Genetic characterization of emerging PRRSV in US: new features of -2/-1 ribosome frameshifting in nsp2 region

by

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Abstract

Recent emergence of PRRSV variants caused increased mortality in growing pigs and reproductive failure in sows. In this study, we characterized two emerging PRRSV variants isolated from infected pigs. Genome sequencing analysis showed that KS 17-C1 and KS 17-C2 have GP5 RFLP cutting pattern of 1-7-4 and 1-18-2, respectively; and they belong to two distinct phylogenetic clades, in which each associated with a group of emerging PRRSV strains that were reported from pigs with severe clinical manifestations. Further in-depth sequence analysis revealed that the -2/-1 programmed ribosome frameshifting (PRF) signal located within nsp2 is the region where these viruses differ the most from historical PRRSV strains. Our previous studies demonstrated that -2/-1 PRF generates two frameshifting products, nsp2TF and nsp2N. In the genome of historical PRRSV strains, the -1 PRF immediately encounters a stop codon, which terminates the translation of -1 reading frame to produce nsp2N. However, KS 17-C1 and KS 17-C2 isolates contain mutations disrupting the -1 PRF stop codon; therefore, extending the translation of nsp2N to generate additional 16 or 23 amino acids at 3'-end (nsp2N+16aa, nsp2N+23aa). The emergence of -1 PRF stop codon mutants was traced back in PRRSV sequences published in the Genbank since 2011 and percentage of the mutants has quickly increased afterward (up to 69% of the sequences). More importantly, these -1 PRF stop codon mutants were reported from swine farms experiencing PRRSV outbreaks with increased mortality/morbidity. To determine whether nsp2N extension correlates with the increased pathogenicity of these viruses, recombinant virus with restored -1 PRF stop codon was generated and compared with wild-type virus. Results showed that the mutant with restored -1 PRF stop codon induced higher levels of innate immune response, suggesting a possible link between nsp2N extension and pathogenicity of this group of newly emerging PRRSV variants in the US.

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Chapter 1 - Literature Review

1.1 Porcine Reproduction and Respiratory Syndrome and the causal agent

Porcine reproductive and respiratory syndrome is one of the most economically significant swine diseases globally. It costs US swine industry more than $600 annually (Holtkamp et al., 2013).The disease causes typical clinical manifestations such as reproductive failure in sows, high fever and respiratory illness in young pigs (Corzo et al., 2010; Rossow, 1998). Infection with highly pathogenic-PRRSV strains is associated with more severe clinical signs, pulmonary lesions and aberrant host immune responses (Hu et al., 2013). PRRS was first reported in North America in 1987 (Keffaber et al., 1989) and described as "mystery swine disease" due to the absence of a recognized cause (Hill H et al., 1990; Reotutar R et al.,1989). In 1991, the similar disease was observed in Europe (G. Wensvoort et al., 1991).

The causal agent of PRRS was found to be porcine reproductive and respiratory syndrome virus (PRRSV) as it was isolated in Netherlands and US in 1990s (Collins et al., 1992; G. Wensvoort et al., 1991). The original European isolate was designated as Lelystad virus (LV) while the North American isolate was named as VR2332 [American Type Tissue Culture (ATCC) collection VR2332] (Benfield et al., 1992; G. Wensvoort et al., 1991). In 2006, highly pathogenic (HP) PRRSV strains were isolated in China and South East Asia (Fang et al., 2007; Feng et al., 2008; K. Tian et al., 2007). Recently another highly pathogenic East European subtype 3 PRRSV, Lena, was reported in Eastern Europe (Karniychuk et al., 2010).

PRRSV belongs to the *Arteriviridae* family in the order of *Nidovirales* (Lunney et al., 2016; E. J. Snijder, M. Kikkert, & Y. Fang, 2013; Snijder & Meulenberg, 1998), which includes two species, currently named as PRRSV-1 and PRRSV-2. Other members of the *Arteriviridae* family include lactate dehydrogenase-elevating virus (LDV), equine arteritis virus (EAV), and simian hemorrhagic fever virus (SHFV) (Lunney et al., 2016; E. J. Snijder et al., 2013; Snijder & Meulenberg, 1998).

Pigs are the only known natural host of PRRSV. In infected pigs, the fully differentiated porcine alveolar macrophages (PAMs) serves as the primary cell target for PRRSV infection (Duan, Nauwynck, & Pensaert, 1997a, 1997b). Only the African green monkey kidney cell line MA-104 and the derivatives such as MARC-145 are fully permissive to PRRSV infection in vitro (Kim et al., 1993). PRRSV enters PAMs through pH-dependent, receptor-mediated endocytosis (Nauwynck, Duan, Favoreel, Van Oostveldt, & Pensaert, 1999). To date, at least 6 potential cell surface molecules have been proved to be associated with PRRSV attachment and entry, including heparan sulphate, vimentin, CD151, CD163, CD169, and DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; also known as CD209) (Zhang & Yoo, 2015). Recently, a cytoskeleton protein, non-muscle myosin heavy chain 9 (MYH-9), has been identified to be related to PRRSV attachment and cell to cell spread via intercellular nanotubes (Gao et al., 2016; Guo, Katz, Tomich, Gallagher, & Fang, 2016). Among these putative receptors, CD163 and CD169 have been studied most extensively. CD169 gene knocked out pigs didn't show an increased resistance to PRRSV infection, indicating that CD169 is not required for PRRSV attachment (Prather et al., 2013). On the other hand,

accumulating studies demonstrated that CD163 is essential for PRRSV initial infection through binding with the minor structural proteins GP2a and GP4 (Calvert et al., 2007; Das et al., 2010; Welch & Calvert, 2010).

1.2 PRRSV virion and genome structure

The PRRSV virion is enveloped, round-shaped particle with a diameter from 50 nm to 74 nm. The single stranded viral genome is encapsulated by nucleocapsid proteins, surface glycoproteins and membrane proteins are inserted onto the lipid-bilayered envelope, which surrounds the nucleocapsid to form the intact viral particle (Spilman, Welbon, Nelson, & Dokland, 2009) (**Fig 1**).

PRRSV has a linear, positive-sense, single-stranded RNA genome of 12-15 kb in length, which is also capped, polyadenylated and is previously known to contain 10 open reading frames (ORFs). ORF1a and ORF1b occupy the ¾ of the whole genome, encoding two large polyproteins PP1a and PP1ab, which are further processed into 14 individual functional nonstructural proteins (NSPs) through the proteolytic process (Y. Fang & E. J. Snijder, 2010) (**Fig 2**). Recently, our lab discovered a new -1/-2 programmed ribosomal frameshifting (PRF) signal in the NSP2 region, which contains a slippery sequence and a C-rich motif. The -1 frameshifting generates the novel protein nsp2N, while the -2 frameshifting generates a novel protein nsp2TF. These nsp2-related proteins all share the same N-terminal region including a papain-like protease domain PLP2 and a long uncharacterized hypervariable region (Y. Fang et al., 2012; Y. Li et al., 2014). The 3’ end of the genome encodes four glycosylated membrane proteins GP2a, GP3, GP4 and GP5; three

unglycosylated membrane proteins E, ORF5a and M; and a nucleocapsid protein N (Snijder et al., 2013) (**Fig 3**).

1.3 PRRSV infectious cycle

PRRSV has the similar life cycle as other nidoviruses (**Fig. 4**). The clathrin-mediated endocytosis is triggered after the virus binding to the cellular surface receptor, and then the viral membrane fuses with the endosomal membrane to release the viral genomic RNA into the cytoplasm. Subsequently, part of the genome is translated into the pp1a and pp1ab, which are later cleaved by internal viral proteinases into the mature nonstructural proteins and then these NSPs assemble into a replication and transcription complex (RTC). The RTC then engages to synthesize the continuous genome-length minus-strand RNA and discontinuous subgenomic (sg)-length minus-strand RNAs. Next, the full-length minus-strand RNA serves as the template for genome replication, while the sg minus-strand mRNAs serve as templates for the sg mRNAs synthesis. The viral structural proteins translation relies on the nested sg mRNAs. The newly synthesized genome and structural proteins are then assembled together at the endoplasmic reticulum (ER) into an intact virion and released into the extracellular environment by exocytosis to infect other naïve cells (Y. Fang & E. J. Snijder, 2010; E. J. Snijder et al., 2013).

1.4 The interplay between PRRSV and host immune system

PRRSV is “notorious” for its immunosuppression to the host. Generally, PRRSV infection elicits poor host immune responses. The induction of innate immune cytokines such as type I interferons (IFNs), tumor necrosis factor-α (TNFα), and interleukin-1 (IL-1) in PRRSV-

infected pigs are significantly inhibited (M. P. Murtaugh, Xiao, & Zuckermann, 2002; Van Reeth, Labarque, Nauwynck, & Pensaert, 1999). Specifically, many PRRSV nonstructural proteins were determined to inhibit type I IFN synthesis and / or signaling in in vitro expression systems. Previous studies suggested that PRRSV NSP1α/β and NSP2 strongly antagonize IFN-β activation, PRRSV NSP4 and NSP11 also have the inhibitory effect on the IFN-β promoter activation (Beura et al., 2010; Huang et al., 2014). More recently, our lab demonstrated that the newly-identified NSP2-related proteins NSP2TF and NSP2N also play an important role in the suppression of type I IFN production (Y. Li et al., 2018).

The activation of adaptive immunity is also suppressed due to the weak innate immune response, which in turn causes a postponed and insufficient production of neutralizing antibodies. However, in contrast to the weak neutralizing antibody responses, PRRSV infection results in the early and lasting production of antibodies specifically against viral proteins such as nsp1α, nsp1β, nsp2 and nsp7(Ansari, Kwon, Osorio, & Pattnaik, 2006; Brown et al., 2009; Johnson, Yu, & Murtaugh, 2007; Lopez & Osorio, 2004; Ostrowski et al., 2002) .

1.5 PRRSV evolution and re-emergence

Due to the infidelity of RNA polymerase, PRRSV is highly diversified in nature, and is continuously and quickly evolving, generating the new strains with increased virulence. Since the initial outbreak in 1991PRRSV-2 has evolved into many different representative historical variants such as NVSL97-7895 isolated in 1997, MN184C isolated in 2001, Chinese HP-pathogenic strains isolated since 2006 and Chinese NADC30-like strains isolated from 2013-2015 (C. Li et al., 2016). Recently, the PRRSV variants isolated from newly emerging PRRS

outbreaks in the middle US have been identified to have a predominant restriction fragment polymorphism (RFLP) of 1-7-4, with increased mortality in piglets and more sever reproductive failure in sows.(van Geelen et al., 2018).

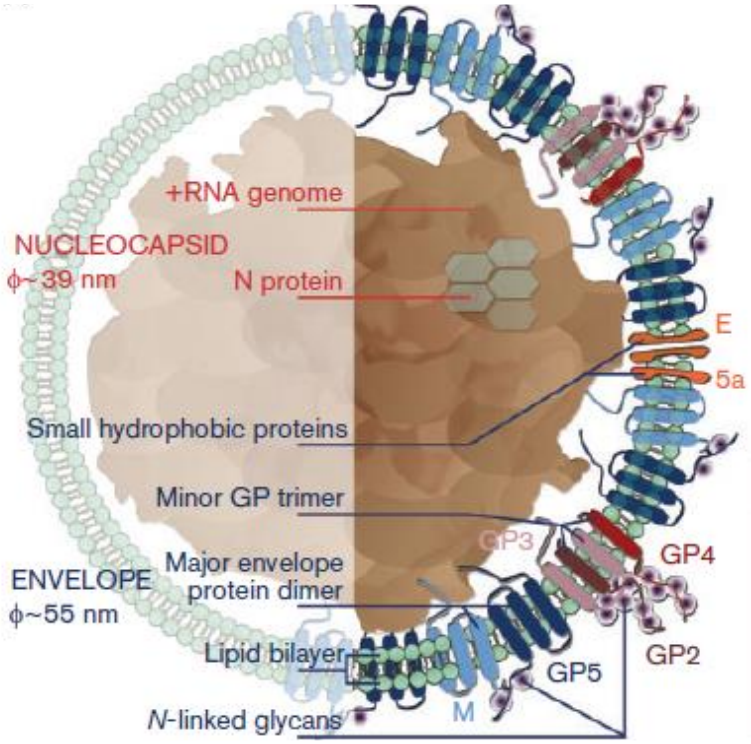


Figure 1. Schematic diagram of PRRSV virion.

The PRRSV virion has a spherical shape with a size about 65 nm, a hollow core of around 40 nm and a smooth outer surface with the envelope protein complexes embedded. The genome is enclosed by nucleocapsid protein. The nucleocapsid core is surrounded by a lipid membrane, the envelope where the structural proteins are embedded. The major protein components of the lipid envelope are GP5 and M, which forma a heterodimer. The minor structural proteins GP2, GP3,

and GP4 forms a multimeric complex incorporated in the lipid envelope. Figure adapted from Snijder et al., 2013.

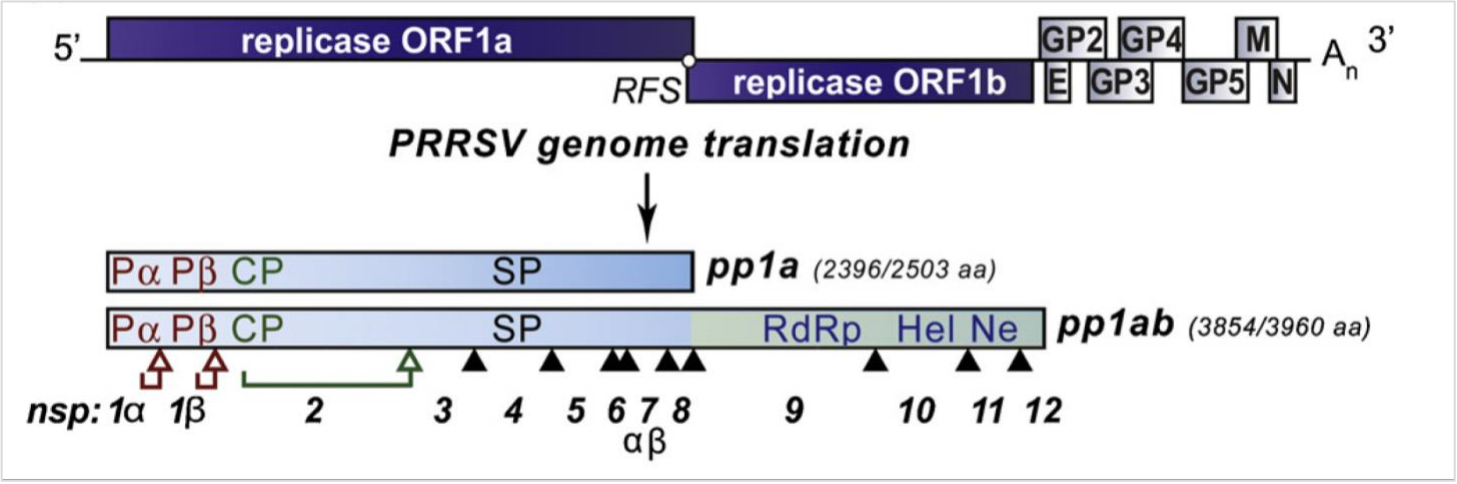


Figure 2. Schematic diagram of previously identified PRRSV genome structure.

The PRRSV is a positive sense, single-stranded RNA virus. The viral genome is about 15 kb in length and is previously known to contain 10 ORFs. The ORF1a and ORF1b occupy ¾ of the whole genome. They encode two large polyproteins pp1a and pp1ab, which are further processed into individual functional nonstructural proteins (NSPs) through the proteolytic processing. Figure adapted Fang & Snijder et al., 2010.

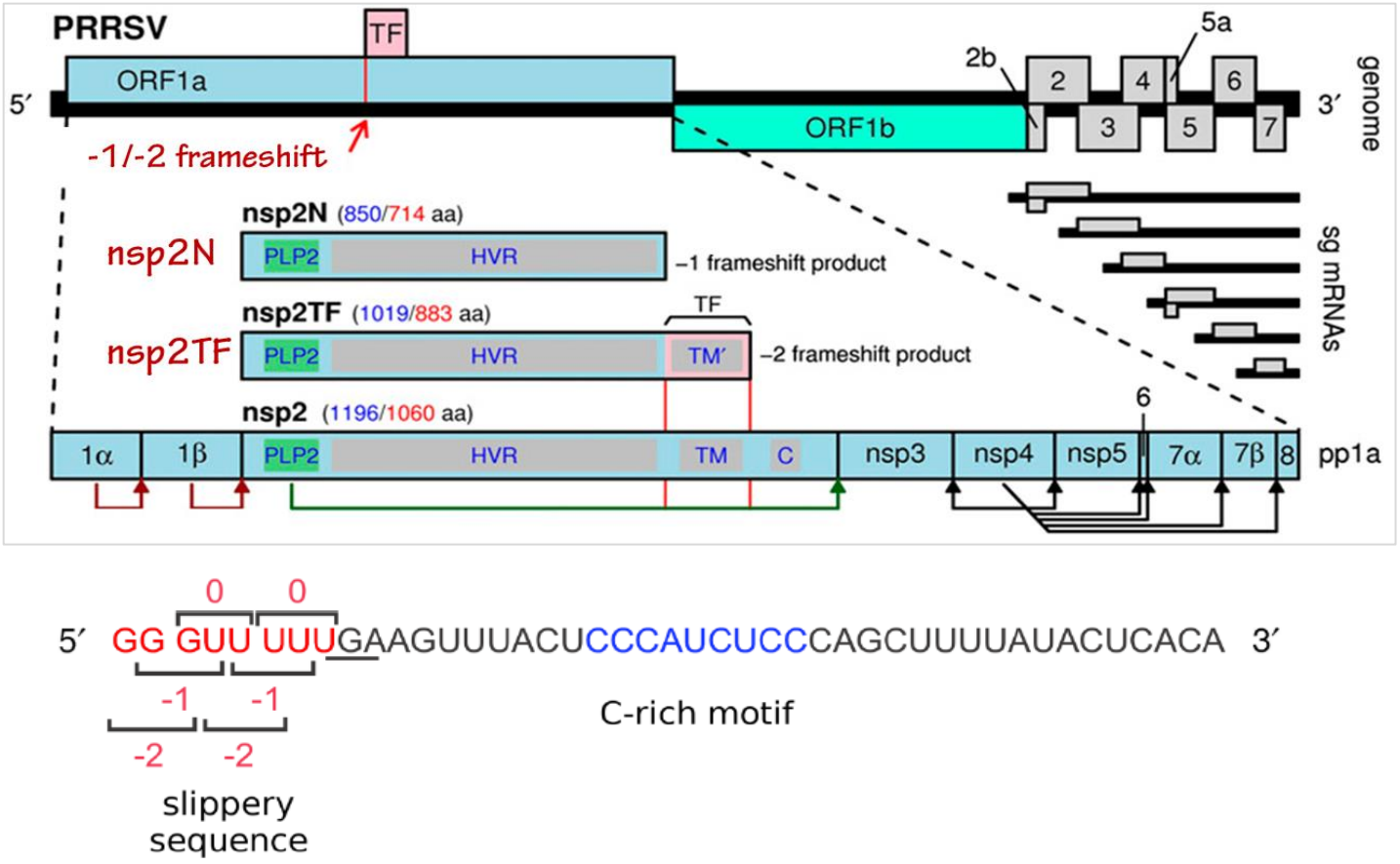


Figure 3. Schematic diagram of newly identified PRRSV genome structure.

In our lab's previously study, a -1/-2 programmed ribosomal frameshifting (PRF) signal was identified in the nsp2 region, which contains a slippery sequence and a C-rich motif. The -1 frameshifting generates the novel protein nsp2N, while the -2 frameshifting generates a novel protein nsp2TF. These nsp2-related proteins all share the same N-terminal region including a papain-like protease PLP2 domain and a long uncharacterized hvpervariable region. Figure adapted from Fang, et al., 2012.

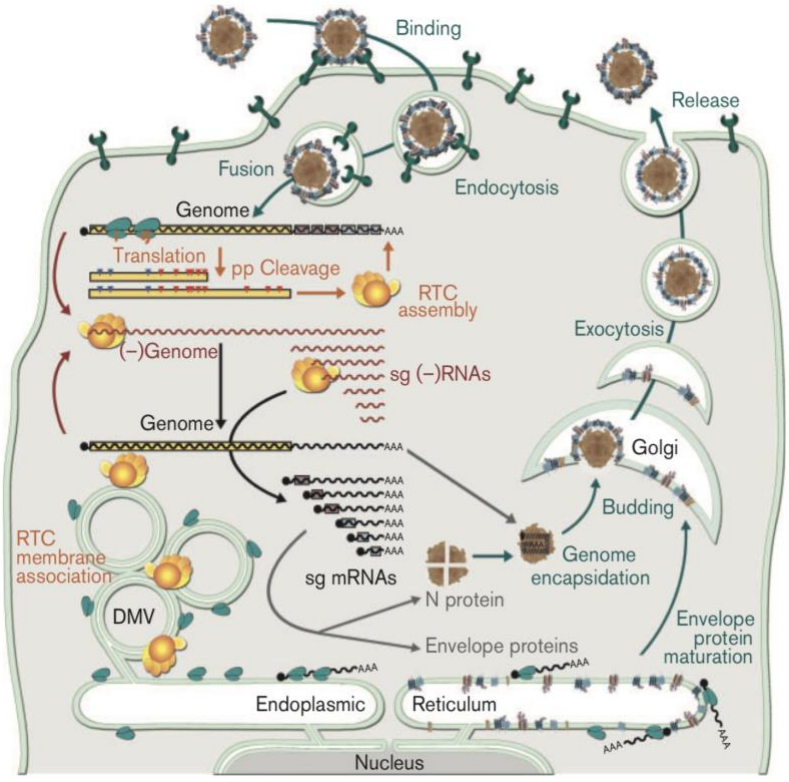


Figure 4. PRRSV infectious cycle.

PRRSV enters the cells by receptor-mediated endocytosis. Genomic RNA released into cytosol and subsequent translation yields replicase polyproteins pp1a and pp1ab. These polyproteins are cleaved by internal proteinases yielding 11 nsps, which are further assembled into a replication and transcription complex (RTC). Both full-length and subgenome-length minus strands are produced with RTCs, the latter serving as templates for the synthesis of sg mRNAs required to express the structural protein genes. Newly synthesized genomes are encapsulated into nucleocapsids that become enveloped by budding from smooth intracellular membranes, after which the new virions leave the cell using the exocytic pathway. Figure adapted from Snijder et al., 2013.

1.6 References

Ansari, I. H., Kwon, B., Osorio, F. A., & Pattnaik, A. K. (2006). Influence of N-linked glycosylation of porcine reproductive and respiratory syndrome virus GP5 on virus infectivity, antigenicity, and ability to induce neutralizing antibodies. *J Virol, 80*(8), 3994-4004. doi:10.1128/JVI.80.8.3994-4004.2006

Benfield, D. A., Nelson, E., Collins, J. E., Harris, L., Goyal, S. M., Robison, D., . . . Chladek, D. (1992). Characterization of swine infertility and respiratory syndrome (SIRS) virus (isolate ATCC VR-2332). *J Vet Diagn Invest, 4*(2), 127-133. doi:10.1177/104063879200400202

Beura, L. K., Sarkar, S. N., Kwon, B., Subramaniam, S., Jones, C., Pattnaik, A. K., & Osorio, F. A. (2010). Porcine reproductive and respiratory syndrome virus nonstructural protein 1beta modulates host innate immune response by antagonizing IRF3 activation. *J Virol, 84*(3), 1574-1584.

Brown, E., Lawson, S., Welbon, C., Gnanandarajah, J., Li, J., Murtaugh, M. P., . . . Fang, Y. (2009). Antibody response to porcine reproductive and respiratory syndrome virus (PRRSV) nonstructural proteins and implications for diagnostic detection and differentiation of PRRSV types I and II. *Clin Vaccine Immunol, 16*(5), 628-635. doi:10.1128/CVI.00483-08

Calvert, J. G., Slade, D. E., Shields, S. L., Jolie, R., Mannan, R. M., Ankenbauer, R. G., & Welch, S. K. (2007). CD163 expression confers susceptibility to porcine reproductive and respiratory syndrome viruses. *J Virol, 81*(14), 7371-7379. doi:10.1128/jvi.00513-07

Chen, Z., Collin, E., Peddireddi, L., Clement, T., Gauger, P., & Hause, B. M. (2017). Genetic diversity in envelope genes of contemporary US porcine reproductive and respiratory syndrome virus strains influences viral antigenicity. *Research in veterinary science, 115*, 432-441.

Collins, J. E., Benfield, D. A., Christianson, W. T., Harris, L., Hennings, J. C., Shaw, D. P., . . . et al. (1992). Isolation of swine infertility and respiratory syndrome virus (isolate ATCC VR-2332) in North America and experimental reproduction of the disease in gnotobiotic pigs. *J Vet Diagn Invest, 4*(2), 117-126. doi:10.1177/104063879200400201

Corzo, C. A., Mondaca, E., Wayne, S., Torremorell, M., Dee, S., Davies, P., & Morrison, R. B. (2010). Control and elimination of porcine reproductive and respiratory syndrome virus. *Virus Res, 154*(1-2), 185-192. doi:10.1016/j.virusres.2010.08.016

Das, P. B., Dinh, P. X., Ansari, I. H., de Lima, M., Osorio, F. A., & Pattnaik, A. K. (2010). The minor envelope glycoproteins GP2a and GP4 of porcine reproductive and respiratory syndrome virus interact with the receptor CD163. *J Virol, 84*(4), 1731-1740. doi:10.1128/JVI.01774-09

Duan, X., Nauwynck, H. J., & Pensaert, M. B. (1997a). Effects of origin and state of differentiation and activation of monocytes/macrophages on their susceptibility to porcine reproductive and respiratory syndrome virus (PRRSV). *Arch Virol, 142*(12), 2483-2497.

Duan, X., Nauwynck, H. J., & Pensaert, M. B. (1997b). Virus quantification and identification of cellular targets in the lungs and lymphoid tissues of pigs at different time intervals after inoculation with porcine reproductive and respiratory syndrome virus (PRRSV). *Vet Microbiol, 56*(1-2), 9-19. doi:10.1016/S0378-1135(96)01347-8

Fang, Y., Schneider, P., Zhang, W. P., Faaberg, K. S., Nelson, E. A., & Rowland, R. R. (2007). Diversity and evolution of a newly emerged North American Type 1 porcine arterivirus: analysis of isolates collected between 1999 and 2004. *Arch Virol, 152*(5), 1009-1017. doi:10.1007/s00705-007-0936-y
Fang, Y., & Snijder, E. J. (2010). The PRRSV replicase: exploring the multifunctionality of an intriguing set of nonstructural proteins. *Virus Res, 154*(1-2), 61-76. doi:10.1016/j.virusres.2010.07.030

Fang, Y., & Snijder, E. J. (2010). The PRRSV replicase: exploring the multifunctionality of an intriguing set of nonstructural proteins. *Virus Research, 154*(1-2), 61-76.

Fang, Y., Treffers, E. E., Li, Y., Tas, A., Sun, Z., Van Der Meer, Y., . . . Snijder, E. J. (2012). Efficient− 2 frameshifting by mammalian ribosomes to synthesize an additional arterivirus protein. *proceedings of the National Academy of Sciences, 109*(43), E2920-E2928.

Fang, Y., Treffers, E. E., Li, Y., Tas, A., Sun, Z., van der Meer, Y., . . . Firth, A. E. (2012). Efficient -2 frameshifting by mammalian ribosomes to synthesize an additional arterivirus protein. *Proc Natl Acad Sci U S A, 109*(43), E2920-2928. doi:10.1073/pnas.1211145109

Feng, Y., Zhao, T., Nguyen, T., Inui, K., Ma, Y., Nguyen, T. H., . . . Gao, G. F. (2008). Porcine respiratory and reproductive syndrome virus variants, Vietnam and China, 2007. *Emerg Infect Dis, 14*(11), 1774-1776. doi:10.3201/eid1411.071676

Gao, J., Xiao, S., Xiao, Y., Wang, X., Zhang, C., Zhao, Q., . . . Zhou, E. M. (2016). MYH9 is an Essential Factor for Porcine Reproductive and Respiratory Syndrome Virus Infection. *Sci Rep, 6*, 25120. doi:10.1038/srep25120

Guo, R., Katz, B. B., Tomich, J. M., Gallagher, T., & Fang, Y. (2016). Porcine Reproductive and Respiratory Syndrome Virus Utilizes Nanotubes for Intercellular Spread. *J Virol, 90*(10), 5163-5175. doi:10.1128/jvi.00036-16

Han, J., Wang, Y., & Faaberg, K. S. (2006). Complete genome analysis of RFLP 184 isolates of porcine reproductive and respiratory syndrome virus. *Virus Research, 122*(1-2), 175-182.

Han, J., Zhou, L., Ge, X., Guo, X., & Yang, H. (2017). Pathogenesis and control of the Chinese highly pathogenic porcine reproductive and respiratory syndrome virus. *Veterinary microbiology*.

Hill, H. (1990). *Overview and history of mystery swine disease (swine infertility/respiratory syndrome)*. Paper presented at the Proceedings of the mystery swine disease committee meeting.

Holtkamp, D. J., Kliebenstein, J. B., Neumann, E. J., Zimmerman, J. J., Rotto, H. F., Yoder, T. K., . . . Haley, C. A. (2013). Assessment of the economic impact of porcine reproductive and respiratory syndrome virus on United States pork producers. *Journal of Swine Health and Production, 21*(2), 72-84.

Hopper, S., White, M., & Twiddy, N. (1992). An outbreak of blue-eared pig disease (porcine reproductive and respiratory syndrome) in four pig herds in Great Britain. *The Veterinary Record, 131*(7), 140-144.

Hu, S. P., Zhang, Z., Liu, Y. G., Tian, Z. J., Wu, D. L., Cai, X. H., & He, X. J. (2013). Pathogenicity and distribution of highly pathogenic porcine reproductive and respiratory syndrome virus in pigs. *Transbound Emerg Dis, 60*(4), 351-359. doi:10.1111/j.1865-1682.2012.01354.x

Huang, C., Zhang, Q., Guo, X. K., Yu, Z. B., Xu, A. T., Tang, J., & Feng, W. H. (2014). Porcine reproductive and respiratory syndrome virus nonstructural protein 4 antagonizes beta interferon expression by targeting the NF-kappaB essential modulator. *J Virol, 88*(18), 10934-10945.

Iseki, H., Morozumi, T., Takagi, M., Kawashima, K., Shibahara, T., Uenishi, H., & Tsunemitsu, H. (2016). Genomic sequence and virulence evaluation of MN184A-like porcine reproductive and respiratory syndrome virus in Japan. *Microbiol Immunol, 60*(12), 824-834. doi:10.1111/1348-0421.12455
Johnson, C. R., Yu, W., & Murtaugh, M. P. (2007). Cross-reactive antibody responses to nsp1 and nsp2 of Porcine reproductive and respiratory syndrome virus. *J Gen Virol, 88*(Pt 4), 1184-1195.

Karniychuk, U. U., Geldhof, M., Vanhee, M., Van Doorsselaere, J., Saveleva, T. A., & Nauwynck, H. J. (2010). Pathogenesis and antigenic characterization of a new East European subtype 3 porcine reproductive and respiratory syndrome virus isolate. *BMC Vet Res, 6*, 30. doi:10.1186/1746-6148-6-30

Kuhn, J. H., Lauck, M., Bailey, A. L., Shchetinin, A. M., Vishnevskaya, T. V., Bào, Y., . . . Gillis, A. (2016). Reorganization and expansion of the nidoviral family Arteriviridae. *Archives of virology, 161*(3), 755-768.

Li, C., Zhuang, J., Wang, J., Han, L., Sun, Z., Xiao, Y., . . . Li, X. (2016). Outbreak Investigation of NADC30-Like PRRSV in South-East China. *Transboundary and Emerging Diseases, 63*(5), 474-479.

Li, C., Zhuang, J., Wang, J., Han, L., Sun, Z., Xiao, Y., . . . Tian, K. (2016). Outbreak Investigation of NADC30-Like PRRSV in South-East China. *Transbound Emerg Dis, 63*(5), 474-479.

Li, X., Bao, H., Wang, Y., & Tian, K. (2016). Widespread of NADC30-like PRRSV in China: Another Pandora's box for Chinese pig industry as the outbreak of highly pathogenic PRRSV in 2006? *Infection, genetics and evolution: journal of molecular epidemiology and evolutionary genetics in infectious diseases, 49*, 12.

Li, X., Wu, J., Tan, F., Li, Y., Ji, G., Zhuang, J., . . . Tian, K. (2016). Genome characterization of two NADC30-like porcine reproductive and respiratory syndrome viruses in China. *SpringerPlus, 5*(1), 1677.

Li, Y., Shang, P., Shyu, D., Carrillo, C., Naraghi-Arani, P., Jaing, C. J., . . . Fang, Y. (2018). Nonstructural proteins nsp2TF and nsp2N of porcine reproductive and respiratory syndrome virus (PRRSV) play important roles in suppressing host innate immune responses. *Virology, 517*, 164-176.

Li, Y., Tas, A., Snijder, E. J., & Fang, Y. (2012). Identification of porcine reproductive and respiratory syndrome virus ORF1a-encoded non-structural proteins in virus-infected cells. *Journal of General Virology, 93*(4), 829-839.

Li, Y., Treffers, E. E., Napthine, S., Tas, A., Zhu, L., Sun, Z., . . . Fang, Y. (2014). Transactivation of programmed ribosomal frameshifting by a viral protein. *Proc Natl Acad Sci U S A, 111*(21), E2172-2181.

Li, Y., Zhu, L., Lawson, S. R., & Fang, Y. (2013). Targeted mutations in a highly conserved motif of the nsp1β protein impair the interferon antagonizing activity of porcine reproductive and respiratory syndrome virus. *Journal of General Virology, 94*(9), 1972-1983.

Liu, J.-k., Zhou, X., Zhai, J.-q., Li, B., Wei, C.-h., Dai, A.-l., . . . Luo, M.-l. (2017). Emergence of a novel highly pathogenic porcine reproductive and respiratory syndrome virus in China. *Transboundary and Emerging Diseases*.

Lopez, O. J., & Osorio, F. A. (2004). Role of neutralizing antibodies in PRRSV protective immunity. *Vet Immunol Immunopathol, 102*(3), 155-163. doi:10.1016/j.vetimm.2004.09.005

Lunney, J. K., Fang, Y., Ladinig, A., Chen, N., Li, Y., Rowland, B., & Renukaradhya, G. J. (2016). Porcine Reproductive and Respiratory Syndrome Virus (PRRSV): Pathogenesis and Interaction with the Immune System. *Annu Rev Anim Biosci, 4*, 129-154. doi:10.1146/annurev-animal-022114-111025

Murtaugh, M. P., Stadejek, T., Abrahante, J. E., Lam, T. T., & Leung, F. C.-C. (2010). The ever-expanding diversity of porcine reproductive and respiratory syndrome virus. *Virus Research, 154*(1), 18-30.

Murtaugh, M. P., Xiao, Z., & Zuckermann, F. (2002). Immunological responses of swine to porcine reproductive and respiratory syndrome virus infection. *Viral Immunol, 15*(4), 533-547. doi:10.1089/088282402320914485

Nauwynck, H. J., Duan, X., Favoreel, H. W., Van Oostveldt, P., & Pensaert, M. B. (1999). Entry of porcine reproductive and respiratory syndrome virus into porcine alveolar macrophages via receptor-mediated endocytosis. *J Gen Virol, 80 (Pt 2)*, 297-305. doi:10.1099/0022-1317-80-2-297

Ostrowski, M., Galeota, J. A., Jar, A. M., Platt, K. B., Osorio, F. A., & Lopez, O. J. (2002). Identification of neutralizing and nonneutralizing epitopes in the porcine reproductive and respiratory syndrome virus GP5 ectodomain. *J Virol, 76*(9), 4241-4250.

Plana, J., Vayreda, M., Vilarrasa, J., Bastons, M., Rosell, R., Martinez, M., . . . Ramos, J. A. (1992). Porcine epidemic abortion and respiratory syndrome (mystery swine disease). Isolation in Spain of the causative agent and experimental reproduction of the disease. *Veterinary microbiology, 33*(1-4), 203-211.

Prather, R. S., Rowland, R. R., Ewen, C., Trible, B., Kerrigan, M., Bawa, B., . . . Green, J. A. (2013). An intact sialoadhesin (Sn/SIGLEC1/CD169) is not required for attachment/internalization of the porcine reproductive and respiratory syndrome virus. *J Virol, 87*(17), 9538-9546. doi:10.1128/jvi.00177-13

Ropp, S. L., Wees, C. E. M., Fang, Y., Nelson, E. A., Rossow, K. D., Bien, M., . . . Christopher-Hennings, J. (2004). Characterization of emerging European-like porcine reproductive and respiratory syndrome virus isolates in the United States. *Journal of Virology, 78*(7), 3684-3703.

Rossow, K. D. (1998). Porcine reproductive and respiratory syndrome. *Vet Pathol, 35*(1), 1-20.

Shang, P., Misra, S., Hause, B., & Fang, Y. (2017). A naturally occurring recombinant enterovirus expresses a torovirus deubiquitinase. *Journal of Virology*, JVI. 00450-00417.

Shi, M., Holmes, E. C., Brar, M. S., & Leung, F. C.-C. (2013). Recombination is associated with an outbreak of novel highly pathogenic porcine reproductive and respiratory syndrome viruses in China. *Journal of Virology, 87*(19), 10904-10907.

Shi, M., Lam, T. T.-Y., Hon, C.-C., Hui, R. K.-H., Faaberg, K. S., Wennblom, T., . . . Leung, F. C.-C. (2010). Molecular epidemiology of PRRSV: a phylogenetic perspective. *Virus Research, 154*(1), 7-17.
Shi, M., Lam, T. T.-Y., Hon, C.-C., Murtaugh, M. P., Davies, P. R., Hui, R. K.-H., . . . Jiang, J.-W. (2010). Phylogeny-based evolutionary, demographical, and geographical dissection of North American type 2 porcine reproductive and respiratory syndrome viruses. *Journal of Virology, 84*(17), 8700-8711.

Snijder, E. J., Kikkert, M., & Fang, Y. (2013). Arterivirus molecular biology and pathogenesis. *J Gen Virol, 94*(Pt 10), 2141-2163. doi:10.1099/vir.0.056341-0

Snijder, E. J., Kikkert, M., & Fang, Y. (2013). Arterivirus molecular biology and pathogenesis. *Journal of General Virology, 94*(10), 2141-2163.

Snijder, E. J., & Meulenberg, J. J. (1998). The molecular biology of arteriviruses. *J Gen Virol, 79 (Pt 5)*, 961-979. doi:10.1099/0022-1317-79-5-961

Spilman, M. S., Welbon, C., Nelson, E., & Dokland, T. (2009). Cryo-electron tomography of porcine reproductive and respiratory syndrome virus: organization of the nucleocapsid. *J Gen Virol, 90*(Pt 3), 527-535. doi:10.1099/vir.0.007674-0

Tian, K., Yu, X., Zhao, T., Feng, Y., Cao, Z., Wang, C., . . . Tian, X. (2007). Emergence of fatal PRRSV variants: unparalleled outbreaks of atypical PRRS in China and molecular dissection of the unique hallmark. *PLoS One, 2*(6), e526.

Tian, K., Yu, X., Zhao, T., Feng, Y., Cao, Z., Wang, C., . . . Gao, G. F. (2007). Emergence of fatal PRRSV variants: unparalleled outbreaks of atypical PRRS in China and molecular dissection of the unique hallmark. *PLoS One, 2*(6), e526. doi:10.1371/journal.pone.0000526

van Geelen, A. G. M., Anderson, T. K., Lager, K. M., Das, P. B., Otis, N. J., Montiel, N. A., . . . Faaberg, K. S. (2018). Porcine reproductive and respiratory disease virus: Evolution and recombination yields distinct ORF5 RFLP 1-7-4 viruses with individual pathogenicity. *Virology, 513*, 168-179.

Van Reeth, K., Labarque, G., Nauwynck, H., & Pensaert, M. (1999). Differential production of proinflammatory cytokines in the pig lung during different respiratory virus infections: correlations with pathogenicity. *Res Vet Sci, 67*(1), 47-52. doi:10.1053/rvsc.1998.0277

Wang, L., & Zhang, Y. (2015). Novel porcine reproductive and respiratory syndrome virus strains in the United States with deletions in untranslated regions. *Archives of virology, 160*(12), 3093-3096.

Wang, L.-j., Xie, W., Chen, X.-x., Qiao, S., Zhao, M., Gu, Y., . . . Zhang, G. (2017). Molecular epidemiology of porcine reproductive and respiratory syndrome virus in Central China since 2014: The prevalence of NADC30-like PRRSVs. *Microbial Pathogenesis, 109*, 20-28.

Wang, L. J., Xie, W., Chen, X. X., Qiao, S., Zhao, M., Gu, Y., . . . Zhang, G. (2017). Molecular epidemiology of porcine reproductive and respiratory syndrome virus in Central China since 2014: The prevalence of NADC30-like PRRSVs. *Microb Pathog, 109*, 20-28. doi:10.1016/j.micpath.2017.05.021

Welch, S. K., & Calvert, J. G. (2010). A brief review of CD163 and its role in PRRSV infection. *Virus Res, 154*(1-2), 98-103. doi:10.1016/j.virusres.2010.07.018

Wensvoort, G., Terpstra, C., Pol, J., Ter Laak, E., Bloemraad, M., De Kluyver, E., . . . Wagenaar, F. (1991). Mystery swine disease in The Netherlands: the isolation of Lelystad virus. *Veterinary Quarterly, 13*(3), 121-130.

Wensvoort, G., Terpstra, C., Pol, J. M., ter Laak, E. A., Bloemraad, M., de Kluyver, E. P., . . . et al. (1991). Mystery swine disease in The Netherlands: the isolation of Lelystad virus. *Vet Q, 13*(3), 121-130.

Wesley, R. D., Mengeling, W. L., Lager, K. M., Clouser, D. F., Landgraf, J. G., & Frey, M. L. (1998). Differentiation of a porcine reproductive and respiratory syndrome virus vaccine strain from North American field strains by restriction fragment length polymorphism analysis of ORF 5. *Journal of Veterinary Diagnostic Investigation, 10*(2), 140-144.

Workman, A. M., Smith, T. P., Osorio, F. A., & Vu, H. L. (2016a). Complete genome sequence of highly virulent porcine reproductive and respiratory syndrome virus variants that recently emerged in the United States. *Genome Announc., 4*(4), e00772-00716.

Workman, A. M., Smith, T. P., Osorio, F. A., & Vu, H. L. (2016). Complete Genome Sequence of Highly Virulent Porcine Reproductive and Respiratory Syndrome Virus Variants That Recently Emerged in the United States. *Genome Announc, 4*(4). doi:10.1128/genomeA.00772-16

Workman, A. M., Smith, T. P., Osorio, F. A., & Vu, H. L. (2016b). Complete genome sequence of highly virulent porcine reproductive and respiratory syndrome virus variants that recently emerged in the United States. *Genome announcements, 4*(4), e00772-00716.

Zhang, Q., & Yoo, D. (2015). PRRS virus receptors and their role for pathogenesis. *Vet Microbiol, 177*(3-4), 229-241. doi:10.1016/j.vetmic.2015.04.002

Zhao, K., Ye, C., Chang, X.-B., Jiang, C.-G., Wang, S.-J., Cai, X.-H., . . . An, T.-Q. (2015). Importation and recombination are responsible for the latest emergence of highly pathogenic porcine reproductive and respiratory syndrome virus in China. *Journal of Virology, 89*(20), 10712-10716.

Chapter 2 - Genetic characterization of emerging variants of PRRSV in the United States: new features of -2/-1 programmed ribosome frameshifting in nsp2-coding region

2.1 Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is the etiological agent of porcine reproductive and respiratory syndrome (PRRS), characterized by respiratory diseases in growing pigs and reproductive failure in sows (Michael P Murtaugh, Stadejek, Abrahante, Lam, & Leung, 2010; Shi, Lam, Hon, Hui, et al., 2010; Shi, Lam, Hon, Murtaugh, et al., 2010). PRRSV is an enveloped, positive-stranded RNA virus, which belongs to the order *Nidovirales*, family *Arteriviridae* (Eric J Snijder, Marjolein Kikkert, & Ying Fang, 2013). Historically, PRRSV isolates have been divided into two distinct genotypes, European genotype (Type 1) and North American genotype (Type 2), which were recently promoted to the species level, named as PRRSV-1 and PRRSV-2, respectively (Kuhn et al., 2016).

The PRRSV genome is a positive sense RNA, about 15kb in length. It contains 5'- and 3'-untranslated region (UTR) that flank with 11 known open reading frames. The 3’ end of the genome encodes four membrane-associated glycoproteins (GP2a, GP3, GP4 and GP5), three

unglycosylated membrane proteins (E, ORF5a and M) and a nucleocapsid protein (N) (Ying Fang & Eric J Snijder, 2010; Eric J Snijder et al., 2013). The replicase-associated genes, ORF1a and ORF1b, situated at the 5' end and represent nearly 75% of the viral genome. The ORF1a and ORF1b encode two long nonstructural polyproteins, pp1a and pp1ab, with expression of the latter depending on a -1 ribosomal frame shift signal in the ORF1a/ORF1b overlap region. Following their synthesis from the genomic mRNA template, the pp1a and pp1ab replicase polyproteins are processed into at least 14 nonstructural proteins (nsps) by a complex proteolytic cascade that is directed by four proteinase domains encoded in ORF1a, which includes two papain-like cysteine proteases (PCPα and PCPβ) located in the nsp1α and nsp1β, a papain-like protease (PLP2) domain located at the N-terminal of nsp2, and a serine protease located in nsp4. The PCPα autocleaves between nsp1α/1β, PCPβ autocleaves between nsp1β/2, and PLP2 cleaves between nsp2/3, which mediate nsp1α, nsp1β and nsp2 rapid releasing from the polyprotein (Y. Li, Tas, Snijder, & Fang, 2012). The nsp2 is the largest replicase cleavage product, containing multidomains for multifunctions (Ying Fang & Eric J Snijder, 2010). Besides cleaving the nsp2/3 site, PLP2 domain functions as a cofactor for the nsp4 serine protease during proteolytic processing of the C-terminal half of pp1a and pp1ab (Y. Li et al., 2012). Recently, a new ORF (TF) was discovered in the central region of ORF1a, which expresses a novel protein, nsp2TF (Ying Fang et al., 2012). The nsp2TF is expressed by -2 ribosomal frameshift mechanism to access an alternative open reading frame (ORF) through a frameshifting site that overlaps the nsp2-encoding region of their replicase ORF1a in the +1 frame, which results a transframe fusion protein consisting of the N-terminal two thirds of nsp2 followed by a unique C-terminal domain that is specified by the novel TF ORF. Remarkably, the same frameshift site was also found to direct an efficient -1 PRF, which is followed by a stop codon, thus yielding a second truncated

nsp2 variant named nsp2N (Ying Fang et al., 2012; Eric J Snijder et al., 2013). Besides their potential roles involved in viral replication, our recent studies showed that nsp2TF and nsp2N may play important roles in suppressing host innate immune responses (Li et al., 2018).

PRRS was initially recognized in the United States at late 1980s, then in Europe at early 1990s (Hill, 1990; Hopper, White, & Twiddy, 1992; Plana et al., 1992; G Wensvoort et al., 1991). Since then, PRRSV has rapidly evolved into one of the leading pathogens threatening global swine industry. It causes numerous acute respiratory disease outbreaks and abortion storms, including the 'porcine high fever disease' outbreaks in South-East Asia (Kegong Tian et al., 2007), the emergence of the highly virulent MN184 strain in the US (Han, Wang, & Faaberg, 2006), and the pathogenic NADC30-like strains emergence in China during 2013-2014 (Han, Zhou, Ge, Guo, & Yang, 2017; C Li et al., 2016; X. Li, Bao, Wang, & Tian, 2016; X. Li, J. Wu, et al., 2016; Liu et al., 2017; Shi, Holmes, Brar, & Leung, 2013; L.-j. Wang et al., 2017; Zhao et al., 2015). Since 2014, occurrence of pathogenic PRRSV-2 variants has been frequently reported from swine farms in United States. The epidemic strains were characterized as 1-7-4 cutting pattern with the restriction fragment length polymorphism (RFLP) analysis on the ORF5 region (Wesley et al., 1998; Aspen M Workman, Timothy PL Smith, Fernando A Osorio, & Hiep LX Vu, 2016b). Recently, we have isolated two emerging PRRSV variants (KS17-C1 and KS17-C2) from pigs in swine farms at Midwest of United States, which had been experiencing outbreaks since the end of 2016. In this study, we performed detailed genetic characterization of these PRRSV variants. These new strains were determined to possess ORF5 RFLP 1 -7-4 and 1-18-2 pattern, respectively; and remarkably, they contain a unique -2/-1 PRF signal that expresses the C-terminus extended nsp2N protein. The potential correlation of nsp2N extension and pathogenicity of these group of PRRSV variants was further explored using reverse genetics and

a nursery pig model. This study provides an insight into the molecular pathogenic mechanisms of the newly emerging PRRSV variants in the US swine herd.

2.2 Materials and methods

2.2.1 Cells and viruses

BHK-21 cells were cultured in Minimum Essential Medium (MEM) (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma Aldrich, St. Louis, MO), antibiotics [100 units/ml of penicillin (Gibco, Carlsbad, CA) and 100 ug/ml of streptomycin (Gibco, Carlsbad, CA)] and 0.25 ug/ml fungizone (Gibco, Carlsbad, CA) at 37 °C with 5% CO_2. Primary porcine alveolar macrophages (PAM) were cultured in RPMI 1640 medium (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum and antibiotics at 37°C with 5% CO2. Infected PAM cells were maintained in MEM supplemented with 2% horse serum (HyClone, Logan, UT) at 37°C with 5% CO2.

The PRRSV positive serum samples were obtained from a swine farm in Midwest US, in which pigs were experiencing respiratory diseases and abortion. KS17-C1 and KS17-C2 strains were isolated by inoculating the serum samples into the cell culture of PAMs as described previously (Ropp et al., 2004). PRRSV infection was confirmed by observation of cytopathic effect and indirect immunofluorescence assay (IFA) as described in previous studies (Ropp et al., 2004; Shang, Misra, Hause, & Fang, 2017). Viruses (cell culture supernatants) were harvested between 24 to 48 hours post infection (hpi).

2.2.2 Full-length genome sequencing and sequence analysis

The passage 1 of KS17-C1 and KS17-C2 isolates from the PAM were subjected to Sanger sequencing by Genscript (Piscataway, NJ). The genome sequences were completed by GeneRacer (Invitrogen). The genome sequence identity of KS17-C1 and KS17-C2 was initially compared with that of prototypic PRRSV-2 strain VR-2332 (Genbank accession No. AY150564.1). Complete PRRSV genome sequences were further aligned with representative PRRSV genome sequences obtained from GenBank using the ClustalW algorithm in MEGA 7.0 software. Phylogeny was inferred with maximum likelihood algorithm using the best-fitting model with gamma distribution. The topology of the phylogenetic tree was assessed with 500 bootstrap replicates.

2.2.3 Construction of full-length cDNA clones of KS17-C1 and PRF site mutants

The full-length cDNA clone of KS17-C1 was constructed based on previously built infectious clone of PRRSV SD95-21 strain(Y. Li et al., 2014)(**Fig 5**). The viral genome of SD95-21 strain was removed by restrictions enzymes-SphI and XbaI, in order to generate an empty vector-pACYC177 containing cytomegalovirus (CMV) promoter. SphI recognition site is located between CMV promoter and SD95-21 genome. XbaI restriction site is located at the immediately 3' end of Hepatitis delta virus (HDV) ribozyme. The four fragments of KS17-C1 genome and HDV element were amplified by Polymerase chain reaction (PCR) with Phusion® High-Fidelity DNA Polymerase (New England BioLabs, Ipswich, MA), and ligated into pACYC177-CMV

vector with NEBuilder® HiFi DNA Assembly Cloning Kit (New England BioLabs, Ipswich, MA). To construct the PRF site mutants, the upstream and downstream regions of the -2/-1 PRF slippery site were amplified and assembled using NEBuilder® HiFi DNA Assembly Cloning Kit (**Fig 6**).

2.2.4 Recovery of recombinant viruses from full-length cDNA clones

The recombinant viruses were launched by transfecting BHK-21 cells as described previously (Y. Li, Zhu, Lawson, & Fang, 2013). Briefly, BHK-21 cells (70-80% confluency) were transfected with 2 μg of the full-length cDNA clone pKS17-C1 or its mutants (pKS17-M1 and pKS17-M2) using FuGENE HD reagent (Promega, Madison, WI). At 48 h post transfection, cell culture supernatant was harvested and passaged onto PAM cells. The viability of recombinant viruses was confirmed by indirect immunofluorescence assay using mAb SDOW17. The recombinant viruses were harvested at 24-48 hpi and serially passaged on PAM cells. The passage three viruses were used for subsequent experiments.

2.2.5 In vitro growth characterization of recombinant viruses in cell culture

Growth kinetics of the wild-type and recombinant viruses were examined by infecting PAM cells at MOI of 0.01. Infected cells were collected at 0, 12, 24, 36, and 48 hours post-infection (hpi). Viral titers were determined by $TCID_{50}$ method and calculated as fluorescent focus unit (FFU)/ml as we described previously(Y. Li et al., 2013).

2.2.6 Quantitative RT-PCR for immune gene detection

PAMs were infected with WT virus or recombinant virus at MOI of 0.1. At 18 hpi, cells were harvested and the expression levels of selected immune genes were determined by quantitative RT-PCR. Briefly, viral RNA was extract using SV Total RNA Isolation System (Promega, Madison, WI). The first strand cDNA was generated with 1 μg total RNA using SuperScript VILO cDNA Synthesis Kit (Life Technologies, Carlsbad, CA). PCR reaction contains 10 μl TaqMan Fast Advanced Master Mix (Applied Biosystems, Foster City, CA), 1 μl cDNA, and 1 μl of predesigned primer/probe sets (Applied Biosystems, Foster City, CA) for IFN-α, IFN-β, IRF-7, IL-28B, IFIT1, IFITM1 and ISG15. Reactions were completed on CFX96 Real-Time PCR system (Bio-Rad) under the following conditions: 2 min of 50 ^{o}C for UNG activation, 20 sec of 95 ^{o}C for polymerase activation, 40 amplification cycles of 30 sec of 95 ^{o}C and 30 sec of 60 ^{o}C. The mRNA expression levels of IFN-α, IFN-β, IRF-7, IL-28B, IFIT1 and IFITM1 were normalized to the endogenous Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) mRNA level.

To assess the ability of mutant viruses to stimulate innate immune responses in virus-infected swine macrophages, PAMs were infected with WT virus, vKO1 or vKO2 at MOI of 1.0. At 12 hpi, cells were harvested in TRIzol LS (Life Technologies, Carlsbad, CA) and total cellular RNA was extracted according to the manufacturer's instruction. The mRNA expression levels of IFN-α, IFN-β, IRF-7, IL-28B, IFIT1, IFITM1 were quantified by quantitative RT-PCR using predesigned probe/primer sets (Applied Biosystems, Foster City, CA) and normalized to the housekeeping gene GAPDH mRNA.

2.3 Results

2.3.1 Isolation and genetic characterization of newly emerging PRRSV variants

In spring of 2017, serum samples were obtained from 7-week old pigs in a swine farm at the Midwest of the US, in which pigs had been experienced PRRSV outbreaks since the end of 2016. Over 40% mortality in growing pigs and 20-30% reproductive failure in sows have been observed. Two PRRSV strains (KS17-C1 and KS17-C2) were isolated by inoculating swine serum on cell culture of porcine alveolar macrophages (PAMs) (**Figure 7**). Cytopathic effect on the PAMs was observed in 48 hours post infection. The full-length genome sequencing was performed with the first passage of the virus from PAMs. The full-length genome of KS17-C1 is 15110 nucleotides (nt) in length, while the genome of KS17-C2 contains 15023-nt, excluding the poly(A) tail. The KS 17-C1 and KS 17-C2 have ORF5 RFLP cutting pattern of 1-7-4 and 1-18-2, respectively. In comparison with the prototypic PRRSV-2 strain VR-2332, the full-length genomic sequences of KS17-C1 and KS17-C2 showed 82.18% and 82.45% nt identity with that of VR-2332, respectively (**Table 2.1**). Phylogenetic analysis showed that they each belongs to a distinct phylogenetic clade (**Figure 8**), which includes newly emerging US PRRSV strains that reported to cause severe clinical manifestations in infected pigs (Chen et al., 2017; Wang & Zhang, 2015; Aspen M Workman, Timothy PL Smith, Fernando A Osorio, & Hiep LX Vu, 2016a).

The most variable region in the genome of KS17-C1 and KS17-C2 is nsp2, in which KS17-C1 contains 300-nt deletion at genome position of 2323 to 2622 nt of VR2332, while KS17-C2 contains 333-nt deletion at genome position of 2306 to 2638 of VR2332 (**Figure 9**). Therefore,

KS17-C1 and KS17-C2 encode nsp2 proteins of 1096 amino acid (aa) and 1065aa, which are 65.42% and 66.92% aa identity with nsp2 from VR-2332, respectively. Further in depth sequence analysis revealed that the -2/-1 programmed ribosome frameshifting (PRF) signal located within nsp2 of KS 17-C1 and KS 17-C2 differs from traditional signal sequences (**Figure 10**). As we described previously (Ying Fang et al., 2012), at the traditional slippery sequence (G_GUU_UUU) site, -2 frameshifting generated a transframe fusion protein, nsp2TF, with the N-terminal two thirds of nsp2 fused to a 169-aa C-terminal region encoded by the TF ORF. At the same slippery site, -1 PRF also occurs. The -1 frameshifting immediately encounters a stop codon (UGA), which terminates the translation of -1 reading frame to produce nsp2N. The KS 17-C1 and KS 17-C2 isolates contain the slippery sequences of G_GUU_UUC (UG) and G_GUU_UUU (GG). The substitution of G_GUU_UU**U** to G_GUU_UU**C** in KS 17-C1 could reduce the -2 PRF efficiency. Most remarkably, mutations immediately downstream of the slippery sequence disrupt the -1 PRF stop codon (UGA to CUG or UGG; Fig. 3), which extends the translation of nsp2N that generates additional 16 or 23 amino acids at 3'-end (nsp2N+16aa, nsp2N+23aa). The emergence of -1 PRF stop codon mutants was traced back in the PRRSV sequences published in the Genbank since 2011 and percentage of such mutants has quickly increased afterward (up to 69% of the sequences; **Table 2.1**). More importantly, these -1 PRF stop codon mutants were mostly reported from swine farms experiencing PRRSV outbreaks with

increased mortality/morbidity (Iseki et al., 2016; L. J. Wang et al., 2017; A. M. Workman, T. P. Smith, F. A. Osorio, & H. L. Vu, 2016).

2.3.2 Recombinant viruses with restored PRF signal shared the similar growth kinetics with the parental virus in cell culture

To explore the potential contribution of the mutations in the -2/-1 PRF site to the pathogenicity of these emerging PRRSV variants, we generated a full-length cDNA infectious clone of KS17-C1 (pKS17-C1; **Fig 5**). The pKS17-C1 construct contains a CMV promoter at the 5′ terminus of the viral genome, the 15111-nt full-length genome of KS17-C1, a 20 nt poly(A) tail and incorporated at the 3′ end of the genome (**Fig 5**). To rescue the cloned virus, plasmid DNA of pKS17-C1 was transfected into BHK-21 cells, and cell culture supernatant from the transfected cells were passaged onto PAM cells at 48 hours post transfection. At 18 hours post infection (hpi), infected cells were stained using N protein-specific mAbs (**Fig 11**). The result indicates that viable cloned virus (vKS17-C1) was recovered from the full-length cDNA infectious clone pKS-C1. Sequence analysis confirmed that vKS17-C1 genome contains 8-nt differences at the following genome positions: the G_{514} to A mutation located within the ORF1a-NSP1a-coding region; the A_{1942} to G, the C_{2582} to T, the A_{2646} to G and the G_{2654} to A mutations located within the ORF1a-NSP2-coding region; the C_{8939} to T mutation within the OFR1ab-NSP9-coding region; the T_{12174} to C mutation located within the ORF2a-GP2a-coding region and the G_{14191} to C mutation within the ORF6-M-coding region, which differentiate vKS17-C1 from original isolate KS17-C1.

Using the pKS17-C1 infectious clone, two mutants with modified PRF signal site were generated and successfully recovered from PAMs (**Figure 11**). Mutations were designed to

restore the traditional slippery sequence G_GUU_UUU (mutant vKS17-M1) and also the immediate stop codon after -1 PRF (mutant vKS17-M2; **Figure 6b&c**). Growth kinetics of the parental virus KS17-C1, clone virus (vKS17-C1) and PRF mutants (vKS17-C1-M1 and vKS17-C1-M2) were compared. PAMs were infected with each of the virus and the supernatant was harvested at 0, 12, 24, 36, and 48 hpi. The result showed that the cloned virus vKS-C1, vKS-C1-M1 and vKS17-C1-M2 exhibit growth kinetics similar to the parental virus KS17-C1 (**Fig. 12**). The parental virus, vKS17-C1 and vKS17-C1-M2 all reached a titer of 10^7 FFU/ml at 24 hpi, while vKS17-C1-M1 reached a titer of a$10^{6.67}$ FFU/ml at 24 hpi (**Fig. 12**).

2.3.3 The -2/-1 PRF mutant stimulated higher level of innate immune gene expression in porcine alveolar macrophages

In our previous studies (Li et al., 2018), the nsp2TF and nsp2N were determined to function as innate immune antagonists, and recombinant viruses with impaired nsp2TF/nsp2N stimulated higher level of innate immune gene expression. Based on these previous findings, the WT, cloned virus, and PRF slippery site mutants were tested on their ability to stimulate the expression of a panel of representative genes in PAMs. The swine macrophages were infected with each virus at MOI of 1, and the viruses were harvested at 12 hpi. Swine gene specific qRT-PCR assays were used to analyze the expression level of the selected innate immune genes, including interferon beta (IFNB), interferon regulatory factor 7 (IRF7), interferon induced with helicase C Domain 1 (IFIH1), interferon induced transmembrane protein 1 (IFITM1), and interferon-stimulated gene 15 (ISG15).

As we expected, all recombinant viruses stimulated limited level of innate immune gene expression. There is no significant difference on the immune gene expression levels in the cells

infected with vKS17-C1-M1 (restored traditional slippery sequence), in comparison with those cells infected with vKS17-C1. In contrast, the nsp2N stop codon mutant vKS17-C1-M2 consistently induced about 2-fold of increase in expression levels for IFN-α, IFN-β, IRF7, IFIH1, IFITM1, in ISG15 in PAM cells (**Fig. 13**) suggesting that the extension of nsp2N may be enhanced innate immune suppression ability of the virus. On the other hand, we cannot exclude the possibility that this is due to the effect of mutations on viral growth ability.

2.4 Discussions

- **Isolation and characterization of two newly emerged PRRSV variants**. Two PRRSV strains (KS17-C1 and KS 17-C2) were isolated in serum samples from swine farms in the Midwest of US, where PRRSV outbreak was reported. Sequence analysis revealed that KS 17-C1 and KS 17-C2 have a RFLP cutting pattern of 1-7-4 and 1-18-2, respectively. They each belongs to a distinct phylogenetic clade, which includes newly emerging US PRRSV strains that reported to cause severe clinical manifestations in infected pigs.

- **Mutations identified in PRF signal region of KS 17-C1 and KS 17-C2 strains.** Both KS17-C1 and KS 17-C2 contain mutations disrupting the -1 PRF stop codon in the slippery sequence region that extends the translation of nsp2N with an additional 16 and 23 amino acids C-terminal peptide, respectively.

- **The emergence of -1 PRF mutants has been rapidly increased in the US since 2011.** The frequency of mutants with mutated -1 PRF stop codon has been increased more than 5 times since 2011.

- **The elongation of nsp2N may be correlated with increased pathogenicity of the newly emerging PRRSV variant.** Recombinant KS17-C1 mutants with restored -1 stop codon and/or PRF signal were generated. Compared with that of WT cloned virus, nsp2N stop codon mutants consistently showed a higher expression level of innate immune genes. The data indicate that the extension of nsp2N may be enhanced innate immune suppression ability of the nsp2N protein, which further linked to the pathogenicity of these strains in infected pigs.

Table 2.1. Genome comparison of PRRSV new isolates with prototype strain VR-2332

	VR-2332	KS17-C1		KS17-C2	
Genetic regions	**Length (nt)**	**Length (nt)**	**% of identity (vs VR-2332)**	**Length (nt)**	**% of identity (vs VR-2332)**
Full-length	15,412	15110	82.18	15023	82.45
5' UTR	190	189	94.24	194	91.79
ORF1a	7,512	7,212	77.96	7,119	77.63
ORF1ab	11,882	11,582	80.71	11,489	80.63
ORF1a'-TF	3907	4207	81.92	3814	83.43
ORF1a'-N	3449	3701	81.92	3377	83.43
ORF2a	771	771	88.72	771	89.75
ORF2b	222	222	89.19	222	89.64
ORF3	765	765	83.53	765	85.62
ORF4	537	537	86.78	537	88.83
ORF5a	156	141	80.77	156	87.18
ORF5	603	603	87.23	603	86.07
ORF6	525	525	89.71	525	91.24
ORF7	372	372	92.47	372	90.32
3' UTR	151	151	94.70	151	92.72
Proteins	**Length (aa)**	**Length (aa)**	**% of identity (vs VR-2332)**	**Length (aa)**	**% of identity (vs VR-2332)**
nsp1a	180	180	95.00	180	95.56
nsp1b	203	203	75.86	203	79.31
nsp2	1,196	1,096	65.42	1,065	66.92
nsp2TF	1,019	919	59.34	888	61.77
nsp2N	850	766	54.97	742	56.80
nsp3	230	230	92.17	230	91.30

nsp4	204	204	92.16	204	92.16
nsp5	170	170	88.82	170	85.29
nsp6	16	16	93.75	16	100
nsp7a	149	149	95.30	149	92.62
nsp7b	110	110	83.64	110	79.09
nsp8	45	45	91.11	45	91.11
nsp9	685	685	95.91	685	95.91
nsp10	441	441	94.78	441	94.56
nsp11	223	223	95.07	223	95.07
nsp12	153	153	89.54	153	91.50
pp1a	2,503	2,403	77.76	2,372	78.16
pp1ab	3,960	3,860	84.06	3,829	84.36
GP2a	256	256	87.11	256	86.72
E	73	73	89.04	73	84.93
GP3	254	254	83.46	254	85.04
GP4	178	178	87.64	178	88.20
GP5a	51	46	82.35	51	82.35
GP5	200	200	85.50	200	85.00
M	174	174	91.38	174	92.53
N	123	123	95.12	123	91.06

Table 2.2. Emergence of -1 PRF mutants in the US since 2011

Slippery sequences	Prior to 2011	2011	2012	2013	2014	2015	2016	2017	-1 PRF		-2 PRF	
	No. of genomes	No. of genomes	No. of genomes	No. of genomes	No. of genomes	No. of genomes	No. of genomes	No. of genomes	Length	No. of genomes	Length	No. of genomes
NN_NUU_UUU AAN AGN GAN	23	1	10	3	25			1	0	63		
NN_NUU_UUU UAN					1				23aa	1		
GCN			1						23aa	1		
GGN			5	4	33	3	8	1	23aa 16aa	13 39	169aa	145
NN_NUU_UUc TGN							1		16aa	1		
GAN			3	1					16aa	4		
GGN	1	3	6	3	10				23aa 16aa	21 2		
Total No. of genomes	24	4	25	11	69	3	9	2				

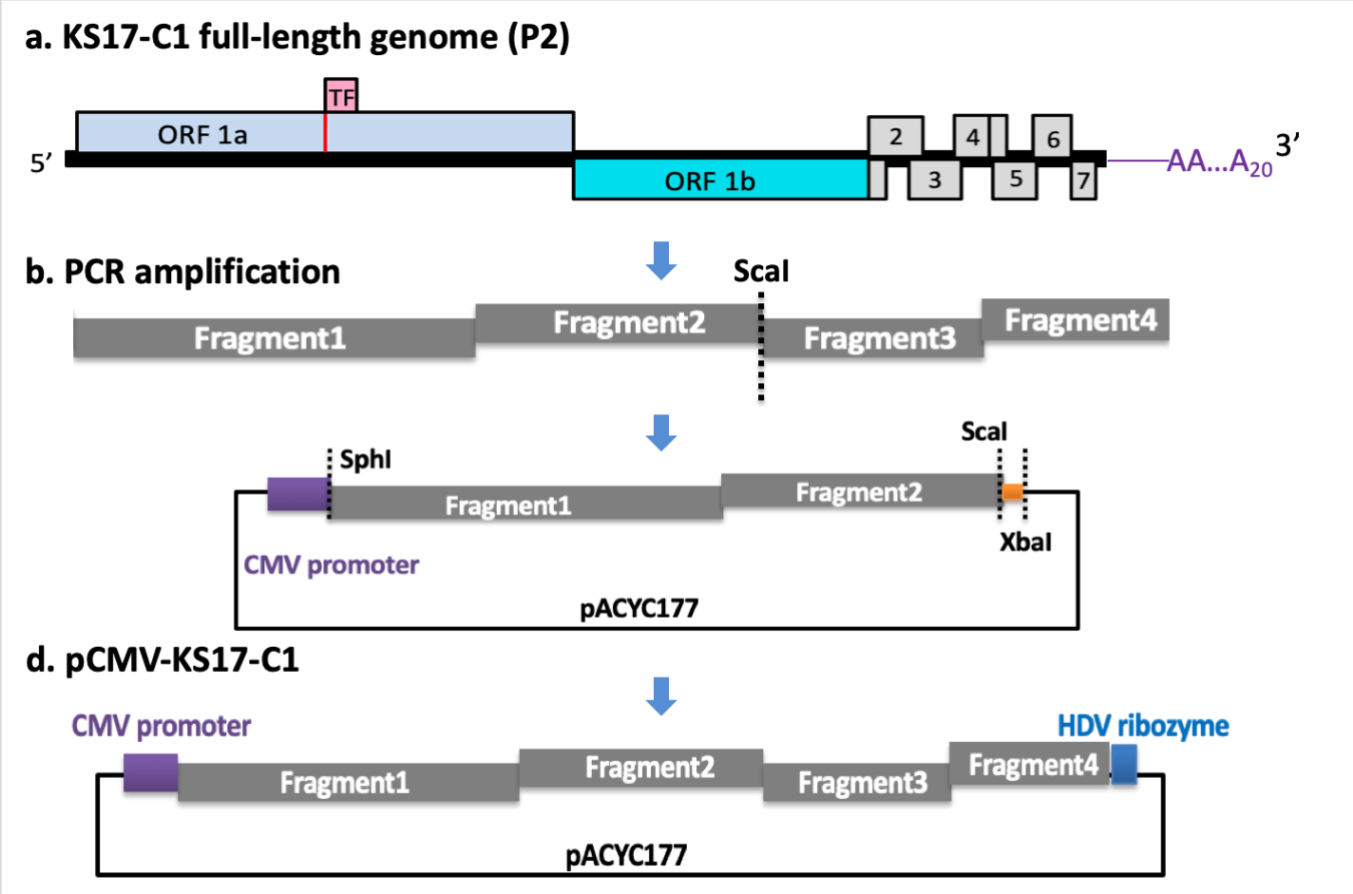


Figure 5. Construction of the full-length cDNA of KS17-C1 wild-type strain.

The full-length cDNA clone of KS17-C1 was constructed based on previously built infectious clone of PRRSV SD95-21 strain. The viral genome of SD95-21 was removed by restrictions enzymes-SphI and XbaI to generate the empty vector-pACYC177 containing cytomegalovirus (CMV) promoter. The four fragments of KS17-C1 genome and HDV element were amplified by PCR and assembled into pACYC177-CMV vector via HiFi DNA Assembly Cloning kit.

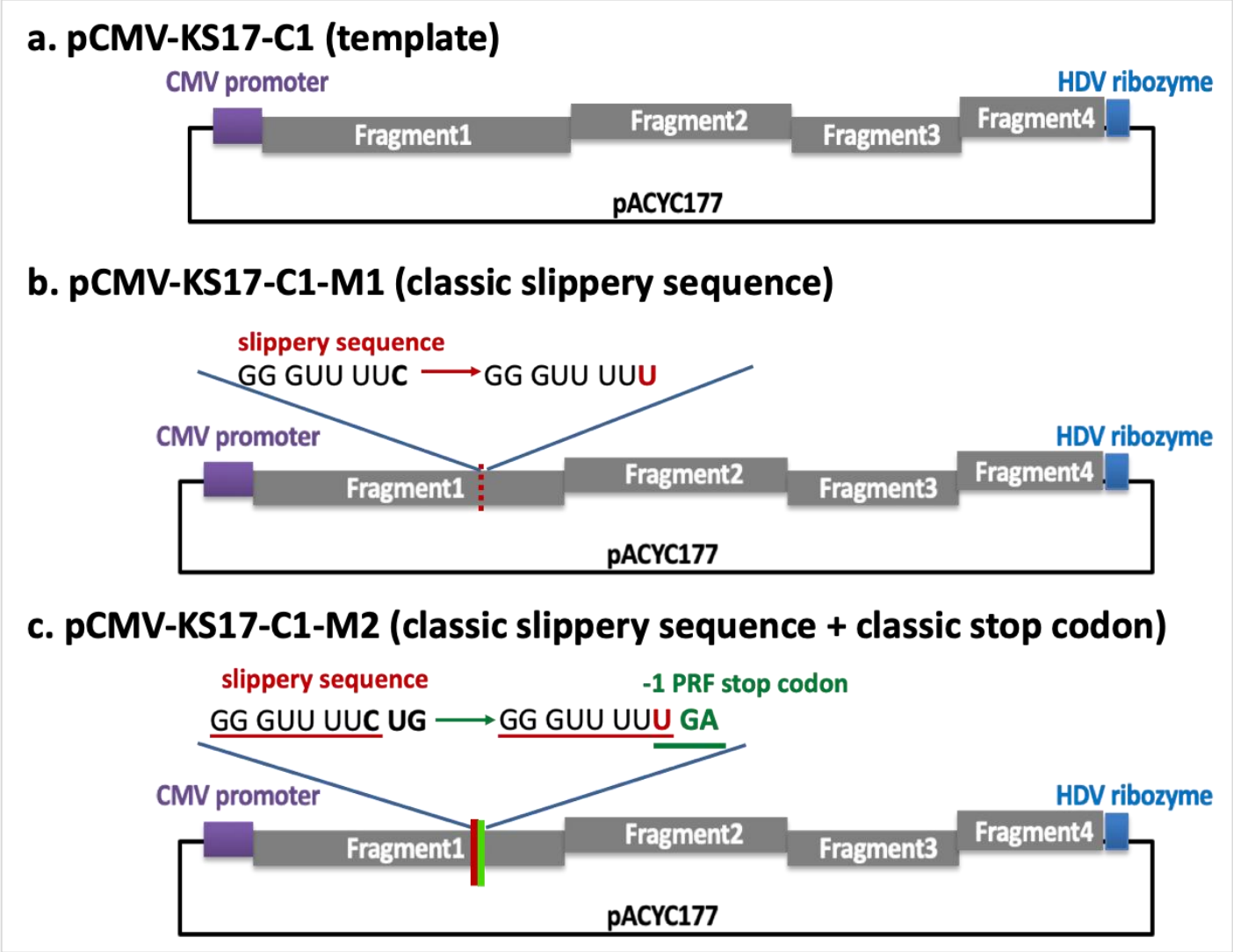


Figure 6. Construction of the full-length cDNA of KS17-C1 PRF mutants.

To construct the PRF site mutants, the pCMV-KS17-C1-WT was used as the template, the upstream and downstream regions of the -2/-1 PRF slippery site were amplified and assembled using via DNA Assembly Cloning Kit.

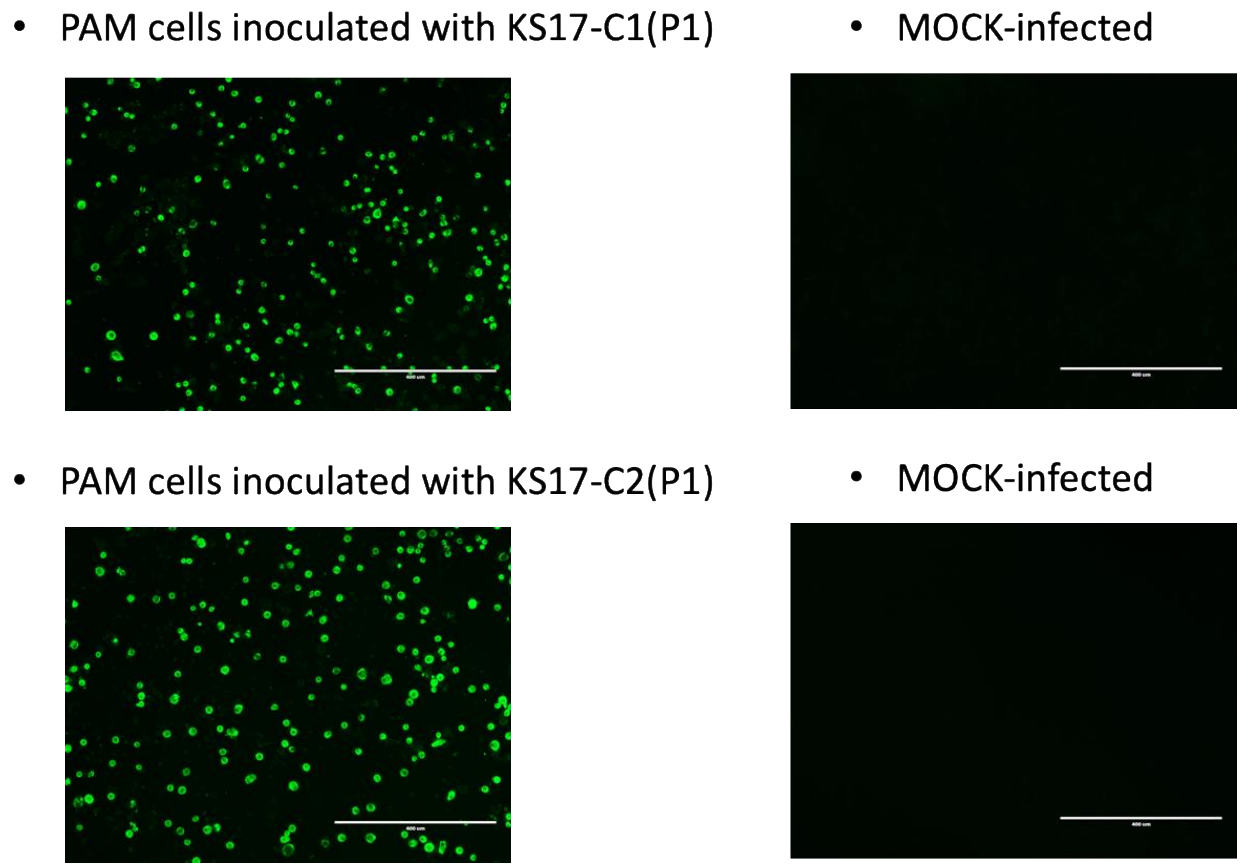


Figure 7. Isolation and genetic characterization of newly emerging PRRSV variants.

The PRRSV positive serum samples were obtained from a swine farm in Midwest US. KS17-C1 and KS17-C2 strains were isolated by inoculating the serum samples into the cell culture of PAMs. PRRSV infection was confirmed by observation of cytopathic effect and indirect immunofluorescence assay (IFA). Viruses (cell culture supernatants) were harvested between 24 to 48 hours post infection (hpi).

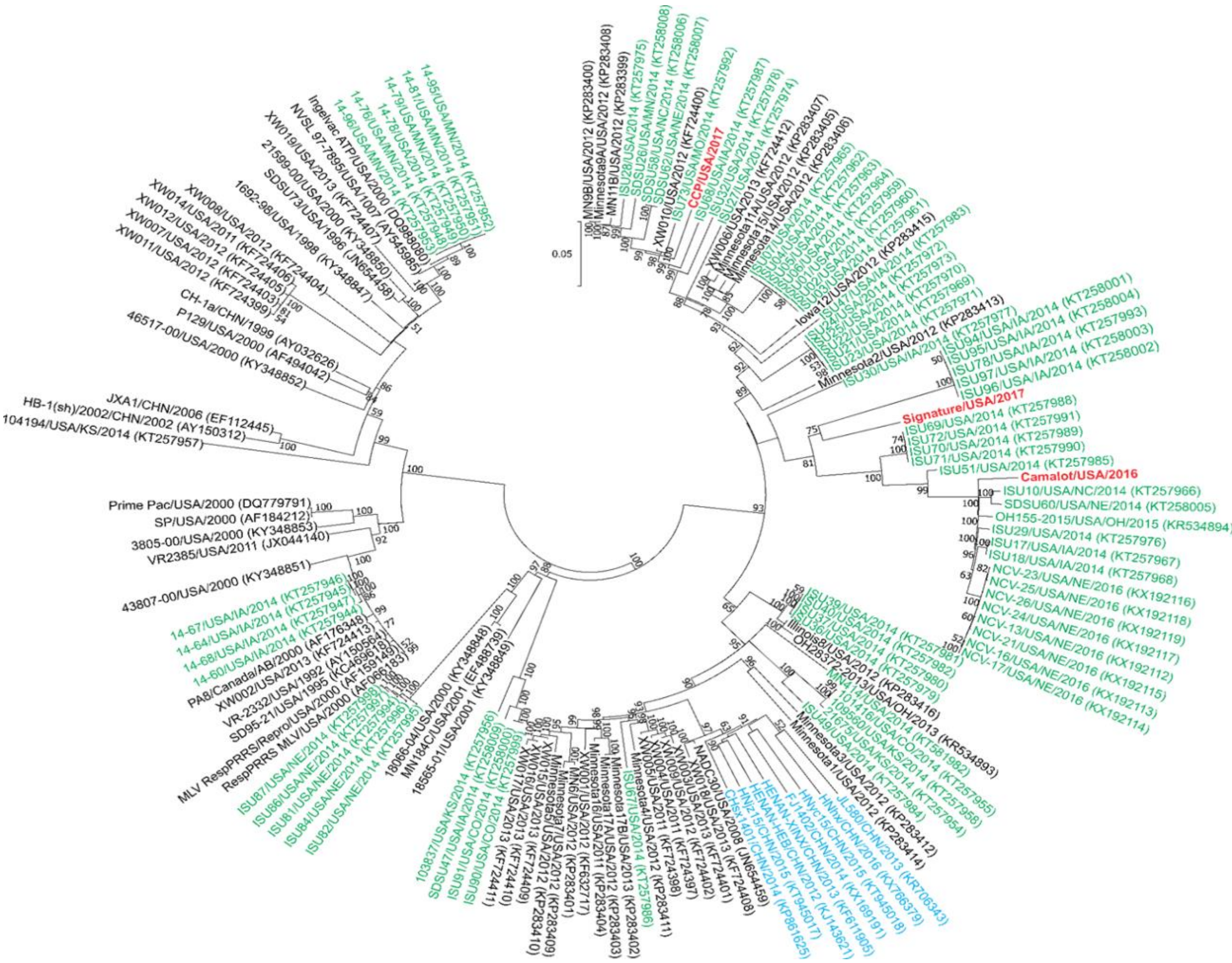


Figure 8. Phylogenetic analysis of newly emerging isolates KS17-C1 and KS17-C2 with other epidemic and historical strains.

The phylogenetic tree was constructed by maximum likelihood method using full-length genome sequences. Green: US variants identified after 2011; blue: Chinese variants identified after 2011; Red: KS17-C1 and KS17-C2 isolates.

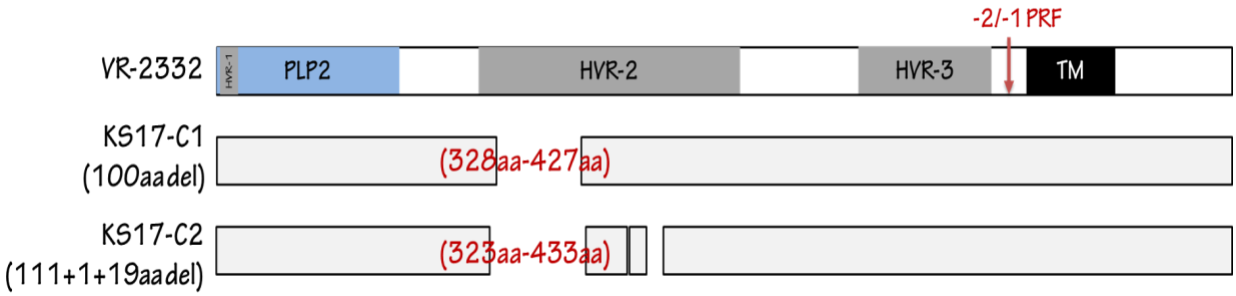
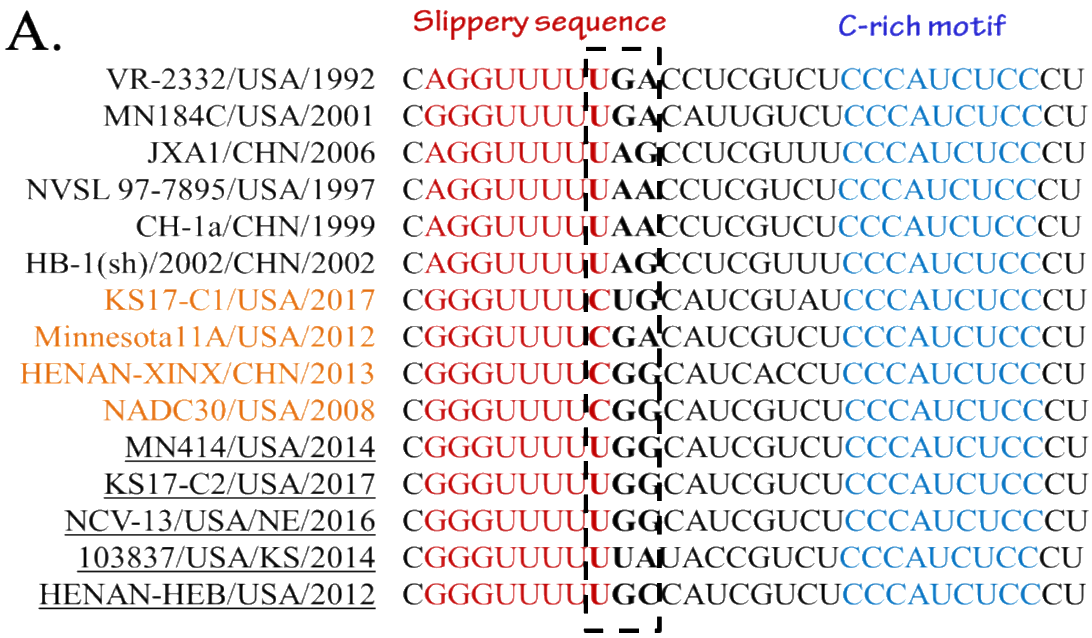


Figure 9. Sequence comparison between KS17-C1 and KS17-C2 with PRRSV-2 prototypic strain VR2332.

The most variable region in the genome of KS17-C1 and KS17-C2 is nsp2, in which KS17-C1 contains 300-nt deletion at genome position of 2323 to 2622 nt of VR2332, while KS17-C2 contains 333-nt deletion at genome position of 2306 to 2638 of VR2332.



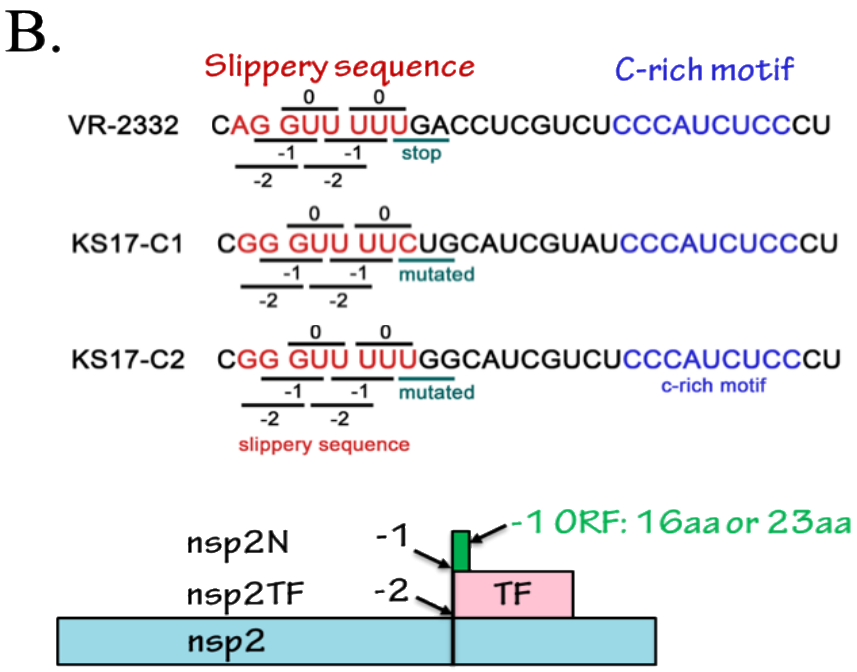
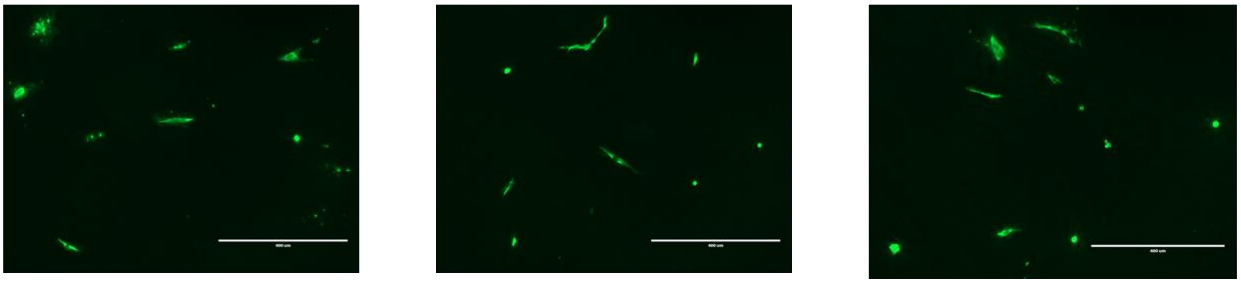


Figure 10. Sequence analysis of -2/-1 PRF signal region.

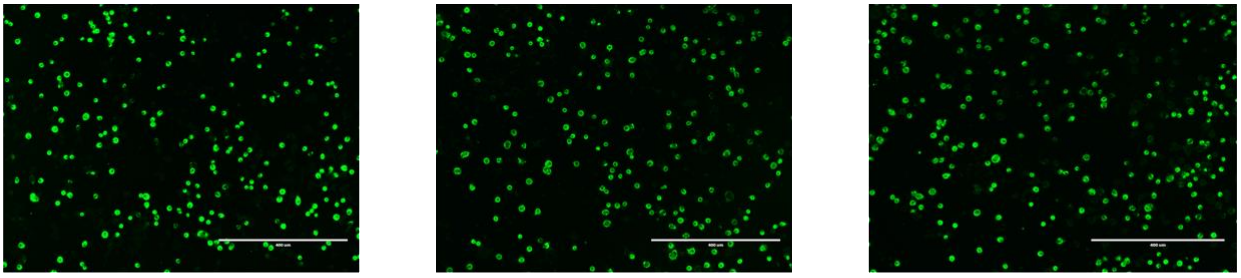
A). Sequence alignment of -2/-1 PRF signal region between traditional PRRSV strains and emerging PRRSV variants since 2011. The PRF slippery sequence is underlined in red while the C-rich motif is underlined in blue. Sequences of KS17-C1 and KS17-C2 were generated from this study. Sequences of traditional and other emerging strains were obtained from GenBank. **B). Sequence mutations of KS 17-C1 and KS17-C2 in the -2/-1 PRF region.** At the traditional slippery sequence G/A_GUU_UUU(GA) site, the -1 PRF immediately encounters a stop codon (UGA), which terminates the translation of -1 reading frame to produce nsp2N. However, The KS 17-C1and KS17-C2 isolates contain different slippery sequences sites of G_GUU_UUC(UG) and G_GUU_UUU(GG), mutations immediately downstream of the slippery sequence disrupt the -1 PRF stop codon (UGA to CUG or UGG), which extends the translation of nsp2N that generates additional 16 or 23 amino acids at 3'-end.

A. Full-length cDNA transfection of KS17-C1 in BHK-21 cells



pKS17-C1 pKS17-C1-M1 pKS17-C1-M2

B. Recovery of recombinant viruses in PAM cells



pKS17-C1 pKS17-C1-M1 pKS17-C1-M2

Figure 11. Recovery of recombinant viruses of pKS17-CI and its PRF mutants.

A.) Transfection of full-length cDNA of pKS17-C1 and its PRF mutants. 2 ug/well of each plasmid was transfected into BHK-21 cells on 6-well plates. The supernatant was collected 48 hours post transfection and the cell plates were stained with mAb against PRRSV-N protein. **B.) Recovery of recombinant viruses on PAMs.** The P0 supernatant of each clone was inoculated into PAMs on 24-well plates. Part of the wells were stained with mAb against PRRSV-N protein 24 hours post infection and the P1 supernatant was collected 24-48 hours post inoculation.

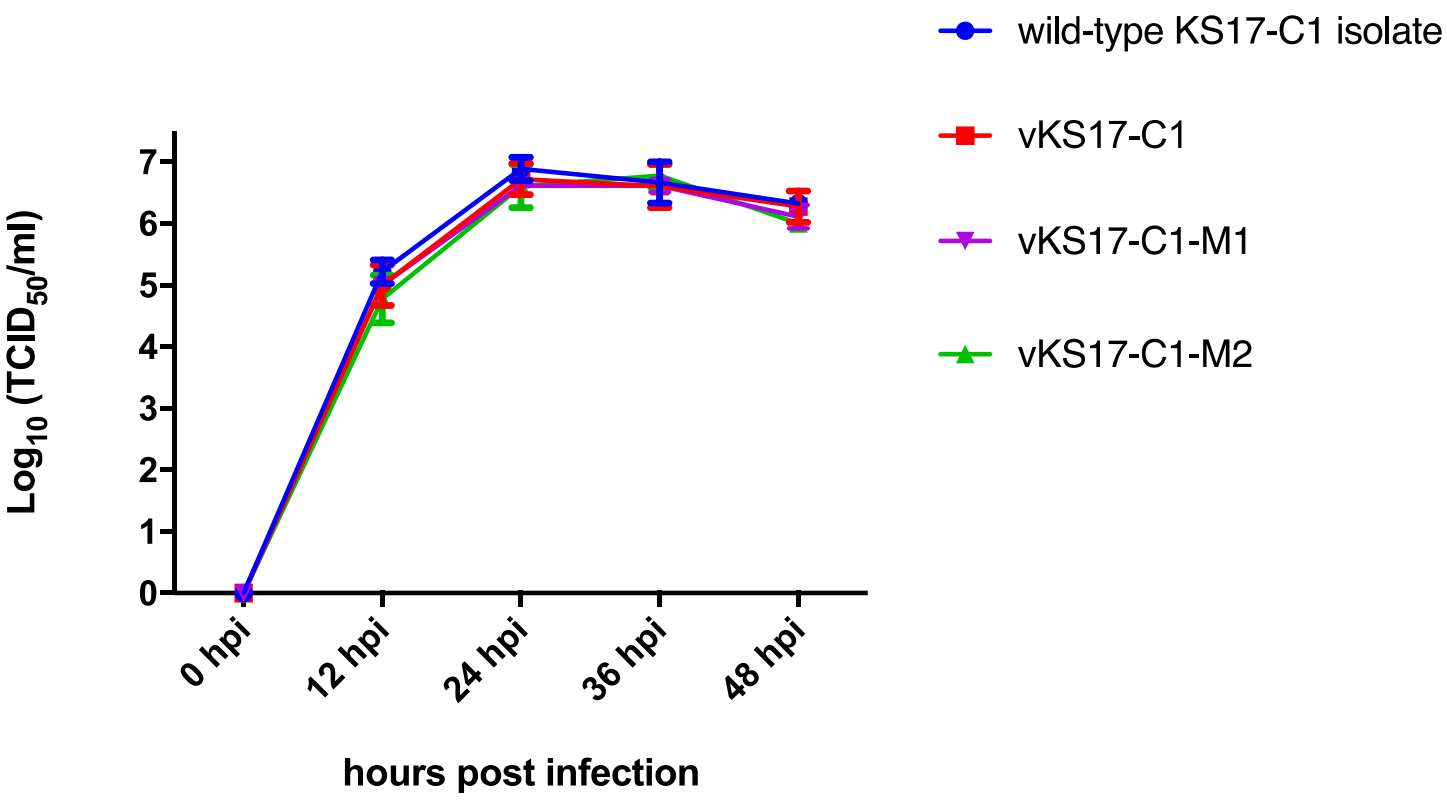


Figure 12. Growth kinetics comparison of the wild-type KS17-C1 isolate and its recovered recombinant viruses.

Growth kinetics of the wild-type and recombinant viruses were examined by infecting PAM cells at MOI of 0.01. Infected cells were collected at 0, 12, 24, 36, and 48 hours post-infection (hpi). Viral titers were determined by TCID50 method and calculated as fluorescent focus unit (FFU)/ml.

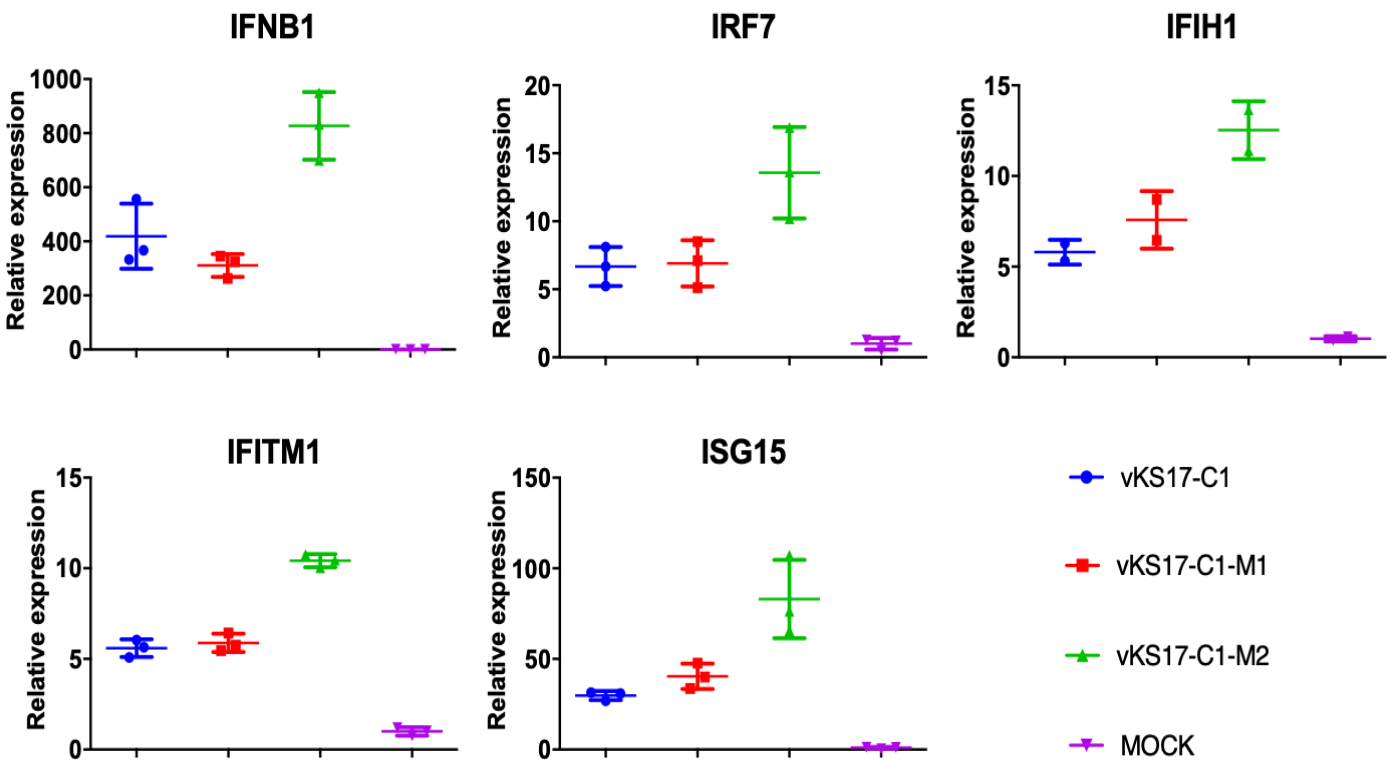


Figure 13. Immune gene expression in recombinant KS 17-C1 viruses-infected porcine alveolar macrophages (PAMs).

PAMs were infected or mock infected by wild type pKS17-C1 virus, mutant viruses pKS17-C1-M1 of "normal" shiftsite and pKS17-C2-M2 of "normal" shiftsite + "normal" nsp2N stop codon. The relative expression levels of immune genes IFNB1, IRF7, IFIH1, IFITM1 and ISG15 were quantified by qRT-PCR.

2.5 Reference

ADDIN EN.REFLIST Ansari, I. H., Kwon, B., Osorio, F. A., & Pattnaik, A. K. (2006). Influence of N-linked glycosylation of porcine reproductive and respiratory syndrome virus GP5 on virus infectivity, antigenicity, and ability to induce neutralizing antibodies. *J Virol, 80*(8), 3994-4004. doi:10.1128/JVI.80.8.3994-4004.2006

Benfield, D. A., Nelson, E., Collins, J. E., Harris, L., Goyal, S. M., Robison, D., . . . Chladek, D. (1992). Characterization of swine infertility and respiratory syndrome (SIRS) virus (isolate ATCC VR-2332). *J Vet Diagn Invest, 4*(2), 127-133. doi:10.1177/104063879200400202

Beura, L. K., Sarkar, S. N., Kwon, B., Subramaniam, S., Jones, C., Pattnaik, A. K., & Osorio, F. A. (2010). Porcine reproductive and respiratory syndrome virus nonstructural protein 1beta modulates host innate immune response by antagonizing IRF3 activation. *J Virol, 84*(3), 1574-1584. doi:10.1128/JVI.01326-09

Brown, E., Lawson, S., Welbon, C., Gnanandarajah, J., Li, J., Murtaugh, M. P., . . . Fang, Y. (2009). Antibody response to porcine reproductive and respiratory syndrome virus (PRRSV) nonstructural proteins and implications for diagnostic detection and differentiation of PRRSV types I and II. *Clin Vaccine Immunol, 16*(5), 628-635. doi:10.1128/CVI.00483-08

Calvert, J. G., Slade, D. E., Shields, S. L., Jolie, R., Mannan, R. M., Ankenbauer, R. G., & Welch, S. K. (2007). CD163 expression confers susceptibility to porcine reproductive and respiratory syndrome viruses. *J Virol, 81*(14), 7371-7379. doi:10.1128/jvi.00513-07

Chen, Z., Collin, E., Peddireddi, L., Clement, T., Gauger, P., & Hause, B. M. (2017). Genetic diversity in envelope genes of contemporary US porcine reproductive and respiratory syndrome virus strains influences viral antigenicity. *Research in veterinary science, 115*, 432-441.

Collins, J. E., Benfield, D. A., Christianson, W. T., Harris, L., Hennings, J. C., Shaw, D. P., . . . et al. (1992). Isolation of swine infertility and respiratory syndrome virus (isolate ATCC VR-2332) in North America and experimental reproduction of the disease in gnotobiotic pigs. *J Vet Diagn Invest, 4*(2), 117-126. doi:10.1177/104063879200400201

Corzo, C. A., Mondaca, E., Wayne, S., Torremorell, M., Dee, S., Davies, P., & Morrison, R. B. (2010). Control and elimination of porcine reproductive and respiratory syndrome virus. *Virus Res, 154*(1-2), 185-192. doi:10.1016/j.virusres.2010.08.016

Das, P. B., Dinh, P. X., Ansari, I. H., de Lima, M., Osorio, F. A., & Pattnaik, A. K. (2010). The minor envelope glycoproteins GP2a and GP4 of porcine reproductive and respiratory syndrome virus interact with the receptor CD163. *J Virol, 84*(4), 1731-1740. doi:10.1128/JVI.01774-09

Duan, X., Nauwynck, H. J., & Pensaert, M. B. (1997a). Effects of origin and state of differentiation and activation of monocytes/macrophages on their susceptibility to porcine reproductive and respiratory syndrome virus (PRRSV). *Arch Virol, 142*(12), 2483-2497.

Duan, X., Nauwynck, H. J., & Pensaert, M. B. (1997b). Virus quantification and identification of cellular targets in the lungs and lymphoid tissues of pigs at different time intervals after inoculation with porcine reproductive and respiratory syndrome virus (PRRSV). *Vet Microbiol, 56*(1-2), 9-19. doi:10.1016/S0378-1135(96)01347-8

Fang, Y., Schneider, P., Zhang, W. P., Faaberg, K. S., Nelson, E. A., & Rowland, R. R. (2007). Diversity and evolution of a newly emerged North American Type 1 porcine arterivirus: analysis of isolates collected between 1999 and 2004. *Arch Virol, 152*(5), 1009-1017. doi:10.1007/s00705-007-0936-y

Fang, Y., & Snijder, E. J. (2010). The PRRSV replicase: exploring the multifunctionality of an intriguing set of nonstructural proteins. *Virus Res, 154*(1-2), 61-76. doi:10.1016/j.virusres.2010.07.030

Fang, Y., & Snijder, E. J. (2010). The PRRSV replicase: exploring the multifunctionality of an intriguing set of nonstructural proteins. *Virus Research, 154*(1-2), 61-76.

Fang, Y., Treffers, E. E., Li, Y., Tas, A., Sun, Z., Van Der Meer, Y., . . . Snijder, E. J. (2012). Efficient− 2 frameshifting by mammalian ribosomes to synthesize an additional arterivirus protein. *proceedings of the National Academy of Sciences, 109*(43), E2920-E2928.

Fang, Y., Treffers, E. E., Li, Y., Tas, A., Sun, Z., van der Meer, Y., . . . Firth, A. E. (2012). Efficient -2 frameshifting by mammalian ribosomes to synthesize an additional arterivirus protein. *Proc Natl Acad Sci U S A, 109*(43), E2920-2928. doi:10.1073/pnas.1211145109

Feng, Y., Zhao, T., Nguyen, T., Inui, K., Ma, Y., Nguyen, T. H., . . . Gao, G. F. (2008). Porcine respiratory and reproductive syndrome virus variants, Vietnam and China, 2007. *Emerg Infect Dis, 14*(11), 1774-1776. doi:10.3201/eid1411.071676

Gao, J., Xiao, S., Xiao, Y., Wang, X., Zhang, C., Zhao, Q., . . . Zhou, E. M. (2016). MYH9 is an Essential Factor for Porcine Reproductive and Respiratory Syndrome Virus Infection. *Sci Rep, 6*, 25120. doi:10.1038/srep25120

Guo, R., Katz, B. B., Tomich, J. M., Gallagher, T., & Fang, Y. (2016). Porcine Reproductive and Respiratory Syndrome Virus Utilizes Nanotubes for Intercellular Spread. *J Virol, 90*(10), 5163-5175. doi:10.1128/jvi.00036-16

Han, J., Wang, Y., & Faaberg, K. S. (2006). Complete genome analysis of RFLP 184 isolates of porcine reproductive and respiratory syndrome virus. *Virus Research, 122*(1-2), 175-182.

Han, J., Zhou, L., Ge, X., Guo, X., & Yang, H. (2017). Pathogenesis and control of the Chinese highly pathogenic porcine reproductive and respiratory syndrome virus. *Veterinary microbiology*.

Hill, H. (1990). *Overview and history of mystery swine disease (swine infertility/respiratory syndrome)*. Paper presented at the Proceedings of the mystery swine disease committee meeting.

Holtkamp, D. J., Kliebenstein, J. B., Neumann, E. J., Zimmerman, J. J., Rotto, H. F., Yoder, T. K., . . . Haley, C. A. (2013). Assessment of the economic impact of porcine reproductive and respiratory syndrome virus on United States pork producers. *Journal of Swine Health and Production, 21*(2), 72-84.

Hopper, S., White, M., & Twiddy, N. (1992). An outbreak of blue-eared pig disease (porcine reproductive and respiratory syndrome) in four pig herds in Great Britain. *The Veterinary Record, 131*(7), 140-144.
Hu, S. P., Zhang, Z., Liu, Y. G., Tian, Z. J., Wu, D. L., Cai, X. H., & He, X. J. (2013). Pathogenicity and distribution of highly pathogenic porcine reproductive and respiratory syndrome virus in pigs. *Transbound Emerg Dis, 60*(4), 351-359. doi:10.1111/j.1865-1682.2012.01354.x

Huang, C., Zhang, Q., Guo, X. K., Yu, Z. B., Xu, A. T., Tang, J., & Feng, W. H. (2014). Porcine reproductive and respiratory syndrome virus nonstructural protein 4 antagonizes beta interferon expression by targeting the NF-kappaB essential modulator. *J Virol, 88*(18), 10934-10945.

Iseki, H., Morozumi, T., Takagi, M., Kawashima, K., Shibahara, T., Uenishi, H., & Tsunemitsu, H. (2016). Genomic sequence and virulence evaluation of MN184A-like porcine reproductive and respiratory syndrome virus in Japan. *Microbiol Immunol, 60*(12), 824-834. doi:10.1111/1348-0421.12455

Johnson, C. R., Yu, W., & Murtaugh, M. P. (2007). Cross-reactive antibody responses to nsp1 and nsp2 of Porcine reproductive and respiratory syndrome virus. *J Gen Virol, 88*(Pt 4), 1184-1195.

Karniychuk, U. U., Geldhof, M., Vanhee, M., Van Doorsselaere, J., Saveleva, T. A., & Nauwynck, H. J. (2010). Pathogenesis and antigenic characterization of a new East European subtype 3 porcine reproductive and respiratory syndrome virus isolate. *BMC Vet Res, 6*, 30. doi:10.1186/1746-6148-6-30

Kuhn, J. H., Lauck, M., Bailey, A. L., Shchetinin, A. M., Vishnevskaya, T. V., Bào, Y., . . . Gillis, A. (2016). Reorganization and expansion of the nidoviral family Arteriviridae. *Archives of virology, 161*(3), 755-768.

Li, C., Zhuang, J., Wang, J., Han, L., Sun, Z., Xiao, Y., . . . Li, X. (2016). Outbreak Investigation of NADC30-Like PRRSV in South-East China. *Transboundary and Emerging Diseases, 63*(5), 474-479.

Li, C., Zhuang, J., Wang, J., Han, L., Sun, Z., Xiao, Y., . . . Tian, K. (2016). Outbreak Investigation of NADC30-Like PRRSV in South-East China. *Transbound Emerg Dis, 63*(5), 474-479.

Li, X., Bao, H., Wang, Y., & Tian, K. (2016). Widespread of NADC30-like PRRSV in China: Another Pandora's box for Chinese pig industry as the outbreak of highly pathogenic PRRSV in 2006? *Infection, genetics and evolution: journal of molecular epidemiology and evolutionary genetics in infectious diseases, 49*, 12.

Li, X., Wu, J., Tan, F., Li, Y., Ji, G., Zhuang, J., . . . Tian, K. (2016). Genome characterization of two NADC30-like porcine reproductive and respiratory syndrome viruses in China. *SpringerPlus, 5*(1), 1677.

Li, Y., Shang, P., Shyu, D., Carrillo, C., Naraghi-Arani, P., Jaing, C. J., . . . Fang, Y. (2018). Nonstructural proteins nsp2TF and nsp2N of porcine reproductive and respiratory syndrome virus (PRRSV) play important roles in suppressing host innate immune responses. *Virology, 517*, 164-176.

Li, Y., Tas, A., Snijder, E. J., & Fang, Y. (2012). Identification of porcine reproductive and respiratory syndrome virus ORF1a-encoded non-structural proteins in virus-infected cells. *Journal of General Virology, 93*(4), 829-839.

Li, Y., Treffers, E. E., Napthine, S., Tas, A., Zhu, L., Sun, Z., . . . Fang, Y. (2014). Transactivation of programmed ribosomal frameshifting by a viral protein. *Proc Natl Acad Sci U S A, 111*(21), E2172-2181.

Li, Y., Zhu, L., Lawson, S. R., & Fang, Y. (2013). Targeted mutations in a highly conserved motif of the nsp1β protein impair the interferon antagonizing activity of porcine reproductive and respiratory syndrome virus. *Journal of General Virology, 94*(9), 1972-1983.

Liu, J.-k., Zhou, X., Zhai, J.-q., Li, B., Wei, C.-h., Dai, A.-l., . . . Luo, M.-l. (2017). Emergence of a novel highly pathogenic porcine reproductive and respiratory syndrome virus in China. *Transboundary and Emerging Diseases*.

Lopez, O. J., & Osorio, F. A. (2004). Role of neutralizing antibodies in PRRSV protective immunity. *Vet Immunol Immunopathol, 102*(3), 155-163. doi:10.1016/j.vetimm.2004.09.005

Lunney, J. K., Fang, Y., Ladinig, A., Chen, N., Li, Y., Rowland, B., & Renukaradhya, G. J. (2016). Porcine Reproductive and Respiratory Syndrome Virus (PRRSV): Pathogenesis and Interaction with the Immune System. *Annu Rev Anim Biosci, 4*, 129-154. doi:10.1146/annurev-animal-022114-111025

Murtaugh, M. P., Stadejek, T., Abrahante, J. E., Lam, T. T., & Leung, F. C.-C. (2010). The ever-expanding diversity of porcine reproductive and respiratory syndrome virus. *Virus Research, 154*(1), 18-30.

Murtaugh, M. P., Xiao, Z., & Zuckermann, F. (2002). Immunological responses of swine to porcine reproductive and respiratory syndrome virus infection. *Viral Immunol, 15*(4), 533-547.

Nauwynck, H. J., Duan, X., Favoreel, H. W., Van Oostveldt, P., & Pensaert, M. B. (1999). Entry of porcine reproductive and respiratory syndrome virus into porcine alveolar macrophages via receptor-mediated endocytosis. *J Gen Virol, 80 (Pt 2)*, 297-305. doi:10.1099/0022-1317-80-2-297

Ostrowski, M., Galeota, J. A., Jar, A. M., Platt, K. B., Osorio, F. A., & Lopez, O. J. (2002). Identification of neutralizing and nonneutralizing epitopes in the porcine reproductive and respiratory syndrome virus GP5 ectodomain. *J Virol, 76*(9), 4241-4250.

Plana, J., Vayreda, M., Vilarrasa, J., Bastons, M., Rosell, R., Martinez, M., . . . Ramos, J. A. (1992). Porcine epidemic abortion and respiratory syndrome (mystery swine disease). Isolation in Spain of the causative agent and experimental reproduction of the disease. *Veterinary microbiology, 33*(1-4), 203-211.

Prather, R. S., Rowland, R. R., Ewen, C., Trible, B., Kerrigan, M., Bawa, B., . . . Green, J. A. (2013). An intact sialoadhesin (Sn/SIGLEC1/CD169) is not required for attachment/internalization of the porcine reproductive and respiratory syndrome virus. *J Virol, 87*(17), 9538-9546. doi:10.1128/jvi.00177-13

Ropp, S. L., Wees, C. E. M., Fang, Y., Nelson, E. A., Rossow, K. D., Bien, M., . . . Christopher-Hennings, J. (2004). Characterization of emerging European-like porcine reproductive and respiratory syndrome virus isolates in the United States. *Journal of Virology, 78*(7), 3684-3703.

Rossow, K. D. (1998). Porcine reproductive and respiratory syndrome. *Vet Pathol, 35*(1), 1-20. doi:10.1177/030098589803500101

Shang, P., Misra, S., Hause, B., & Fang, Y. (2017). A naturally occurring recombinant enterovirus expresses a torovirus deubiquitinase. *Journal of Virology*, JVI. 00450-00417.

Shi, M., Holmes, E. C., Brar, M. S., & Leung, F. C.-C. (2013). Recombination is associated with an outbreak of novel highly pathogenic porcine reproductive and respiratory syndrome viruses in China. *Journal of Virology, 87*(19), 10904-10907.
Shi, M., Lam, T. T.-Y., Hon, C.-C., Hui, R. K.-H., Faaberg, K. S., Wennblom, T., . . . Leung, F. C.-C. (2010). Molecular epidemiology of PRRSV: a phylogenetic perspective. *Virus Research, 154*(1), 7-17.

Shi, M., Lam, T. T.-Y., Hon, C.-C., Murtaugh, M. P., Davies, P. R., Hui, R. K.-H., . . . Jiang, J.-W.

(2010). Phylogeny-based evolutionary, demographical, and geographical dissection of North American type 2 porcine reproductive and respiratory syndrome viruses. *Journal of Virology, 84*(17), 8700-8711.

Snijder, E. J., Kikkert, M., & Fang, Y. (2013). Arterivirus molecular biology and pathogenesis. *J Gen Virol, 94*(Pt 10), 2141-2163. doi:10.1099/vir.0.056341-0

Snijder, E. J., Kikkert, M., & Fang, Y. (2013). Arterivirus molecular biology and pathogenesis. *Journal of General Virology, 94*(10), 2141-2163.

Snijder, E. J., & Meulenberg, J. J. (1998). The molecular biology of arteriviruses. *J Gen Virol, 79 (Pt 5)*, 961-979. doi:10.1099/0022-1317-79-5-961

Spilman, M. S., Welbon, C., Nelson, E., & Dokland, T. (2009). Cryo-electron tomography of porcine reproductive and respiratory syndrome virus: organization of the nucleocapsid. *J Gen Virol, 90*(Pt 3), 527-535. doi:10.1099/vir.0.007674-0

Tian, K., Yu, X., Zhao, T., Feng, Y., Cao, Z., Wang, C., . . . Tian, X. (2007). Emergence of fatal PRRSV variants: unparalleled outbreaks of atypical PRRS in China and molecular dissection of the unique hallmark. *PLoS One, 2*(6), e526.

Tian, K., Yu, X., Zhao, T., Feng, Y., Cao, Z., Wang, C., . . . Gao, G. F. (2007). Emergence of fatal PRRSV variants: unparalleled outbreaks of atypical PRRS in China and molecular dissection of the unique hallmark. *PLoS One, 2*(6), e526. doi:10.1371/journal.pone.0000526

van Geelen, A. G. M., Anderson, T. K., Lager, K. M., Das, P. B., Otis, N. J., Montiel, N. A., . . . Faaberg, K. S. (2018). Porcine reproductive and respiratory disease virus: Evolution and recombination yields distinct ORF5 RFLP 1-7-4 viruses with individual pathogenicity. *Virology, 513*, 168-179.

Van Reeth, K., Labarque, G., Nauwynck, H., & Pensaert, M. (1999). Differential production of proinflammatory cytokines in the pig lung during different respiratory virus infections: correlations with pathogenicity. *Res Vet Sci, 67*(1), 47-52. doi:10.1053/rvsc.1998.0277

Wang, L., & Zhang, Y. (2015). Novel porcine reproductive and respiratory syndrome virus strains in the United States with deletions in untranslated regions. *Archives of virology, 160*(12), 3093-3096.

Wang, L.-j., Xie, W., Chen, X.-x., Qiao, S., Zhao, M., Gu, Y., . . . Zhang, G. (2017). Molecular epidemiology of porcine reproductive and respiratory syndrome virus in Central China since 2014: The prevalence of NADC30-like PRRSVs. *Microbial Pathogenesis, 109*, 20-28.

Wang, L. J., Xie, W., Chen, X. X., Qiao, S., Zhao, M., Gu, Y., . . . Zhang, G. (2017). Molecular epidemiology of porcine reproductive and respiratory syndrome virus in Central China since 2014: The prevalence of NADC30-like PRRSVs. *Microb Pathog, 109*, 20-28. doi:10.1016/j.micpath.2017.05.021
Welch, S. K., & Calvert, J. G. (2010). A brief review of CD163 and its role in PRRSV infection. *Virus Res, 154*(1-2), 98-103. doi:10.1016/j.virusres.2010.07.018

Wensvoort, G., Terpstra, C., Pol, J., Ter Laak, E., Bloemraad, M., De Kluyver, E., . . . Wagenaar, F. (1991). Mystery swine disease in The Netherlands: the isolation of Lelystad virus. *Veterinary Quarterly, 13*(3), 121-130.

Wensvoort, G., Terpstra, C., Pol, J. M., ter Laak, E. A., Bloemraad, M., de Kluyver, E. P., . . . et al. (1991). Mystery swine disease in The Netherlands: the isolation of Lelystad virus. *Vet Q, 13*(3), 121-130. doi:10.1080/01652176.1991.9694296

Wesley, R. D., Mengeling, W. L., Lager, K. M., Clouser, D. F., Landgraf, J. G., & Frey, M. L. (1998). Differentiation of a porcine reproductive and respiratory syndrome virus vaccine strain from North American field strains by restriction fragment length polymorphism analysis of ORF 5. *Journal of Veterinary Diagnostic Investigation, 10*(2), 140-144.

Workman, A. M., Smith, T. P., Osorio, F. A., & Vu, H. L. (2016a). Complete genome sequence of highly virulent porcine reproductive and respiratory syndrome virus variants that recently emerged in the United States. *Genome Announc., 4*(4), e00772-00716.
Workman, A. M., Smith, T. P., Osorio, F. A., & Vu, H. L. (2016). Complete Genome Sequence of Highly Virulent Porcine Reproductive and Respiratory Syndrome Virus Variants That Recently Emerged in the United States. *Genome Announc, 4*(4). doi:10.1128/genomeA.00772-16

Workman, A. M., Smith, T. P., Osorio, F. A., & Vu, H. L. (2016b). Complete genome sequence of highly virulent porcine reproductive and respiratory syndrome virus variants that recently emerged in the United States. *Genome announcements, 4*(4), e00772-00716.

Zhang, Q., & Yoo, D. (2015). PRRS virus receptors and their role for pathogenesis. *Vet Microbiol, 177*