then incubated with polyclonal antibody specific to GP5 (Biomatik) or monoclonal antibody specific to Nsp9 (provided by Dr. Hanchun Yang), N protein (SR30 from Rural Technologies, Inc.) or GAPDH (Thermo Scientific) diluted in PBS with 0.05% Tween 20 (PBST) and 10% blocking buffer at 4 °C overnight. After three washes of 10 min each with PBST, the membrane was incubated again with either IRDye 680RD Goat anti-Mouse IgG or IRDye 800CW Goat anti-Rabbit IgG according to the respective primary antibody (Licor Biosciences) diluted in 0.05% PBST with 10% blocking buffer for 1 hr at room temperature. After three washes of 10 min each with PBST, the proteins were visualized via the Licor Odyssey imaging device (Licor Biosciences).

Pathogenicity study of the codon-pair deoptimized viruses in specific-pathogen-free (SPF) pigs. To determine the virulence of the two codon-pair deoptimized viruses, a total of 32 SPF pigs were divided into 4 groups of 8 pigs each. The animal study was approved by the Institutional Animal Care and Use (IACUC) Committees from Virginia Tech and Iowa State University. Pigs in groups 2 and 3 were each intramuscularly inoculated with 2 ml of SAVE5 and SAVE9 viruses (2×10^5 TCID₅₀/pig), respectively. Pigs in group 1 were mock-infected with 2 ml of the cell culture media as negative

controls, and pigs in group 4 were intramuscularly inoculated with 2 ml of the wt VR2385 virus (2×10^5 TCID₅₀/pig) as positive controls. Serum samples from each pig were collected prior to inoculation and weekly thereafter and tested for viral loads by quantitative RT-PCR and for infectious virus titers by a TCID₅₀ infectivity assay in MARC-145 cells. All pigs were euthanized at 14 dpi, and the gross lung lesions were evaluated and scored at the time of necropsy according to a published method (Halbur et al., 1995). Lung tissues from each pig were also collected and fixed in 10% neutral buffered formalin and processed for histological examination of microscopic lung lesions (Halbur et al., 1995).

Quantitation of viral RNA loads using SYBR-Green based qRT-PCR. To quantify the viral RNA loads in serum and lung tissues from each pig after infection with virus or DMEM, a SYBR-Green based quantitative RT-PCR was used. Total RNAs were extracted from serum or lung tissue samples using TRI Reagent (MRC) and reverse transcribed to cDNA using the Superscript II kit (Invitrogen) with primer RTspR51 (Table 1). The subsequent qPCR assay was conducted in a CFX96 real-time PCR system (Bio-Rad). A pair of primers located in the conserved regions of PRRSV N gene (realtime2F and realtime2R, Table 1) was designed by utilizing the Beacon software and used in the PCR reactions as described (Ni et al., 2013). A dissociation curve analysis was performed using the instrument's default setting immediately after each PCR run to confirm the specificity of PCR product. Reaction was performed in triplicates.

Quantification of infectious virus titers in serum samples of infected pigs by an infectivity assay. To determine the infectious virus titers in serum samples, a TCID₅₀ assay were performed as described previously (Ni et al., 2013). Briefly, 10-fold serially diluted serum samples (10^{-1} to 10^{-4}) collected at different dpi's from pigs were used to infect MARC-145 cells seeded in 96-well plates. After 1 hr incubation at 37 °C, the inocula were removed and the cells were washed twice with PBS. The cells were maintained in fresh DMEM media supplemented with 2% fetal bovine serum for 48 h at 37°C before being fixed with 80% acetone (Sigma). The infectious titer of each serum sample was subsequently quantified using an immunofluorescence-based assay (Ni et al., 2013). Titration was performed in triplicates in this study.

Evaluation of gross and histological lesions in the lungs of infected pigs. The gross and histological lung lesions were evaluated by a veterinary pathologist who was blind to the treatment groups of the pigs. The scoring system for estimating the macroscopic lung lesions was based on the approximate volume that each lung lobe contributes to the entire lung as described previously (Halbur et al., 1995; Ni et al., 2013). Histological lung sections representative of all lobes from each pig were scored for the presence and severity of interstitial pneumonia ranging from 0 (normal) to 6 (severe diffuse interstitial pneumonia) as previously described (Halbur et al., 1995; Ni et al., 2013).

RESULTS

Characteristics of codon-pair deoptimized PRRSV GP5 and nsp9 genes. The distribution of the CPB score calculated from the available swine genes

(ftp://ftp.ensembl.org/pub/release-66/fasta/sus_scrofa/cdna/) is similar to that of human genes, which is in the range of 0 to 0.1 (data not shown). The CPB scores of PRRSV GP5 and nsp9 genes were each calculated and the original CPB scores for these two genes were in the range from -0.1 to 0.1. A computer algorithm was used to switch the existing codons in the GP5 and nsp9 genes to more underrepresented codon-pairs in order to decrease the CPB scores of the viral genes to approximate -0.3. The characteristics of the codon-pair deoptimized gene segments are shown in Table 2. The alignment of the GP5 gene sequences between the wt VR2385 virus and the codon-pair deoptimized SAVE5 virus is shown in Fig. 1.

Codon-pair deoptimized viruses were successfully rescued from recombinant infectious clones *in vitro*: The recombinant DNA-launched infectious clones with codon-pair deoptimized GP5 gene and codon-pair deoptimized nsp9 gene were designated as pIR-VR2385-SAVE5 and pIR-VR2385-SAVE9 respectively. The SAVE5 virus with codon-pair deoptimized GP5 gene and the SAVE9 virus with codon-pair deoptimized nsp9 gene were successfully rescued from infectious clones pIR-VR2385-SAVE5 and pIR-VR2385-SAVE9, respectively. The infectivity of the rescued viruses was confirmed by IFA with an anti-PRRSV N monoclonal antibody in the infected MARC-145 cells (Fig. 2).

Codon-pair deoptimization of GP5 or nsp9 gene reduces the corresponding viral protein synthesis in the rescued viruses. To investigate if the codon-pair

deoptimization of the GP5 or nsp9 gene affected the translation of the corresponding gene, western blot analyses were performed to assess the viral protein expression levels *in vitro*. The results showed that, at 24 hpi, the expression levels of codon-pair deoptimized GP5 or nsp9 genes from the respective SAVE5 and SAVE9 virus-infected cells were significantly reduced when compared to wt VR2385 virus-infected cells (Fig. 3). However, the expression levels of the viral nucleocapsid protein N or internal control GAPDH in cells infected with the codon-pair deoptimized viruses were not significantly different from that of wt virus-infected cells (Fig. 3).

Codon-pair deoptimization of GP5 or nsp9 gene decreased the growth kinetics of PRRSV *in vitro*. To compare the growth characteristics between the two codon-pair deoptimized viruses (SAVE5 and SAVE9) and the wt VR2385 virus, the growth kinetics of the three viruses were analyzed by infecting PK15-CD163 with each virus at the same m.o.i. of 0.1. The results showed that both SAVE5 and SAVE9 viruses had lower infectious titers and reduced levels of replication at all time points with statistically significant differences observed after 36 hpi (Fig. 4). When compared the growth kinetics between SAVE5 and SAVE9, both viruses showed a similar growth capacity *in vitro*.

Experimental infection of pigs with the codon-pair deoptimized SAVE5 virus resulted in significantly lower viral RNA loads and lower infectious titers in sera and lung tissues compared to wt virus-infection. The viral RNA loads in pig sera were quantified by qPCR. The group of pigs infected with the codon-pair deoptimized SAVE5

virus had significantly decreased viral RNA loads in sera at 7 dpi and 14 dpi when compared to the group of pigs infected with wt VR2385 ($p = 0.032$, $p = 0.036$) (Fig. 5A and 5B). The viral infectious titers in sera were also measured and, similar to viral RNA loads, there was also a significantly lower infectious virus titer in the SAVE5 virus-infected pigs compared to wt VR2385 virus-infected pigs at both time points ($p = 0.0097$, $p = 0.048$) (Fig. 6A and 6B). Likewise, there were also significantly lower viral RNA loads in the lung tissues from the SAVE5-infected pigs compared to wt VR2385 virus-infected pigs ($p = 0.047$).

The SAVE9 virus with codon-pair deoptimized nsp9 gene failed to replicate in pigs. The SAVE9 virus was successfully rescued in BHK-21 cells and infectious in MARC-145 cells *in vitro*. However, the group of pigs inoculated with the SAVE9 virus did not show detectable viral RNA or infectious virus in the sera (Fig. 5 and 6). Similarly, viral RNA was also not detected either in the lung tissues from pigs inoculated with the SAVE9 virus (Fig. 5C).

Pigs infected with the codon-pair deoptimized SAVE5 virus had significantly reduced macroscopic and microscopic lung lesions compared to the pigs infected with the wt VR2385 virus. Pigs inoculated with the GP5 gene deoptimized virus SAVE5 or DMEM showed absent or only very mild macroscopic lung lesions, whereas pigs inoculated with the wt VR2385 virus had very pronounced macroscopic lung lesions. The control pigs inoculated with DMEM showed no lesions. The difference of

the macroscopic lung lesions between the codon-pair deoptimized SAVE5 virus group and wt VR2385 virus group was significant ($p = 0.0057$) (Fig. 7A). The mean scores of the microscopic lung lesions were 1.38 ± 0.42 for the SAVE5 virus-infected pigs, and 3.88 ± 0.30 for the wt VR2385 virus-infected pigs, and 0.87 ± 0.23 for the negative control pigs. Similar to the gross lung lesions, the codon-pair deoptimized SAVE5 virus-infected group had also a significantly reduced mean interstitial pneumonia score compared to the wt VR2385 virus-infected group ($p = 0.0001$) (Fig. 7B).

The codon-pair deoptimized GP5 gene in the SAVE5 virus is genetically stable *in vivo*. To determine if the codon-pair deoptimized GP5 gene is stable during SAVE5 virus replication in pigs, we amplified and sequenced the GP5 gene region of the SAVE5 virus recovered from the infected pigs at 14 dpi. Sequence analysis revealed that the GP5 gene sequences of the SAVE5 virus recovered from the infected pigs at 14 dpi were identical to that of the original SAVE5 virus inocula, indicating that the codon-pair deoptimized GP5 gene was genetically stable in pigs during *in vivo* virus replication.

DISCUSSION

The frequent emergences of novel and more virulent strains of PRRSV worldwide necessitate the need for rapid development of vaccines to combat against these emerging variant strains of PRRSV. The SAVE approach based on codon-pair deoptimization is an emerging new technology that can rapidly attenuate viruses for further development of

potential vaccines. Unfortunately this promising technology has only been used for poliovirus and influenza virus attenuation and has not been broadly explored for rapid attenuation of other important viruses, especially important veterinary viruses (Coleman et al., 2008; Mueller et al., 2010). In this study, we successfully attenuated PRRSV, an important veterinary pathogen, by using the SAVE approach. PRRSV structural gene GP5 and non-structural gene nsp9 were selected in this study for codon-pair deoptimization because of their critical roles in viral assembly and replication, respectively. After codon-pair deoptimization of these two genes with a computer-aided algorithm, we successfully rescued two viruses, SAVE5 with codon-pair deoptimized GP5 gene and SAVE9 with codon-pair deoptimized nsp9 gene. Both SAVE5 and SAVE9 were shown to be viable *in vitro*.

In vitro characterization of the SAVE5 and SAVE9 viruses revealed that, as expected, codon-pair deoptimization led to a significantly reduced translation of the corresponding genes in the SAVE5 and SAVE9 viruses while the production of PRRSV N protein in these two codon-pair deoptimized viruses as well as the internal control GAPDH levels in the same cells were not affected, indicating the specificity of the impact of codon-pair deoptimization on viral protein translation by SAVE. For the SAVE5 virus, there was also a significantly decreased level of nsp9 expression, which may be due to the reduced level of viral assembly and limiting further virus spread in cultures or the use of a monoclonal antibody against nsp9 in the Western blot analysis. In the porcine kidney cell line PK15 expressing the PRRSV receptor CD163, the two codon-pair deoptimized viruses SAVE5 and SAVE9 replicated to significantly lower titers after

36 hr post-infection when compared to that of the wt VR2385 virus, an indication of attenuation phenotype *in vitro*.

To determine if the two codon-pair deoptimized viruses were attenuated *in vivo*, we conducted a comparative pathogenicity study in SPF pigs. The results demonstrated that infection of pigs with the codon-pair deoptimized SAVE5 virus resulted in significantly lower viral RNA loads in sera and lung tissues when compared to the wt VR2385 virus-infected pigs. Similarly, the codon-pair deoptimized SAVE5 virus also produced significantly lower infectious virus titers in sera of infected pigs. Importantly, both the macroscopic and microscopic lung lesion scores were significantly reduced in SAVE5 virus-infected pigs compared to those in wt VR2385 virus-infected pigs, indicating that the SAVE5 virus is attenuated *in vivo*. The mechanism of attenuation of SAVE5 virus seems to be due to the impact of SAVE approach on the GP5 protein expression level, which correlated to the apoptosis of infected cells thus affecting the virus pathogenicity *in vivo*. In addition, the GP5 involves in the assembly of PRRSV particles, therefore less expression of the GP5 proteins may decrease the yield of complete assembled viral particles which affect the generation of infectious viral titer thus leading to the attenuation of the SAVE5 virus.

Interestingly, although the nsp9 gene codon-pair deoptimized SAVE9 virus was viable *in vitro* in two different permissive cell lines, it failed to replicate in pigs. There was no detectable viremia or viral RNA in the lung tissues from the SAVE9 virus-inoculated pigs. It is likely that the codon-pair deoptimization of the critical non-structural nsp9 gene in PRRSV drastically reduced the translation of the nsp9 protein in

animals. Since nsp9 is critical for virus replication, the reduced nsp9 translation subsequently affects the replication of PRRSV in pigs, which brings an unwanted decreased viral replication in geometric ratio. This study provides the first evidence that SAVE produces a viable virus *in vitro* that could no longer be fit to replicate in its natural animal host. Therefore, selection of the appropriate viral gene(s) for rapid attenuation by SAVE is important in order to generate an attenuated infectious virus.

The codon-pair deoptimized GP5 sequence in the SAVE5 virus was genetically stable up to 14 dpi *in vivo*. The large numbers of nucleotide changes in the deoptimized GP5 gene sequence (Fig. 1) not only significantly decrease the chance of reversion to virulence phenotype of the attenuated SAVE5 virus also but reduce the chance of homologous recombination with other field strains of PRRSV as well. Compared to the traditional virus attenuation approach which typically requires serial passages in cell cultures, the SAVE technology is much more rapid for virus attenuation based on direct synthesis of codon-pair deoptimized viral gene sequence.

In summary, we successfully used SAVE to rapidly attenuate PRRSV. The SAVE5 virus with a codon-pair deoptimized GP5 gene was successfully rescued and displayed an attenuation phenotype both *in vitro* and *in vivo*. A unique advantage of the SAVE approach is that it effectively attenuates PRRSV without altering the antigenicity of the viral proteins on the whole virion. Therefore, the attenuated SAVE5 virus should retain the same antigenicity as the wild-type virus. The results from this study demonstrated the feasibility of rapid attenuation of PRRSV by the SAVE approach. Whether or not the attenuated SAVE5 could be useful for further development into a

potential candidate MLV against PRRSV requires future in-depth immunogenicity and challenge studies, which is not the scope of the present study aiming to assess the PRRSV attenuation feasibility by SAVE. Nevertheless, the SAVE approach appears to have a broad application in rapidly attenuating important viruses.

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Fig. 1. An alignment of the GP5 nucleotide sequences between the wild-type (wt) VR2385 virus and the codon-pair deoptimized SAVE5 virus. The GP5 sequence of the wt virus VR2385 was shown on top. Only differences were indicated for the SAVE5 virus.

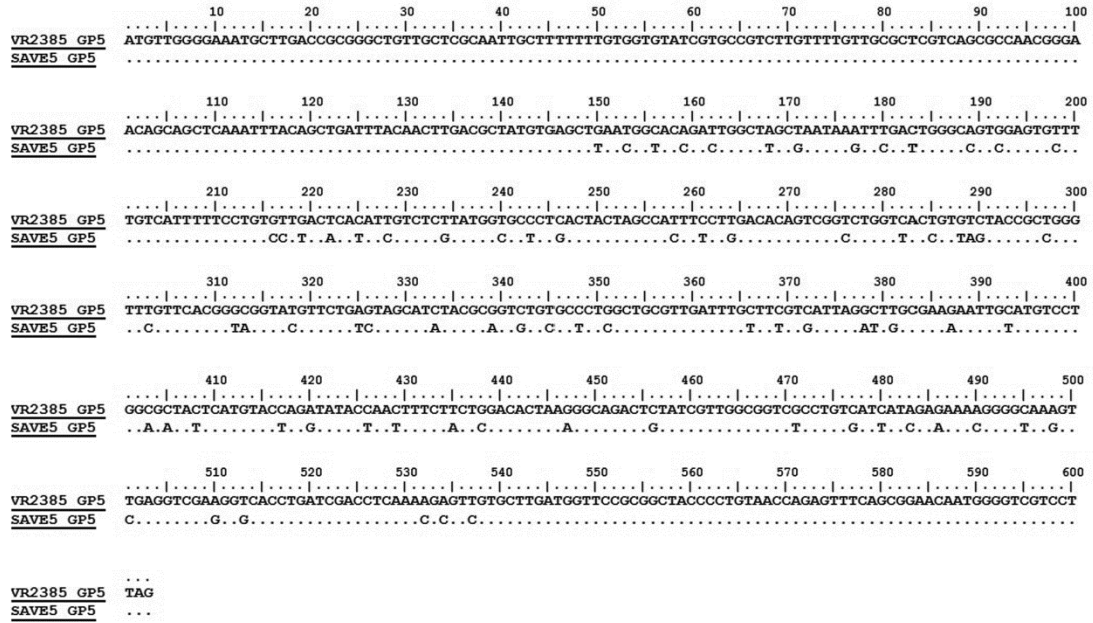


Fig. 1

Fig. 2. Rescue and passage of cloned GP5 or nsp9 gene codon-pair deoptimized viruses by synthetic attenuated virus engineering (SAVE). (A) Immunofluorescence assay (IFA) confirmation of the rescue of the wt PRRSV VR2385 from MARC-145 cells infected with the suspension of cells transfected with a DNA-launched wt PRRSV infectious clone pIR-VR2385-CA. **(B)** There was no fluorescent signal by IFA in mock-infected MARC-145 cells. **(C)** IFA confirmation of the rescue of the GP5 gene codon-pair deoptimized SAVE5 virus. **(D)** IFA confirmation of the rescue of the nsp9 gene codon-pair deoptimized SAVE9 virus.

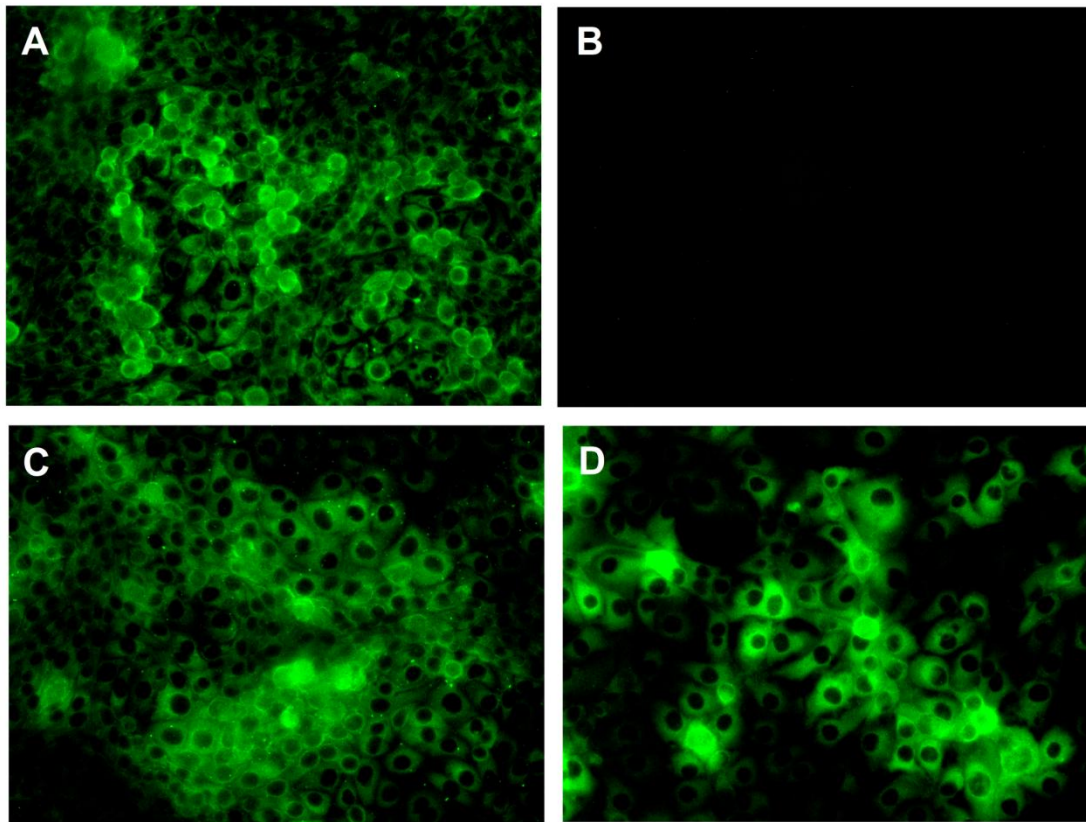


Fig. 2

Fig. 3. Western blot analysis of PRRSV protein expression in infected cells. PK15-CD163 cells were infected with the same 0.1 m.o.i of wt virus VR2385 or codon-pair deoptimized viruses SAVE5, and SAVE9, respectively. Total proteins were extracted from cell lysates and stained with PRRSV-specific antibodies against GP5 protein (Biomatik), Nsp9 protein (provided by Dr. Hanchun Yang), N protein (SR30 from Rural Technologies, Inc.) or GAPDH (Thermo Scientific). GAPDH was used as an internal control.

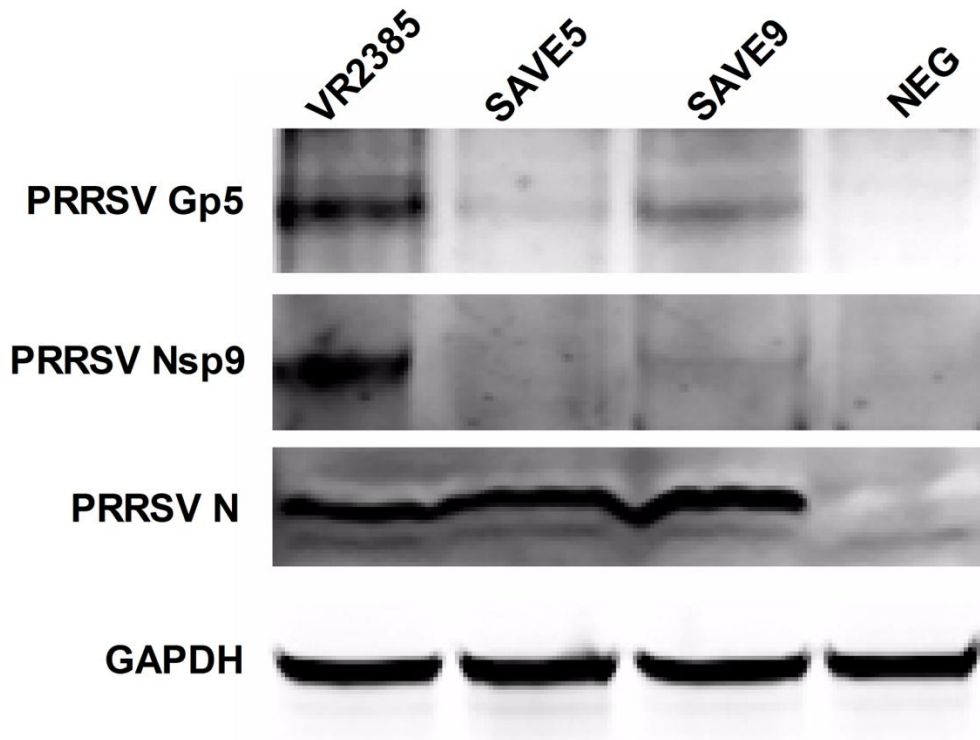


Fig. 3

Fig. 4. *In vitro* growth kinetics of the GP5 and nsp9 gene codon-pair deoptimized SAVE5 and SAVE9 viruses. The PK15-CD163 cells were infected with each of the SAVE5, SAVE9 or wt VR2385 viruses at the same m.o.i of 0.1, and mock-infected cells were also included in the experiment as a control. The viruses from the infected cells were harvested every 12 hr until 72 hr post-infection. The virus titers (TCID₅₀/ml) were determined at the indicated time points post-infection. The symbols “*” (SAVE5) and “#” (SAVE9) indicate a statistically significant difference between the corresponding codon-pair deoptimized virus and the wt VR2385 virus at that time point. The standard error bars are indicated.

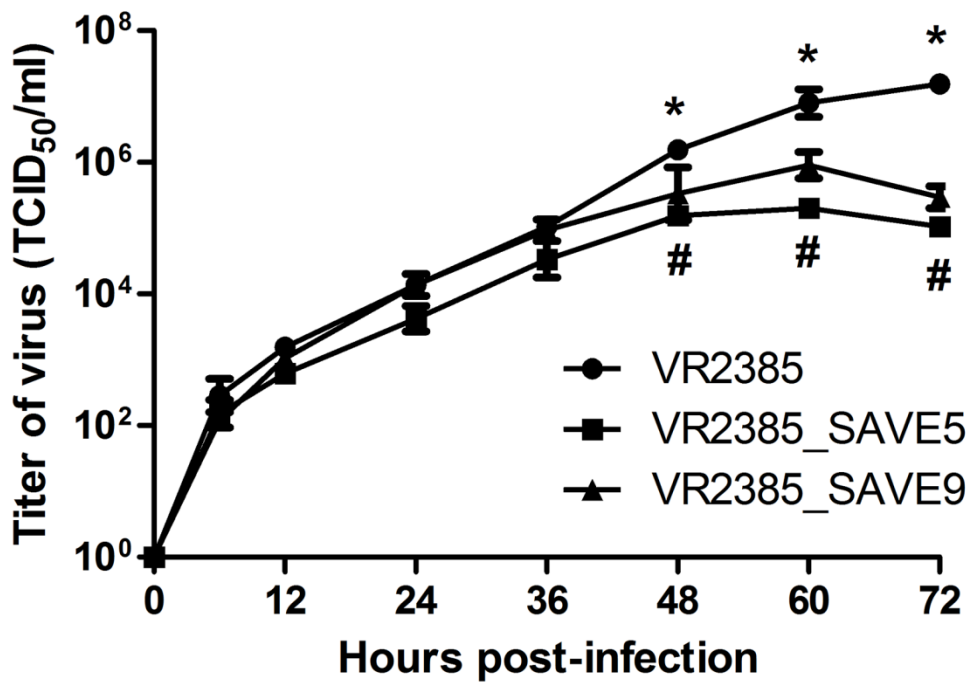


Fig. 4

Fig. 5. PRRSV RNA loads in serum samples and lung tissues from pigs experimentally inoculated with codon-pair deoptimized SAVE5 and SAVE9 viruses respectively or with the wt VR2385 virus. (A) PRRSV RNA loads in serum samples at 7 dpi from the pigs (n=8) inoculated with SAVE5, SAVE9, VR2385 or media (“NEG”), respectively. There was a significant difference in serum viral RNA loads between the GP5 codon-pair deoptimized SAVE5 virus-infected group and wt VR2385 virus-infected group (indicated with *) at that time point. **(B)** PRRSV RNA loads in serum samples at 14 dpi. There was a significant difference in serum viral RNA loads between the GP5 codon-pair deoptimized SAVE5 virus-infected group and wt VR2385 virus-infected group (indicated with *). **(C)** Viral RNA loads in the lung tissues collected during necropsy at 14 dpi. A significant difference between the SAVE5 virus-infected group and the wt VR2385 virus-infected group are observed (indicated with *). The standard error bars are indicated. No viral RNA was detected in serum or lung tissue samples from SAVE9 virus or media (“NEG”)-inoculated pigs, indicating that the SAVE9 virus, although viable *in vitro*, is non-infectious in pigs.

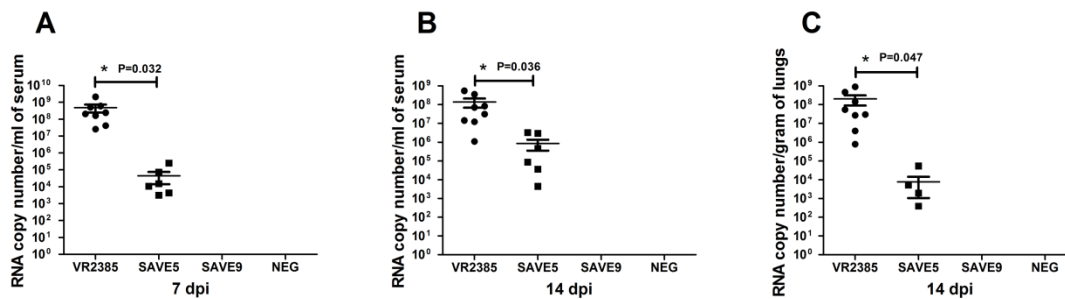


Fig. 5

Fig. 6. Viral infectious titers in serum samples of pigs experimentally infected with the GP5 codon-pair deoptimized SAVE5 virus or wt VR2385 virus. Viral infectious titers in serum samples of pigs were quantified as TCID₅₀/ml. There are significant differences at both 7 dpi and 14 dpi between the SAVE5 virus-infected group and wt VR2385 virus-infected group (indicated with *). The standard error bars are indicated.

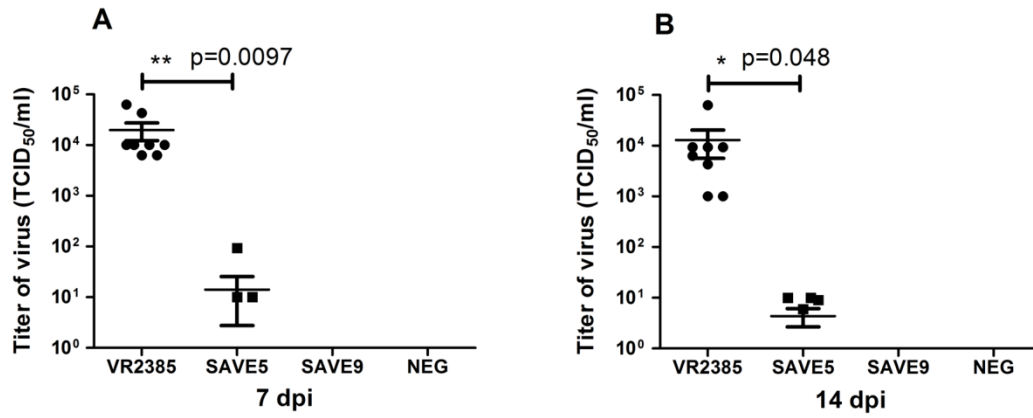


Fig. 6

Fig. 7. Macroscopic and microscopic lesions of the lung tissues from pigs experimentally infected with the GP5 codon-pair deoptimized SAVE5 virus as well as wt VR2385 virus during necropsy at 14 dpi. (A) Gross lesion scores of the lung tissues from the 8 pigs in each group experimentally inoculated with SAVE5, SAVE9, VR2385 viruses or culture media (“NEG”), respectively. **(B)** Microscopic lesion scores of the lung tissues from 8 pigs in each group experimentally inoculated with SAVE5, SAVE9 and VR2385 viruses, respectively. Mock-infected pigs with cell culture media were included as negative control (“NEG”). Significant differences are indicated as asterisks (**, $P < 0.01$; ***, $P < 0.0001$).

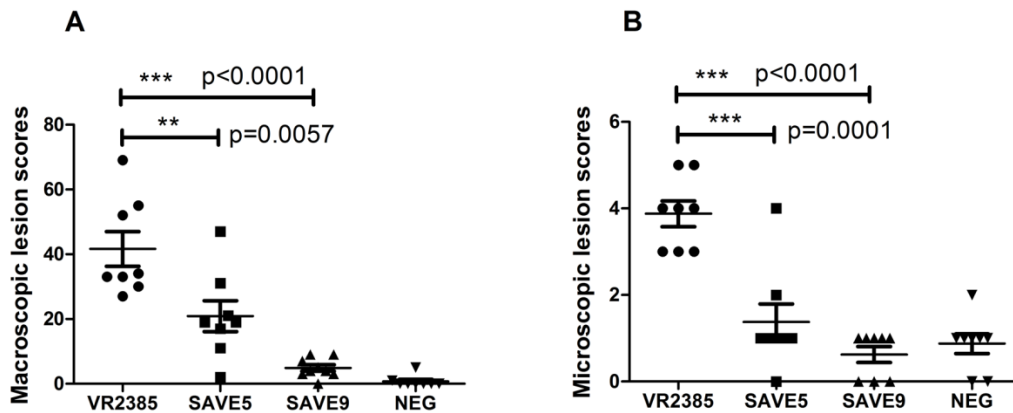


Fig. 7

Table 1. Oligonucleotide primers used in this study

Primer ID	Primer sequence (5'>3')	nt position in wt PRRSV VR2385 genome
For codon-pair deoptimized genes		
CpD5F	ATGTTGGGGAAATGCTTGACCG	13354-13375
CpD5R	CTAAGGACGACCCCATTTGTTCCG	13934-13956
CpD9F	ACTGAGACAGCGGTGAAAATCGTTAAATTTTCAT	7312-7344
CpD9R	CACTCTCGACTTCTTCCCCTCGTAATTCGACCTCAA	9166-9201
For fusion PCR with codon-pair deoptimized GP5 gene		
fu5F	GGCAATTTTCTTTCGAACTCAC	12382-12404
fu5R	CGGTCAAGCATTTCCCCAACAT	13354-13375
5fuF	CGGAACAATGGGGTCGTCCTTAG	13934-13956
5fuR	AAACCCTCTAGAATAAGAGACAATGTGAGTC	Vector
For fusion PCR with codon-pair deoptimized nsp9 gene		
fu9F	GCCTGACTAAGGAGCAGTGTTT	7236-7257
fu9R	TTCACCGCTGTCTCAGTAACAA	7307-7328
9fuF	GAGGGGAAGAAGTCGAGAGTGT	9181-9202
9fuR	TTGGGGAGCGGAGAGCTCGA	10510-10529
For real-time PCR		
RTspR51	GCCCTAATTGAATAGGTGAC	14909-14928
realtime2F	TTAAATATGCCAAATAACAACGG	14449-14471
realtime2R	TGCCTCTGGACTGGTT	14554-14569

Table 2. Characteristics of codon-pair deoptimized PRRSV gene sequences

Deoptimized gene (length in bp)	Deoptimized coding region of gene (nt position)	Number of silent mutations introduced	CPB* of original gene fragment	CPB of deoptimized gene fragment	MFE [¶] of original gene fragment (kcal/mol)	MFE of deoptimized gene fragment (kcal/mol)
gp5 (603)	148 to 546	78	-0.049	-0.354	-134.8	-122.6
nsp9 (1938)	82 to 1938	459	0.016	-0.317	-628.7	-585.0

*CPB: Codon Pair Bias

[¶]MFE: Minimum free energy

Addendum

Construction and rescue of a DNA-launched infectious clone of a modified live-attenuated vaccine (ML) strain of PRRSV

Yan-Yan Ni, and Xiang-Jin Meng

ABSTRACT

Suvaxyn® PRRSV is a MLV vaccine virus that was attenuated by serial passages of a North American type 2 strain (ISU-55) of PRRSV originally isolated from a swine farm in Iowa during the outbreak of PRRS in early 90's. To facilitate the further genetic study to identify genetic determinants for PRRSV virulence, we determined the full-length genomic sequence of this MLV virus and successfully established a DNA-launched infectious clone of the Suvaxyn MLV (pIR-SV). The full-length viral genome engineered with two ribozyme elements at both 5' and 3' ends was placed under the control of the eukaryotic CMV promoter. The infectious virus was successfully rescued from the pIR-SV DNA-transfected BHK-21 cells. The availability of this DNA-launched infectious clone of an attenuated MLV now affords us the opportunity to delineate the critical viral genetic elements that are important for virulence in the future.

INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped, positive-sense, single-strand RNA virus in the family *Arteriviridae* of the order *Nidovirale*. It can be classified into two distinct genotypes: The European type (type 1)

and North American type (type 2) (3, 8). The disease caused by PRRSV is known as porcine reproductive and respiratory syndrome (PRRS) which can be classified into two distinct clinical types: respiratory disease and reproductive failure (1, 4).

The full-length genome of PRRSV is approximately 15 kb in size, which is capped at 5' end and polyadenylated at 3' end. There are at least 15 non-structural proteins derived from polyproteins pp1a and pp1ab which are encoded by the open reading frame (ORF) 1a and 1b of PRRSV. Among the non-structural proteins, Nsp9, which contains a putative RNA-dependent RNA polymerase (RdRp), is associated with PRRSV RNA synthesis (2, 11). Nsp2, the largest non-structural protein, is also located in the most variable region of PRRSV genome. Natural deletions, mutations have been reported in field strains of PRRSV (5, 7, 12). At least eight ORFs including two small ORFs (ORF2b and ORF5a) encode the structural proteins of PRRSV.

PRRSV strain ISU-55 was isolated from pig lungs during PRRS outbreaks in Iowa in early 1990's (8). PRRSV ISU-55 is of moderate pathogenicity in pigs (6) which belongs to genetic lineage 5 of type 2 PRRSV, that was phylogenetically close to PRRSV VR2385 (9). PRRSV Suvaxyn MLV is an attenuated vaccine virus strain derived from ISU-55 by serials passage *in vitro*. Suvaxyn PRRS MLV was originally called Cyblue®, and was the first inactivated PRRSV vaccine made available (April 1994) for commercial use. In this study, in order to facilitate future genetic research to delineate the virulence determinants of PRRSV, we construct a DNA-launched full-length infectious clone of PRRSV Suvaxyn MLV and infectious virus was successfully rescued *in vitro*.

MATERIALS AND METHODS

Virus and cells: A vaccine strain of PRRSV, Suvaxyn PRRS MLV, was attenuated from wild type PRRSV strain ISU-55 isolated from pig lungs during PRRS outbreaks in Iowa in the early 90's (6). The virus stock was provided by Pfizer Inc. BHK-21 cells were used for transfection, and MARC-145 cells were used for infection of Suvaxyn PRRSV. BHK-21 and MARC-145 cells were grown at 37°C in Dulbecco's minimum essential medium (DMEM) supplemented with 10% FBS and antibiotics.

Determination of the full-length genomic sequence of Suvaxyn PRRS MLV and sequence analyses: The sequences of the ORFs 2 to 7 genes, but not ORF1, of the ISU-55 isolate have been published (9). To determine the complete genomic sequence of Suvaxyn PRRSV MLV, total RNAs were isolated using TRI Reagent (MRC). Reverse transcription and cDNA synthesis were performed at 42 °C for 60 min in a 20-µl reaction mixture containing 100 U of Superscript II reverse transcriptase (Invitrogen), 10mM deoxyribonucleoside triphosphate, 100 mM of DTT, 1 U of RNasin (Promega), and 0.5 µg of oligo dT primers (Invitrogen). A total of 10 overlapping PCR fragments covering the entire genome of the Suvaxyn PRRS virus were amplified from the viral cDNA (Table 1), and subsequently cloned into a pCR-2.1 vector (Invitrogen). Three individual clones of each fragment were selected for sequencing. The consensus sequences were assembled and used for sequence analysis utilizing the lasergene software (Version 8, DNA STAR, Inc.). The extreme 5'-end sequence of viral genome was determined using the GeneRACER kit (Invitrogen) with two reverse primers SV5RACE1 and SV5RACE2

(Table 1). The extreme 3'-end sequence of viral genome was determined by a nested PCR using the same forward primer SV10F with two reverse primers SV3RACE adaptor_T and SV3RACE primer (Table 2).

Construction of aDNA-launched infectious clone of the Suvaxyn PRRS MLV:

After determining the sequence at the extreme 5'-end and 3'-end of the viral genome, a total of 5 overlapping fragments (AX, XP, PN, NE, EX) with unique restriction enzyme sites were amplified from the viral cDNA of Suvaxyn PRRSV MLV (Fig. 1). A 20-poly(A) nucleotides were introduced immediately downstream of the 3'-end of the viral genome. A nested PCR was used to introduce the ribozyme elements to the both end of viral genome for construction of DNA-launched final infectious clone as previous described (10). Two restriction sites AscI and XbaI were artificially added to the upstream and downstream of viral genome for assembly, respectively. A total of 6 individual clones for each fragment were selected for sequencing, and the clone containing the consensus sequence was used for the assembly of the full-length clone. The fragment ACYC-XA from the plasmid pACYC-177 was amplified using primers pACYC-XbaI

5'-
AAACCCGATATCAAACCCTCTAGAGCCCTTCGCCCTTCCGGCTGGCT -3' and
pACYC-AscI 5'-
GGGTTTCATATGGGGTTTGTTTAAACGGGTTTGGCGCGCCGGATCCTCCGGCG
TT -3'

, incorporating the unique restriction sites AscI, PmeI, NdeI, EcoRV and XbaI, which were then used to ligate this fragment upstream to the viral genome. Among the five fragments, each of PN, NE, and EX fragments was used to sequentially replace the fragments with the same restriction enzyme sites on the modified expression vector pIRES-EGFP2 and a three-fragments ligation was used to assemble fragments AX, XP into the vector using restriction sites AscI and PmeI, resulting in the assembly of a full-length clone of the Suvaxyn PRRS MLV in modified vector pACYC-177. The assembled full-length genome of Suvaxyn PRRS MLV was then used to replace the VR2385 viral genome in pIR-VR2385-CA infectious clone plasmid with restriction sites AscI and XbaI. The final full-length clone of Suvaxyn PRRS MLV was designated as pIR-SV.

***In vitro* transfection and recovery of infectious viruses from the DNA-launched infectious clone:** For rescue of infectious Suvaxyn PRRS MLV virus from the DNA-launched infectious clone, BHK-21 cells at a 60% confluency in 6-well plates were transfected with 3 µg of pIR-SV DNA using 8 µl of Lipofectamine LTX (Invitrogen) according to the manufacturer's instruction. At 48 h post-transfection, the cell culture supernatant was harvested and passaged onto MARC-145 cells. The rescue of virus was confirmed by IFA with the anti-PRRSV N monoclonal antibody (SDOW17, Rural Technologies).

RESULTS AND DISCUSSION

Construction of DNA-launched clone of PRRSV Suvaxyn PRRS MLV: The complete genomic sequence of Suvaxyn PRRS MLV was determined using primers based on the published sequence of PRRSV ISU-55 and conserved sequences in type 2 PRRSV. 5' RACE and 3' RACE were used to determine the extreme 5' and 3' end sequences of Suvaxyn PRRS MLV. Strategies for construction of DNA-launched infectious clone of PRRSV VR2385 reportedly previously were used to construct the DNA-launched infectious clone of Suvaxyn PRRS MLV. The Suvaxyn PRRS viral genome fused with ribozyme fragments were successfully assembled in a modified vector pIRES-EGFP2 downstream of a CMV promotor, which is used for transcription *in vitro*.

Rescue of a PSuvaxyn PRRS MLV virus from the DNA-launched infectious clone: The Suvaxyn PRRS MLV virus was successfully rescued from the modified DNA-launched infectious clone and confirmed by IFA with an anti-PRRSV N monoclonal antibody in the transfected BHK-21 cells (Fig. 2C) and the infected MARC-145 cells as previously described (Fig. 2F). The sequence of rescued virus was confirmed from an RT-PCR fragment obtained from supernatant of MARC-145 cells infected with rescued Suvaxyn virus.

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FIG. 1. Construction of a DNA-launched full-length clone of Suvaxyn PRRS MLV pIR-SV. An AscI restriction site, ribozyme sequence, and two guanosines were introduced at the 5' end of fragment AX. An XbaI restriction site, ribozyme sequence, as well as 20 poly(A) were introduced to the 3' end of fragment EX. All five fragments overlapping with each other contain unique restriction sites for full-length viral genome assembly. First, fragments were orderly cloned to a modified vector pACYC177-AX with stuffer fragment to generate a full-length clone. The full-length Suvaxyn PRRS MLV genome was then transferred to a modified pIRES-EGFP2 vector downstream of the CMV promotor, and this newly generated DNA-launched Suvaxyn PRRS MLV clone was designated as pIR-SV.

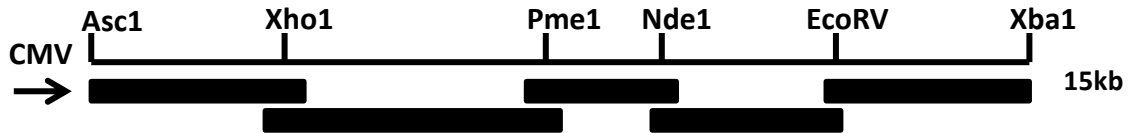


Fig. 1

FIG. 2. Immunofluorescence staining with anti-PRRSV N antibody of BHK-21 cells transfected with DNA-launched Suvaxyn PRRS MLV clone and MARC-145 cells infected with transfected BHK-21 cell supernatant. (A) Mock transfected; (B) BHK-21 cells transfected with pIR-SV; and (C) Mock infected; (D) MARC-145 cells infected with supernatant of BHK-21 cells transfected with pIR-SV;

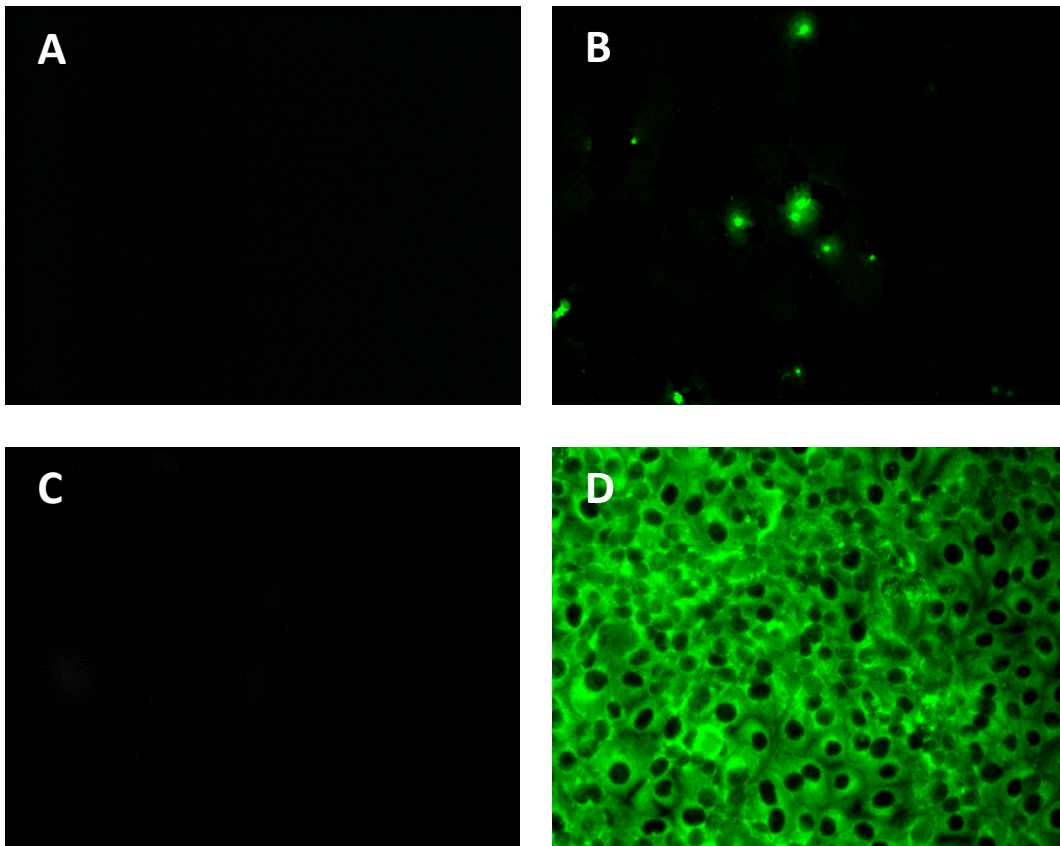


Fig. 2

Table 1. Oligonucleotide primers used in this study

Primer ID	Sequence (5'>3')	nt position in Suvaxyn virus genome
For determine the full-length genome of Suvaxyn (10 small fragments)		
SV1F	ATGACGTATAGGTGTTGGCTCTA	1-22
SV1R	TTGTGACTGCCAAACCGG	1312-1329
SV2F	GACCTCCTCAGAATAAGGGTTGA	1247-1269
SV2R	TCGGCTGCCCAGGCCATCA	2559-2579
SV3F	AAAGACCAGATGGAGGAGGA	2501-2520
SV3R	AGAAAATACACCCAAGAGGGGA	3595-3616
SV4F	GATTGGGGTTTTGCAGCTTTTAC	3524-3546
SV4R	GTCAACGCAGCGCGGCGGAC	5114-5133
SV5F	TGGCCCCCGCGGTGTT	5057-5071
SV5R	ACTCTAAGTGCTTTAGCATAGG	6588-6610
SV6F	AGCTTTTGCTGATACCGTGGA	6563-6583
SV6R	AACGTCGATTTCAGGGACGCTCT	8165-8187
SV7F	TTCTATGGCTGGAATAAATGGG	8118-8139
SV7R	GGAGCCAGTATGTTTTCCAGC	9640-9661
SV8F	GCGCAGCAGGTTTCATCATCGG	9609-9629
SV8R	CTTTGCATAGACCCCATTTTCAT	11639-11660
SV9F	TCATTGAACCAACTTTAGGCC	11609-11629
SV9R	ACTCCACTGCCCAGTCAAATT	13529-13549
SV10F	TGAGCTGAATGGCACAGATTGG	13497-13518
SV10R	TCATGCTGAGGGTGACGCTG	14807-14826
For 5' RACE and 3' RACE		
SV5RACE1	GGCAACCCCGTTTCGTAAA	
SV5RACE2	CTCGGGAGTCTGGAGATTCA	
SV3RACE	GGATCCGGTACCTCTAGATCAGATTTTTTTTTTTTTTTTTT	
adaptor_T		
SV3RACE primer	GGATCCGGTACCTCTAGATCAGA	
For Assembly (5 long fragments)		
SV_AXF_rz	<u>TGAGGACGAAACGGTACCCGGTACCGTC</u> ATGACGTATA GGTGT*	1-16
SV_AXR	ATCATCACAAACTCACACGG	3512-3531
SV_XPF	GATAGCAGGTTTGAGTTTCT	3458-3477
SV_XPR	GGGTTTTCTAGATTTACCGCTGTCTCAGTGAC	7744-7768
SV_PNF	TTGGTATGATGAACGTCGAC	7593-7612
SV_PNR	TTCTTCAAACCATTCAGGAT	9497-9516
SV_NEF	TAACCGTGACAGGATTCTCG	9381-9400
SV_NER	CACCATTTTCATCAATCAGGG	12387-12406
SV_EXF	GCCACCAGCATGAAGTTTAA	12007-12026
SV_EXR	TACATATGTTTTTTTTTTTTTTTTTTTAAATTCGGC CGCG	15400-15412
PRV-ASCIF_rz	AAGGATCCGGCGCGCC <u>ACGTCATCTGATGAGTCCGTGA</u> <u>GGACGAAAC</u>	

* Letters with underline represent ribozyme sequences