APPLICATION OF HIGH-THROUGHPUT SEQUENCING FOR THE ANALYSES OF PRRSV-HOST INTERACTIONS

by

NANHUA CHEN

D.V.M., Fujian Agriculture and Forestry University, Fuzhou, China, 2006;
M.S., China Agricultural University, Beijing, China, 2009.

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine and Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2014

Abstract

Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) is the most costly virus to the swine industry, worldwide. This study explored the application of deep sequencing techniques to understand better the virus-host interaction. On the virus side, PRRSV exists as a quasispecies. The first application of deep sequencing was to investigate amino acid substitutions in hypervariable regions during acute infection and after virus rebound. The appearance and disappearance of mutations, especially the generation of a new N-glycosylation site in GP5, indicated they are likely the result of immune selection. The second application of deep sequencing was to investigate the quasispecies makeup in pigs with severe combined immunodeficiency (SCID) that lack B and T cells. The results showed the same pattern of amino acid substitutions in SCID and normal littermates and no different mutations were identified between SCID and normal littermates. This suggests the mutations that appear during the early stages of infection are the product of the virus becoming adapted to replication in pigs. The third application of deep sequencing was to investigate the locations of recombination events between GFP-expressing PRRSV infectious clones. The results identified different cross-over occurred within three conserved regions between EGFP and GFPm genes. And finally, the fourth goal was applied to develop a set of sequencing tools for analyzing the host antibody repertoire. A simple method was developed to amplify swine VDJ repertoires. Shared and abundant VDJ sequences that are likely expressed by PRRSV-activated B cells were determined in pigs that had different neutralization activities. These sequences are potentially correlated with different antibody responses.

APPLICATION OF HIGH-THROUGHPUT SEQUENCING FOR THE ANALYSES OF PRRSV-HOST INTERACTIONS

by

NANHUA CHEN

D.V.M., Fujian Agriculture and Forestry University, Fuzhou, China, 2006;
M.S., China Agricultural University, Beijing, China, 2009.

A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine and Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2014

Approved by:

Major Professor
Dr. Raymond R. R. Rowland

Copyright

NANHUA CHEN

2014

Abstract

Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) is the most costly virus to the swine industry, worldwide. This study explored the application of deep sequencing techniques to understand better the virus-host interaction. On the virus side, PRRSV exists as a quasispecies. The first application of deep sequencing was to investigate amino acid substitutions in hypervariable regions during acute infection and after virus rebound. The appearance and disappearance of mutations, especially the generation of a new N-glycosylation site in GP5, indicated they are likely the result of immune selection. The second application of deep sequencing was to investigate the quasispecies makeup in pigs with severe combined immunodeficiency (SCID) that lack B and T cells. The results showed the same pattern of amino acid substitutions in SCID and normal littermates and no different mutations were identified between SCID and normal littermates. This suggests the mutations that appear during the early stages of infection are the product of the virus becoming adapted to replication in pigs. The third application of deep sequencing was to investigate the locations of recombination events between GFP-expressing PRRSV infectious clones. The results identified different cross-over occurred within three conserved regions between EGFP and GFPm genes. And finally, the fourth goal was applied to develop a set of sequencing tools for analyzing the host antibody repertoire. A simple method was developed to amplify swine VDJ repertoires. Shared and abundant VDJ sequences that are likely expressed by PRRSV-activated B cells were determined in pigs that had different neutralization activities. These sequences are potentially correlated with different antibody responses.

Table of Contents

List of Figures ix
List of Tables x
Acknowledgements xi
Chapter 1 - Introduction: PRRSV infection, antibody repertoire and deep sequencing 1
1.1. PRRSV and PRRSV replication 1
1.1.1 PRRSV genome 1
1.1.2 PRRSV replication cycle 3
1.2. PRRSV origin, divergence and subtyping 4
1.2.1 The origin of PRRSV 5
1.2.2 The divergence time of PRRSV 5
1.2.3 The subtyping of PRRSV 6
1.3 Mechanisms of PRRSV diversity 7
1.3.1 Mutation 7
1.3.2 Recombination 8
1.4 PRRSV-host interactions 9
1.4.1 Protective immune responses 9
1.4.1.1 Innate immune response to PRRSV 9
1.4.1.2 Humoral immune response to PRRSV 10
1.4.1.3 Cellular immune response to PRRSV 11
1.4.2 Mechanisms of immune evasion 12
1.5. Control and elimination of PRRSV infection 13
1.5.1 Current PRRSV vaccines 13
1.5.2 Next-generation PRRSV vaccines 14
1.6. Swine antibody repertoire 15
1.7. Next-generation sequencing 16
Chapter 2 - Analysis of mutations within hypervariable regions of the PRRSV genome during acute infection and rebound 34
2.1 Introduction 35

2.2 Materials and Methods ... 35
2.2.1 Sample sources ... 35
2.2.2 Library preparation and 454 sequencing ... 36
2.3 Results ... 36
2.3.1 PRRSV rebound ... 36
2.3.2 Mutations identified by deep sequencing ... 37
2.4 Discussion ... 38
Chapter 3 - PRRSV replication and quasispecies evolution in pigs that lack adaptive immunity 48
3.1 Introduction ... 49
3.2 Materials and Methods ... 49
3.2.1 Animals and viruses ... 49
3.2.2 PRRSV real-time RT-PCR ... 50
3.2.3 Fluorescent microsphere immunoassay (FMIA) ... 50
3.2.4 Preparation of the amplicon library and deep sequencing ... 51
3.3 Results ... 52
3.3.1 PRRSV viremia in normal and SCID pigs ... 52
3.3.2 PRRSV-specific IgM and IgG responses ... 52
3.3.3 Nucleotide and amino acid substitutions at 11 and 21 dpi ... 53
3.4 Discussion ... 54
Chapter 4 - Identification of recombination patterns between two PRRSV infectious clones ... 62
4.1 Introduction ... 63
4.2 Materials and Methods ... 63
4.2.1 Sample preparation ... 63
4.2.2 Library preparation and deep sequencing ... 64
4.3 Results and Discussion ... 64
4.3.1 Identification of cross-over events ... 64
4.3.2 Potential cross-over patterns ... 65
Chapter 5 - Analysis of B-cell repertoire related to PRRSV neutralization ... 73
5.1. Introduction ... 74
5.2. Materials and Methods ... 75
5.2.1 Primer design and sample selection ... 75

5.2.2 Cloning and sequencing 75
5.2.3 Phylogenetic analyses 76
5.2.4 Selection of lineages potentially associated with different antibody responses 76
5.3. Results 77
5.3.1 Analysis of heavy chain sequences 77
5.3.2 Identification of an activated B-cell compartment 77
5.4. Discussion 78
References 90

List of Figures

Figure 1.1 Schematic representation of a PRRSV particle. 18

Figure 1.2 PRRSV genome organization 19

Figure 1.3 Overview of PRRSV life cycle in macrophages. 21

Figure 1.4 The phylogenetic tree of *Nidoviruses*. 22

Figure 1.5 PRRSV isolates show high genetic diversity. 24

Figure 1.6 Copy-choice recombination. 25

Figure 1.7 Virus replication and adaptive immune responses against PRRSV. 26

Figure 1.8 Clonal expansion theory. 27

Figure 1.9 454 sequencing system. 29

Figure 2.1 Raw phenotypic data of log-transformed viremia in pigs infected with PRRSV 40

Figure 2.2 Location of regions on the PRRSV genome selected for 454 sequencing 41

Figure 2.3 The nine amplicons from the parental virus NVSL 97-7895. 42

Figure 2.4 The generation of a new N-glycosylation site in GP5 43

Figure 3.1 PCR amplification of the structural protein regions of PRRSV. 56

Figure 3.2 PRRSV viremia for SCID pigs and normal littermates. 57

Figure 3.3 PRRSV-specific IgM and IgG responses in normal and SCID pigs. 58

Figure 3.4 Location of mutations in the structural genes of PRRSV 59

Figure 4.1 Recombination event occurred between two infectious clones. 67

Figure 4.2 Evidence for the occurrence of cross-over events. 68

Figure 4.3 Four potential cross-over patterns for the recombination events. 69

Figure 4.4 The alignment analyses identified two cross-over events. 70

Figure 5.1 The variation of swine VDJ genes is concentrated in the CDRs. 80

Figure 5.2 Alignment of 25 types of swine Ig V_H genes. 81

Figure 5.3 Percentages of major V_H genes comprising adaptive antibody repertoires. 82

Figure 5.4 Phylogenetic analysis of swine VDJ sequences from four pigs. 83

Figure 5.5 Experimental setup and selection criteria. 84

Figure 5.6 Alignment of abundant and shared sequences likely expressed by activated B-cells. 85

List of Tables

Table 1.1 Characteristics and functions of PRRSV nonstructural and structural proteins. 30

Table 1.2 Potential immune evasion strategies utilized by PRRSV. 31

Table 1.3 Commercial PRRS vaccines. 32

Table 1.4 Comparison of commercial sequencing methods. 33

Table 2.1 Primers used in this study. 44

Table 2.2 Total number of substitutions for all four animals. 45

Table 2.3 Mutations identified in serum and tonsil samples collected at 42dpi. 46

Table 2.4 Substitutions that are either increasing or decreasing in frequency during infection. .. 47

Table 3.1 Primers used in this study. 60

Table 3.2 Amino acid substitutions in the structural proteins at 11 and 21 dpi. 61

Table 4.1 Primers used in this study. 71

Table 4.2 Mutations and their percentages in the co-infected sample. 72

Table 5.1 Swine Ig heavy chain VDJ region mRNAs used in this study. 86

Table 5.2 Virus neutralization properties of sera from the pigs used in this study. 87

Table 5.3 Sequences with identical or high similar CDR3 from four pigs. 88

Acknowledgements

I would like to thank my major professor Dr. Bob Rowland for his insight, encyclopedic knowledge, and patience. Thanks to him for inspiring me to think hard, for encouraging me to be an independent researcher, and for giving me the opportunity to participate in several exciting projects. Thanks also to my committee members, Dr. Richard Hesse, Dr. Wenjun Ma, Dr. Stefan Rothenburg, and Dr. Ying Fang for their time and helpful guidance. I would like to thank Dr. Rollie Clem for serving as the outside chair of my program committee. I further thank all the members of Rowland research group and PHGC study team, especially Maureen Kerrigan, Becky Eaves and Ben Trible, for their advice and help. Special thanks to Mal Hoover for helping me prepare the figures in this dissertation. Moreover, I would like to appreciate all my friends in Manhattan, Kansas, who helped me enjoy my time here. I thank Dr. Kegong Tian from OIE PRRS Reference Laboratory, for enlightening me and giving me the first glance at PRRSV research. Finally, I thank my father Kunyin Chen, my mother Zengjin Wang, and my sisters Xiuzhu Chen, Xiufang Chen, Xuefang Chen in China, for their everlasting love and support.

Chapter 1 - Introduction: PRRSV infection, antibody repertoire and deep sequencing

This chapter gives the relevant background for this dissertation, including a review of PRRSV, PRRSV genomics, PRRSV replication cycle, the origin and evolution of PRRSV, mechanisms of PRRSV genetic diversity, PRRSV-host interactions, control and elimination strategies of PRRS, and also an introduction of swine antibody repertoire and next-generation sequencing.

1.1. PRRSV and PRRSV replication

Porcine reproductive and respiratory syndrome (PRRS) is the most economically important swine disease worldwide. In the United States only, the estimated cost of PRRS losses in national breeding and growing pig herds is at $664 million annually. The additional costs attributed to PRRS for veterinary, biosecurity and other outbreak related costs is estimated to be $477.79 million annually (98). PRRS is characterized with either severe reproductive failure in pregnant sows, or respiratory distress in neonatal pigs (107). Some common clinical signs include fever, anorexia, cough, asthma, diarrhea, and blue discoloration of the ears (182). In addition, the disease has been complicated by the patterns of persistent infection and subclinical infection as well as being a co-factor for porcine respiratory disease complex (PRDC) (222). Furthermore, the emergence of highly pathogenic PRRS (HP-PRRS) in China and several neighboring countries from 2006 has posed a significantly threat to the global swine industry (5, 57, 226).

1.1.1 PRRSV genome

The etiological agent of PRRS is PRRS virus (PRRSV). PRRSV is an enveloped, single-strand positive-sense RNA virus (Fig. 1.1) belonging to the family of *Arteriviridae* in the order of *Nidovirales* (202). Within the *Arteriviridae* family, PRRSV, equine arteritis virus (EAV), lactate dehydrogenase-elevating virus of mice (LDV), and simian hemorrhagic fever virus (SHFV), form the only genus *Arterivirus* (46). PRRSV genome is about 15kb in length, containing a 5’ cap structure, 5’ untranslated region (UTR), at least 10 open reading frames (ORFs), 3’ UTR and a 3’ poly (A) tail (Fig. 1.2).

The viral genome RNA (mRNA1) is utilized for the translation of ORF1a and ORF1b. Translation of ORF1a yields the pp1, a replicase polyprotein, whereas ORF1b is expressed through -1 programmed ribosomal frameshifting (PRF) to extend pp1a into pp1ab (72). A short transframe ORF (TF) has been identified to be translated via -2 PRF to yield a transframe protein, nsp2TF (85). Both -1 and -2 PRF are transactivated by nsp1β (126). The pp1a and pp1ab polyproteins are processed and cleaved by four viral proteases (nsp1α, nsp1β, nsp2 and nsp4) to release 14 non-structural proteins (nsps). Ten of them (nsp1-8, including nsp1α/1β and nsp7α/7β) are encoded in ORF1a and the other four (nsp9-12) are encoded in ORF1b (77). Several proteolytic cleavage products of pp1a are important for post-translational processing of replicase polyproteins, whereas nsp9 to nsp12 are involved in viral genome transcription and virus replication (158).

Most of the nsps, if not all, assemble into a replication and transcription complex (RTC) accumulating at the virus-induced endoplasmic reticulum (ER)-derived double-membrane vesicles (DMVs). The RTC directs both genome amplification and subgenomic (sg) mRNAs synthesis. The transmembrane (TM) domains in nsp2, nsp3 and nsp5 appear to play a role in the formation of the membrane-bound RTC and in recruiting other viral components of RTC that lack membrane-spanning domains (231). RNA-dependent RNA polymerase (RdRp) in nsp9 and RNA helicase in nsp10 are two core enzymes for viral RNA synthesis (84). Intriguingly, nsp9 and nsp10 may also contain virulence determinants of HP-PRRSV (127). Nsp11 contains the uridylate-specific endoribonuclease (NendoU), which is a unique genetic marker of nidoviruses (158, 255). The NendoU activity may be associated with the inhibition of interferon (IFN) responses (250), but the exact function of NendoU and other nsps are still unclear.

PRRSV structural proteins are translated from a nested set of six major sg mRNAs. All sg mRNAs are both 5'- and 3'- coterminal with the genomic RNA, containing a common short "leader" sequence corresponding to the 5'-proximal region of the genome fused to different "body" segments that are co-linear with its 3'-proximal region. The leader-body joining relies on the mechanism of discontinuous RNA synthesis (202). PRRSV sg mRNAs are structurally polycistronic but functionally monocistronic with two exceptions that are functionally bicistronic GP2a/E and GP5/ORF5a (87, 103, 204). The six sg mRNAs are translated to produce eight viral structural proteins (GP2a, E, GP3, GP4, GP5, ORF5a, M and N) to constitute an infectious virion.

Three N-glycosylated minor envelope proteins (GP2a, GP3 and GP4) are presented in virions as disulphide-linked GP2a-GP3-GP4 heterotrimers. The non-glycosylated minor envelope protein E is associated with GP2a-GP3-GP4 heterotrimers (68). In addition, E protein is likely an ion channel protein embedded in the viral membrane envelope and may facilitate uncoating of the virion and releasing of the viral genome into the cytoplasm (123). GP3 is heavily glycosylated and can be an integral membrane protein or a non-virion-associated soluble protein (69, 138). GP4 is not only a part of the GP2a-GP3-GP4 heterotrimer, but also interacts with the major glycoprotein GP5 (68). Major envelope proteins GP5 and M form a disulfide-linked heterodimer in the virion (139). The small hydrophobic protein ORF5a is also an enveloped protein and essential for PRRSV viability (213). Nucleocapsid (N) protein is the sole component of PRRSV capsid, which plays dual roles during PRRSV infection: a virion structural function and a non-structural role in the nucleus/nucleolus (183). The GP5-M heterodimer may interact with host receptor proteins heparan sulfate and sialoadhesin (Sn/SIGLEC1/CD169) (71), while the GP2a-GP3-GP4 heterotrimer interacts with the scavenger receptor CD163 (68), resulting in successful PRRSV infection of macrophages (173). The characteristics and functions of PRRSV non-structural and structural proteins are shown in table 1.1.

1.1.2 PRRSV replication cycle

Macrophages are the primary target cells of PRRSV. The life cycle of PRRSV in macrophages includes the following steps: virus attachment and internalization, genome translation and replication, post-translational processing of the replicase, formation of replication complex, subgenomic mRNAs synthesis and translation, virions assembly and release (Fig. 1.3).

During PRRSV entry into the macrophage, the first host receptor CD169 may interact with sialic acids on the GP5-M heterodimer (230), which promotes the attachment and internalization of the virion in the clathrin coated vesicle. The virion then enters porcine alveolar macrophages via the standard endocytotic route (203). However, the CD169 receptor is not required for the attachment and internalization of PRRSV (173). PRRSV can replicate in MARC-145 cells derived from African green monkey kidney cell line (MA104) (110), even though they do not have CD169, indicating the existence of other alternative receptors (104). The second receptor CD163 locates in the endosome compartment, binds to the GP2a-GP3-GP4 heterotrimer and participates in the uncoating of the virion (68, 225, 241). In low pH

microenvironment, the virion fuses with the endosome envelope and releases its genome into the cytoplasm.

Even though three proteins (nsp1α, nsp1β and N) can enter the nucleus/nucleolus (59, 183), the replication cycle of PRRSV is completed entirely in the cytoplasm (202). After uncoating of the virion, the viral genome is translated to produce two replicases pp1a and pp1ab, which comprise all functions required for viral RNA synthesis. During post-translational processing of the replicases, four viral proteases (in nsp1α, nsp1β, nsp2 and nsp4) cleave pp1a and pp1ab to produce 14 nsps, which assemble into a RTC. RTC accumulates at the virus-induced ER-derived DMVs, where viral RNA synthesis occurs. The continuous genome-length minus-strand RNAs (antigenomes) are generated first, then serve as the templates for the plus-strand genome synthesis (genome replication). In addition, the discontinuous sg-length minus-strand RNAs are also produced using a complex transcription mechanism, and serve as the templates for the plus-strand sg mRNAs synthesis (sg mRNAs transcription). Six sg mRNAs express eight structural proteins, including seven envelope proteins residing on the membranes of ER and Golgi complex and the N protein located nearby DMVs. After encapsidation of viral genome into N protein, the complex of genome and N protein becomes enveloped via budding into the lumen of the smooth ER and/or Golgi complex. Virions accumulate in intracellular vesicles to finish the maturation program, and then are transported to the plasma membrane for the release of progeny viruses through the exocytic pathway (203).

1.2. PRRSV origin, divergence and subtyping

PRRSV emerged almost simultaneously in the United States and in Europe in the late 1980s. It evolves at a higher evolutionary rate of 10^{-2}/site/year compared to other RNA viruses of 10^{-3} to 10^{-5}/site/year (96). Phylogenetic analysis of PRRSV isolates indicates the existence of two major genotypes: European PRRSV (genotype 1) and North American PRRSV (genotype 2) strains (149). Lelystad virus (LV) and ATCC VR-2332 strain are considered the prototype viruses for Type 1 and Type 2 PRRSV isolates, respectively (61, 242). The amino acid identity between these two types is less than 60% and the short time period between the appearance of PRRSV in two continents, strongly suggest that these two PRRSV genotypes evolved separately, possibly from non-disease-causing viruses, and are only distantly related to a common ancestor (203).

1.2.1 The origin of PRRSV

PRRSV specific antibody could be detected from swine herds in Ontario as early as 1979 (44). The first report of PRRSV infection was in 1987 (107). PRRSV was isolated almost concurrently in the United States and the Netherlands (61, 242). Currently, both genotypes of PRRSV strains have been isolated in North American, European and Asian countries (55, 83, 208). However, the origin of PRRSV is still a mystery. Several studies proposed the hypotheses for the origin of PRRSV genotypes (96, 170). Phylogenetic analysis showed that PRRSV is closest related to LDV (Fig. 1.4), suggesting that PRRSV might be derived from LDV (170). Since swine is the only known host of PRRSV, the origin of PRRSV may have coincided with a host-species jump from mice to swine (156, 198). A plausible hypothesis is that ancestral LDV-like PRRSV in rodents adapted to Eurasian wild boars, which served as intermediate hosts that brought the virus from rodents to domestic swine population (170). Even though the origin of PRRSV is extremely difficult, if not impossible, to identify due to the absence of retrospective data prior to the epidemic in late 1980s, it's possible to determine the origin of highly pathogenic PRRSV (HP-PRRSV). HP-PRRSV first emerged in China in 2006 (226), and has currently spread to several Asian countries (5). A phylogenetic study has shown that HP-PRRSV probably originated from CH-1a-like Chinese classical PRRSV isolates and that HB-1(sh)/2002, SHB-2005 and GD3-2005 viruses might serve as intermediate PRRSV isolates (6).

1.2.2 The divergence time of PRRSV

Even though type 1 and 2 PRRSVs emerged almost concurrently, they showed about 40% difference in amino acid sequences, which indicated they underwent pre-emergence evolution (197). They might have diverged not long before emergence but went through extremely high substitution rate. Another possibility is they might have diverged long before the emergence then evolved independently for a long time. During the estimation of the most recent common ancestor (MRCA) of all PRRSV isolates, Hanada et al (96) favored the recent divergence of these two genotypes. They estimated that the MRCA of PRRSVs is around 1982-1988 and the rate of PRRSV nucleotide substitution is $4.71\text{-}9.8\times10^{-2}$/site/year, which is the highest among RNA viruses so far reported. However, Forsberg et al (88) pointed out the problems in the study of Hanada et al such as using inappropriate methodology. They re-estimated the MRCA of Type 1 and 2 PRRSVs to be around 1880, which favored the early

divergence of the two types of PRRSV isolates. The scenario of early divergence is consistent with the origin hypothesis proposed by Plagemann, 2003 (170). In addition, the MRCAs of Type 1 and Type 2 PRRSVs have been placed at 1946-1967 and 1977-1981, respectively (198). The results further supported the early divergence because the MRCA of all PRRSV must be ahead of the MRCAs of each genotype.

1.2.3 The subtyping of PRRSV

Type 1 PRRSV was referred to as European type PRRSV, which was first recorded in early 1990s in Western Europe. The first PRRSV isolate, Lelystad virus, was isolated in Netherlands (242). However, Lelystad-like viruses were not likely the ancestors for most of the current Type 1 PRRSV isolates (197) (Fig. 1.5). The virus diversity for ORF7 was 12.0% in Eastern Europe while it was 5.8% in Western countries. The larger diversity indicated longer establishment of Type 1 PRRSV in Eastern Europe than in Western Europe, which supported the hypothesis that Type 1 PRRSV first emerged in Eastern Europe (206). In addition, Type 1 PRRSVs have been introduced to several countries outside of Europe, including the United States, Canada, Thailand, South Korea and China in the last decade (55, 73, 122, 181, 223). The coexistence of Type 1 and Type 2 PRRSV in the same countries, even in the same farms, contributes to PRRSV diversity and makes it more complicated to control PRRS. According to currently available sequence data, the highly diverged Type 1 PRRSV has been grouped into three subtypes based on the size polymorphism of ORF7, including a pan-Europe subtype I, a subtype II with samples from Belarus, Lithuania and Russia, and a subtype III mainly containing Belarus isolates. Most of The type 1 isolates outside of Europe belong to the pan-Europe subtype I. The nucleoprotein sizes of the three subtypes are 128, 125, and 124 amino acids, respectively (207).

Type 2 PRRSV is referred to as North American type because the United States reported the first outbreak of type 2 PRRSV infections and Canada detected the earliest PRRSV positive sera so far (44, 107). Compared to Type 1 PRRSV, Type 2 PRRSV is more international. Besides in North America, Type 2 PRRSVs were detected in several Asian and European countries, and became predominant in Asia. According to a recent study, Type 2 PRRSV isolates could be divided into nine lineages, including four major clusters and five small groups with at least 10% genetic distance in ORF5 between any two of the lineages (198). Besides the small

lineages 3 and 4 were only found in China and Japan, respectively. The other seven lineages could all be detected in North America. The major lineage 1, represented by MN184 (A-C), caused outbreaks in Minnesota in 2001 (95), which likely originated from Eastern Canada (197). The major lineage 5, represented by type 2 PRRSV prototype strain VR-2332 and Ingelvac PRRS modified live vaccine (MLV), is the most cosmopolitan cluster. The major lineages 8 and 9 are also widespread, causing the outbreaks of "acute PRRS" in United States in 1996 (94). In addition, the causative agent of highly pathogenic PRRS in China in 2006 was clustered in lineage 8 (198, 226). Remarkably, swine herds experienced outbreaks of PRRS even though they had been vaccinated with MLVs, including Ingelvac PRRS MLV (lineage 5) and Prime Pac PRRS vaccines (lineage 7) (94, 108, 226). The observations are consistent with the fact that PRRSV MLVs cannot provide sufficient cross-over protection.

1.3 Mechanisms of PRRSV diversity

Mutation and recombination are two evolutionary mechanisms responsible for the genomic diversity of PRRSV (92). High mutation rate is a common trait in RNA viruses due to the lack of proofreading activity of viral RNA polymerase (121). The error-prone replication and quick replication kinetics of PRRSV produce a mutational cloud of variants known as viral quasispecies (18, 91, 187). Recombination is another important genetic mechanism contributing to PRRSV diversity, which is a common phenomenon in the field (144). Both type 1 and type 2 PRRSV have been identified to undergo intra-type recombination (56, 83, 125, 128, 234, 254). The high diversity of PRRSV generated by mutation and recombination allows the viral population to rapidly adapt to dynamic environments and evolve resistance to vaccines (121). Although both mutation and recombination could occur frequently in the course of viral RNA genome replication, only a minority of viable mutants and recombinants could actually be detected in the progeny viruses because natural selection is continuously pruning away unfit mutants and recombinants (78).

1.3.1 Mutation

A mutation is a change of the nucleotide sequence in the viral genome, which is likely to be harmful, but few mutations are either neutral or beneficial. Mutations are from errors in the process of virus replication or unrepaired damage to viral RNA genome. PRRSV has a high mutation rate of $4.71\text{-}9.8\times10^{-2}$/site/year (96). Two main contributors to such a high mutation rate

are: 1) the virus RNA polymerases make mistakes per 10^3 to 10^5 nucleotides copied during RNA synthesis; and 2) they lack proofreading-repair ability (210). The high mutation rate of PRRSV produces a swarm of diverse variants (quasispecies) that interact cooperatively on the functional level and collectively contribute to the characteristics of the virus population. The fitness of the viral quasispecies is likely to be determined more by its freedom to mutate into related sequences than by its own replicative ability (121). Low replicative fidelity generates a diverse population of variants, which are generally more fit in a dynamic environment, while a homogeneous population, generated by high replicative fidelity, may lack the flexibility and be less successful in the dynamic host environment (78).

1.3.2 Recombination

Recombination of RNA viruses is the process to form chimeric molecules from parental genomes of mixed origin. The process is termed recombination if it occurs within a single genomic segment, and is referred to as reassortment in the viruses that possess segmented genomes (199). Recombination has been associated with the expansion of viral host range, increases in virulence, immune evasion and resistance to vaccines and antivirals (19, 109, 137, 199). The PRRSV genome is composed of a single segment. The most widely accepted model of PRRSV recombination is copy-choice recombination (Fig. 1.6). During the synthesis of negative strand viral RNA, the RNA-dependent RNA polymerase switches from the donor template to the acceptor template while remaining bound to the nascent nucleic acid chain, thereby generating an RNA molecule with mixed ancestry (1). The factors that influence template switching include the sequence similarity between RNA templates and secondary structure of the viral RNA (89, 256). According to the sequence similarity, RNA recombination can be divided into homologous and non-homologous types (120). Homologous recombination occurs most often between regions of high sequence identity and also must be present close to, although not necessarily at, the cross-over sites. Non-homologous recombination between different genomic regions or non-related RNA molecules that do not show any sequence homology may also occur, even though it's relatively infrequent. PRRSV could undergo homologous recombination with the frequency from <2% up to 10% *in vitro* and ~38% *in vivo*, whereas non-homologous recombination has not been detected (128, 234, 254). PRRSV recombination is a common phenomenon in the field and

may contribute to the outbreak of novel HP-PRRSV (144, 196); however, very few recombinants can obtain selective advantages to compete with the parental viruses and gain dominance (79).

1.4 PRRSV-host interactions

Exposure of pigs to PRRSV induces immunity that begins with an innate antiviral response principally involving the production of type I interferons (IFNs). PRRSV infection appears to elicit only a minimal interferon and cytokine responses (157, 189, 250). Innate immune cells such as activated macrophages and dendritic cells function in viral recognition, immune surveillance and antigen presentation, which directly bridge innate and adaptive immunity (189). The weak innate response seems to compromise the initiation and elaboration of PRRSV-specific adaptive immune responses (157). Although the humoral and cellular immune responses are vague and delayed, they still can clear the virus from circulation in due course, but not from lymphoid tissues, where the virus persists for 6 months or longer (4). Meanwhile, the virus modulates the host antiviral responses and develops several evasion strategies to survive and replicate in the host cells. Currently, at least four PRRSV proteins, including three nonstructural proteins nsp1, nsp2, nsp11, and the structural protein N, are known to function as the viral antagonists of host defenses (13, 59, 218, 219, 250).

1.4.1 Protective immune responses

Both innate and adaptive immune systems are involved in antiviral immunity. Innate immune response provides immediate frontline protection against viral infections. In addition, innate immune cells, including activated macrophages and DCs, are antigen-presenting cells, which are involved in the development of adaptive immunity and potentiate the adaptive immune system for viral clearance (97). Currently, the elements of protective immunity against PRRSV infection are still far from been fully elucidated. Here is a brief review of current knowledge about swine immune responses to PRRSV infection.

1.4.1.1 Innate immune response to PRRSV

NK cells are one of the most important components of innate antiviral immunity, which can be activated within hours of infection and result in cytotoxicity to virus-infected cells (14). However, NK cytotoxicity toward PRRSV-infected primary alveolar macrophages (PAMs) showed to be suppressed (43). Innate immunity to PRRSV begins in the cytoplasm of an infected

macrophage. The presence of viral dsRNA in infected cells triggers the induction of type I IFNs, such as IFN-α and IFN-β, which are hallmarks of cellular antiviral defense (157). Type I IFNs induce antiviral responses through a heteromeric receptor composed of two subunits, IFN-α/β receptor 1 (IFNAR1) and IFNAR2 (229). Type I IFNs can induce antiviral responses via both autocrine and paracrine mechanisms, resulting with inactivation of viruses and the limit of viral spreading (189). Infection of monocyte-derived DCs (mDCs) by PRRSV significantly suppresses type I IFN production of the IFN-α subtypes but not the IFN-β subtypes (135). This result is likely associated with the notion that IFN-α subtypes seem to play a more important role in anti-PRRSV innate immunity, while IFN-β subtypes display more immunomodulatory activity (39, 105). In addition, antimicrobial molecules, such as antimicrobial peptides (AMPs), are another major group of innate antiviral immune effectors (190). AMPs can exert antiviral activity by distortion of the virion glycoproteins and lipid membranes in enveloped viruses, and the blocking of virus entry into host cells (189). PRRSV infection generally decreases the production of AMPs, and suppresses the antimicrobial activities of both PAMs and NK cells (190). These findings suggest that intervening in the interaction between sugar moieties of the viral envelope proteins and host cells is a target for innate immune molecules to inhibit PRRSV infection (117). IL-10 is an immunosuppressive cytokine, which has shown to be either up-regulated or down-regulated by PRRSV infection. This indicates that PRRSV-regulation of IL-10 production may depends on both pig breeds and virus strains used in the studies (211, 224). Taken together, the initial innate immunity to PRRSV is weak, which is consistent with suboptimal stimulation of antigen-specific humoral and cellular immune responses (157).

1.4.1.2 Humoral immune response to PRRSV

Adaptive immunity against PRRSV has been extensively studied, particularly the development of neutralizing antibodies (nAbs) and cell-mediated immune (CMI) responses (Fig. 1.7). NAbs are now considered an important component of protective immunity against PRRSV (133). The onset of nAbs after experimental infection showed to be accompanied with the clearance of PRRSV from circulation (119). Since nAbs have the potential to clear free virus from circulation, it is presumed to play an important role in prevention or reduction of viral spread from animal to animal (252). A serum passive transfer experiment provided direct evidence that PRRSV nAbs alone could prevent transplacental infection by PRRSV, extinguish PRRSV infection in the pregnant sows, and provide sterilizing immunity *in vivo* (162). The

minimal neutralizing titer in recipient piglets that would fully protect animals against viremia upon challenge at nAb titers was 1:8, whereas sterilizing immunity could be attained at titers of 1:32 (132). Viral epitopes that induce nAbs appear to reside on the GP2a, GP3, GP4, GP5 and M proteins (40, 112, 249). Of these, the neutralizing epitope B (aa 37-44) in GP5 may be most relevant to protection (163, 171). Generally, nAbs appear only at 4 weeks post infection or later and maintain at low levels (119, 130, 133, 252). The observations that PRRSV induces low levels of nAbs and nAbs in sera and may not react with virions due to glycan shielding of the neutralizing epitopes provide indirect evidence for the significance of nAbs in providing protective immunity against PRRSV (114).

1.4.1.3 Cellular immune response to PRRSV

Cell-mediated immunity (CMI) is also extremely important for PRRSV protection. The different capacity of PRRSV strains to induce protective immunity depends on their abilities to induce CMI, involving IFN-γ secreting cells (IFN-γ-SCs) and probably IL-10 (75). IFN-γ-SCs are probably the main factors in protection against PRRSV infection and IL-10 may constrain the development of such IFN-γ-SCs (114). IFN-γ-SCs are mainly CD4+CD8+ cells, with a small proportion of CD4-/CD8αβ+ cytotoxic T cells (148). IFN-γ blocks PRRSV replication in macrophages by inhibiting viral RNA synthesis through a dsRNA inducible protein kinase (12, 186). However, the establishment of long-term persistent infection in the host suggests that CMI, including IFN-γ and IL-2 production, is not potent or effective in curtailing the infection (2, 245). This observation may be due to the weak and delayed CMI against PRRSV (157). PRRSV-specific lymphocyte responses began in peripheral blood at approximately 4 weeks post vaccination (wpv) and virus-specific IFN-γ-SCs became detectable between 4 and 12 wpv (114, 131, 147). T-cell proliferative responses were mainly directed against GP5, M, and N proteins (11). In a vaccinia virus system, M protein showed to be the most potent inducer of T-cell proliferation, followed by GP5, GP3, GP2 and N proteins (12). Intriguingly, two distinct regions of GP5 (Amino acid residues 117-131, LAALICFVIRLAKNC, and 149-163, KGRLYRWRSPVII/VEK) appeared to contain immunodominant T-cell epitopes based on their abilities to stimulate IFN-γ-SCs (235). PRRSV-specific IFN-γ-SCs were only 50-100/10^6 peripheral blood mononuclear cells (PBMCs) at 13 wpv (147). In contrast, virus-specific IFN-γ-SCs were 200-300/10^6 PBMCs at 3 wpv against Aujeszky's disease virus (148). However, the intensity of PRRSV-specific IFN-γ-SCs gradually increased to 400-500/10^6 PBMCs at 48 wpv,

whereas nAbs were barely detectable, indicating CMI might be necessary for the complete elimination of PRRSV at late phase (147, 157).

1.4.2 Mechanisms of immune evasion

The host immune system recognizes and responds to invading pathogens, while the pathogens have successfully evolved a range of immune evasion strategies to overcome both innate and adaptive immune responses. The anti-immune strategies that may be used by PRRSV are shown in table 1.2.

PRRSV can evade, minimize, or block innate antiviral immune responses through no surface expression of viral proteins in infected cells (63), interference with antigen presentation (239), suppression of NK cell-mediated cytotoxicity (43), down-regulation of antimicrobial molecules (190), and inhibition of type I IFN production via at least four viral proteins (nsp1, nsp2, nsp11, and N) (13, 111, 169, 219, 250). Nsp1α inhibits IFN production by degrading CREB-binding protein (CBP) in the nucleus and blocks NF-κB activation in the cytoplasm (111). Nsp1β antagonizes IRF3 activation (13). Nsp2 ovarian tumor (OTU) domain interferes with the NF-κB signal pathway (219). Nsp11 may be associated with the inhibition of IRF3 activation (250). Nuclear localization of nsp1α, nsp1β and N proteins may facilitate PRRSV persistence (59, 169).

PRRSV uses many strategies to evade antibody neutralization. First, the decoy epitope A (aa 27-31) at the upstream of neutralizing epitope B (aa 37-44) in GP5 induces a strong non-nAbs response rapidly after PRRSV exposure, which is consistent with the function of a decoy epitope and may cause the diminishment of immune response against the adjacent neutralizing epitope B (163). Second is the presence of N-linked glycosylation sites in GPs. Glycan shielding of neutralizing epitopes delays nAbs production and diminishes sensitivity of the virus to neutralization. Mutants without the glycan residues in GP5 induced significantly higher titers of nAbs (7). Third is antibody-dependent enhancement (ADE). The non-nAbs increase the association of viral particles with permissive macrophages via binding of virus-antibody complexes to the Fc receptor, thus enhancing virus attachment and internalization in macrophages (40, 251). Enhancement of viral entry into target cells contributes to PRRSV immune evasion and reduces virus neutralization efficacy (114). Fourth is the prevention of normal B-cell repertoire development. PRRSV infection causes biased expansion of a

subpopulation of the pre-immune repertoire with hydrophobic binding sites that normally disappear during antigen-driven repertoire diversification. The subversion of normal repertoire development may contribute to the delayed immune response to PRRSV (37). Fifth, the diversity of viral quasispecies results in incomplete protection against heterologous variations (187).

1.5. Control and elimination of PRRSV infection

Currently, several strategies have been used to control and eliminate PRRS. Control at the farm level is pursued through different management procedures, such as semen monitoring, gilt acclimation, and vaccination. Elimination methods include testing and animal removal, whole herd depopulation and repopulation, and herd closure and rollover (52, 62).

1.5.1 Current PRRSV vaccines

Vaccination is now considered the most effective method for PRRS prevention and control. Many vaccines have been developed to combat PRRSV infection all around the world, including modified live vaccines (MLVs), killed virus vaccines (KVs), and a subunit vaccine (Table 1.3).

PRRSV MLVs protect pigs from PRRSV-mediated reproductive and respiratory diseases. MLVs shorten viremia in gilts and reduce numbers of pre- and post-natal death and congenitally infected piglets (193). Utilization of MLVs in sows reduces abortion and return to oestrus, increases farrowing rates and number of weaning pigs (3). MLV immunization in growing pigs reduces viremia, respiratory signs, and improves growth performance (41, 42). Remarkably, MLV vaccination during acute PRRSV infection reduces virus shedding and respiratory disease (41, 42, 118). PRRSV KVs have less efficacy than MLVs. KVs fail to provide protection in naïve pigs, whereas they improve reproductive performance in PRRSV-infected pigs (166, 192).

There are still several concerns about current commercial PRRSV vaccines. PRRSV MLVs confer delayed protective immunity (260). The protection is genotype-specific and, to the most extent, strain-specific (142). In addition, MLV immunization may interfere with the protective efficacy of other vaccines (80). Another major concern about PRRSV MLVs is reversion to virulence (17). The revert-to-virulence vaccine virus can cause clinical diseases and affect growth performance (159). In addition, MLV vaccinated pigs develop viremia and spread the virus to other naïve animals (52). KVs are safe, but the non-nAbs induced by KVs may contribute to virus infectivity and immune evasion via ADE (40).

To summarize, PRRSV MLVs can provide satisfied protection against homologous or closely related viruses, whereas the efficacies of killed and subunit vaccines are limited or unknown. Currently, none of the vaccines can provide sufficient protection against heterologous PRRSV strains (155). A better vaccine is still urgently needed.

1.5.2 Next-generation PRRSV vaccines

The development of PRRSV next-generation vaccines is the topic of interest among PRRSV researchers. Numerous efforts have been made to develop an ideal PRRSV vaccine. An ideal vaccine against PRRSV should possess high immunogenicity, induce a high level of broad nAbs and specific CMI against PRRSV within a short period of time, establish memorial protection, and is safe (52, 99, 114, 154, 155).

Efforts have been reported to develop more effective PRRSV vaccines, including the use of several adjuvants (51, 53), use of multi-strain vaccines (151, 152), and the generation of alternative vaccines, such as mucosal vaccines (179), DNA vaccines (8), subunit vaccine (174), synthetic peptide vaccine (54), alphavirus-derived replicon (52), bacterial vector vaccine (10), insect cell-derived vaccine (172), plant-derived vaccine (58), recombinant DNA vector vaccines using adenovirus (102), PRV (175), poxvirus (195), vaccinia virus (257), and transmissible gastroenteritis virus (64) as vectors. However, none of these efforts confer significantly better protection when compared to PRRSV MLVs alone (52).

In the guide of the immunological principles for infectious diseases, current thinking about next-generation PRRSV vaccine development can be related to the induction of type I IFNs, the production of high titer of cross-protective nAbs, and the suppression of regulatory T cell activity (99). Several PRRSV non-structural proteins (nsp1, nsp2, nsp4 and nsp11) have been reported to suppress antiviral type I IFN responses (217, 250). Genetic manipulations of PRRSV by deletion of essential factors (nsps as deletion targets) using reverse genetics techniques may generate a suitable MLV candidate that can restore or enhance type I IFN responses. The identification of broad nAbs in our laboratory provides direct evidence for the existence of conserved broad neutralizing epitopes in PRRSV. Determination of the lineages of broad nAbs and the corresponding epitopes will tremendously contribute to the development of a cross-protective PRRSV vaccine. Inhibition of T cell activity during PRRSV persistent infection may be associated with CD25+Foxp3+ regulatory T cells (Tregs) (99). Suppression of Tregs

induction may enhance the efficacy of the next generation PRRSV MLVs. Further work is needed to identify the viral components associated with Tregs induction, and then mutated MLVs can be generated by reverse genetics to block Tregs induction.

Even though there are many knowledge gaps and challenges existing in PRRSV immunology and vaccinology, the recent completion of the porcine genome provides an important resource for further expansion of our molecular and genetic understanding of porcine immunology (93). Along with the increasing knowledge in the field of immune development and regulatory pathways in pigs, the generation of a broadly effective and safe PRRSV vaccine will become a more and more realistic goal.

1.6. Swine antibody repertoire

An antibody is made by the products of two genes, encoding heavy and light chains, which form a cleft for antigen binding. To elicit immune response to unlimited numbers of foreign antigens, the immune system must be able to recognize countless numbers of antigens. However, the unlimited numbers of unique antigen receptors are not genetically encoded. Rather, it is achieved by creating variation in the antigen-recognition regions. The mechanisms of generating variation in the antigen-binding pockets basically involve mixing and matching variable (V), diversity (D), and joining (J) gene segments in a process called V(D)J recombination (141). The heavy chain undergoes V-D-J rearrangement first, then both κ and λ light chain undergo V-J rearrangements. At the end of this process, each B cell contains only a single functional variable-region for its heavy chain and another for its light chain (κ or λ) to form a unique antigen receptor (115). In addition to V(D)J rearrangement, the diversity of the antibody repertoires can be further expanded by somatic hypermutation (SHM) and gene conversion (homologous recombination) (76).

Swine antibodies have about 30 V_H genes, 2 functional D_H genes, and 1 functional J_H gene encoding the heavy chain, and also 11 functional Vκ genes, 5 Jκ genes, 12-13 functional V_λ genes, and two functional J_λ genes encoding light chains (27, 34). For the heavy chain, swine utilize seven major V_H genes (V_HA, V_HB, V_HC, V_HE, V_HF, V_HY, V_HZ), two D_H segments and a single J_H gene to account for nearly the entire (>90%) VDJ pre-immune repertoire (34). The situation for light chains is less well studied, but there is little junctional diversity (small insertions and deletions), less SHM, and mutations are not concentrated in the complementarity

determining regions (CDRs) of light chains (35). There is a hypothesis that light chain functions allow specificity modification and rescue auto-reactive B cells through receptor editing (227).

When a foreign antigen enters the peripheral lymphoid system, any mature B cell displaying an antibody specific to that antigen will bind to it and be activated, then identical B cell clones and antigen-specific antibodies are produced through clonal expansion (Fig. 1.8) (20, 101). Intriguingly, the exposure to environmental antigen does not change the V_H genes that comprise the pre-immune repertoire. The same V_H genes comprise the adaptive repertoire but ~90% of them are somatically mutated (34). SHM of the seven V_H genes results in the diversification of the heavy chain variable regions. SHM is concentrated in heavy chain CDRs but lower and widely distributed in rearranged light chains (27). The antibody binding site is primarily determined by the heavy chain and specifically its CDR3, whereas the light chain may only play a supporting role and its presence primarily affects the conformation of the heavy chain binding site (34, 165). This unique feature provides an opportunity to analyze porcine antibody repertoire by detecting the entire VDJ repertoire.

1.7. Next-generation sequencing

In 1977, Dr. Sanger described a method for determining nucleotide sequences in DNA using dideoxynucleotide analogs as chain-terminating inhibitors of DNA polymerase (191), which became the gold standard for DNA sequencing. In 2005, the commercial launch of the first massively parallel pyrosequencing platform ushered in the new era of high throughput genetic analysis now referred to as next-generation sequencing (NGS) (236). Currently, 5 NGS platforms are commercially available, of which, Roche 454 GS FLX and Illumina MiSeq/HiSeq are most popularly used (129). The properties of these platforms are shown in table 1.4.

In this dissertation, we used 454 sequencing (Fig. 1.9), which included amplicon library preparation, emulsion PCR (emPCR), 454 sequencing and data analysis. The preparation of an amplicon library incorporated two rounds of PCR (65). The first round of PCR was performed using target sequence-specific primers. A second round of amplification was performed using 454-adaptor multiplex identifier (MID) primers. For unidirectional sequencing, MIDs were included only on forward primers for the second round of PCR. The annealing temperature was set at 60 °C for both first and second rounds of PCR. The cycles of PCR amplification were set at 35 cycles. The final concentrations of primers and DNA polymerase were 0.3 μM and

0.02U/μl, respectively. The other reaction components and thermocycling conditions were set according to the recommended protocols provided for the Phusion high fidelity DNA polymerase (New England Biolabs).

The second round amplicons were purified with Agencourt AMPure XP 5mL Kit (Beckman Coulter). The concentration of each amplicon was measured using a NanoDrop ND-8000 Spectrophotometer (Thermo Scientific). Products were converted to molecules/μl using the following formula:

$$\text{Molecules}/\mu l = [\text{sample conc. (ng}/\mu l) \times 6.022 \times 10^{23}]/[656 \times 10^{9} \times \text{amplicon length (bp)}]$$

We normalized each amplicon by dilution and mixed equal volume of each normalized amplicon to create a library including all samples. The amplicon library was diluted to the final concentration at 1×10^7 molecules/μl for each amplicon in 1 × TE buffer and stored at -20 °C.

The amplicon library was sent to the Department of Plant Pathology, Kansas State University for emPCR amplification and 454 sequencing. Lib-L emPCR Kit (Roche) was used for emPCR according to the emPCR Amplification Method Manual. For 454 sequencing, GS FLX Titanium Sequencing Kit XLR70 (Roche) was used following the protocol described in the Sequencing Method Manual. Sequencing was performed at GS FLX+ System and data acquisition, and processing and analysis were performed with 454 Sequencing System Software Version 2.6 packages on the GS FLX+ Instrument. Reads for each sample were sorted according to the MID. Sequence reads were mapped against reference sequences with 454 Life Sciences GS Reference Mapper (Version 2.6). Minimum overlap length was 40, and the minimum overlap identity was 90%. Coverage (number of reads per amplicon) was calculated and variants were called. Variants were further filtered based on the coverage, variant frequency, and homopolymer. Only high confidence single nucleotide variants that had the following features were selected: (1) at least 3 non-duplicate reads with the nucleotide substitution; (2) a substitution frequency greater than 5%; and (3) not located at homopolymer sites. All mutations were further confirmed by sequence assembly visualization using Tablet (153).

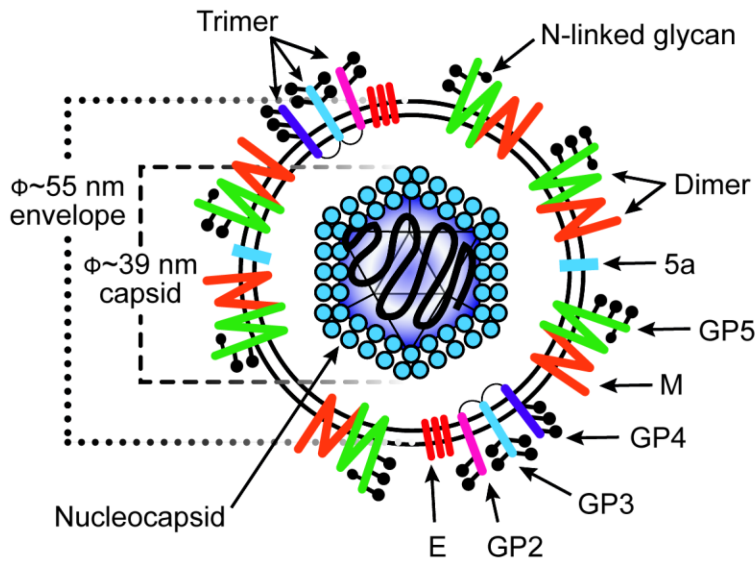


Figure 1.1 Schematic representation of a PRRSV particle.

The PRRSV particle is enveloped, spherical, and ~55 nm in diameter. The envelope proteins include GP2, E, GP3, GP4, GP5, 5a, and M. Minor glycoproteins GP2, GP3, GP4 form a disulfide-linked heterotrimer. Major glycoproteins GP5 and M form a disulfide-linked heterodimer. Glycoproteins have different numbers of N-linked glycosylation sites. The possibly icosahedral nucleocapsid core is ~39 nm in diameter. The ssRNA genome is associated with the N protein to form the nucleocapsid. The figure is modified based on previous studies (202, 203).

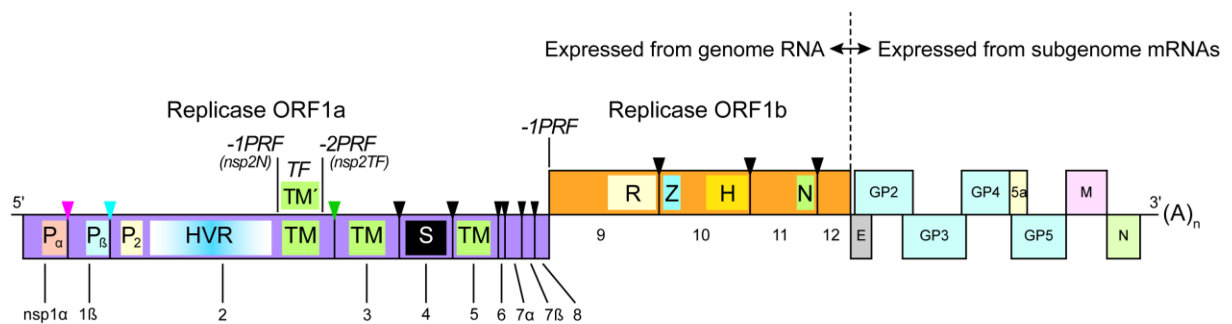


Figure 1.2 PRRSV genome organization

The replicase ORF1a and ORF1b are translated from viral genome RNA (mRNA1) and cleaved into 14 nonstructural proteins (nsps). ORF1b is expressed via -1 programmed ribosomal frameshifting (PRF) to extend pp1a into pp1ab. A short transframe (TF) ORF is expressed via -1/2 PRF to yield nsp2N and nsp2TF. Pα, Pβ and P2 are three proteinases residing in nsp1α, nsp1β and nsp2, whereas the main proteinase S is located in nsp4. The cleaved sites for Pα, Pβ and P2 are shown in pink, light blue and green triangles, respectively, while the cleaved sites for S are shown in black triangles. A hypervariable region (HVR) resides in nsp2. ORF1a encodes three transmembrane domains (TM). ORF1b encodes four highly conserved domains: RNA-dependent RNA polymerase (R), multinuclear zinc-binding domain (Z), RNA helicase (H) and NendoU endoribonuclease domain (N). ORFs 2-7 are expressed from six subgenome mRNAs encoding eight structural proteins, including minor envelope proteins (GP2a, GP3, GP4, E and ORF5a), major envelope proteins (GP5 and M) and the nucleocapsid protein (N).

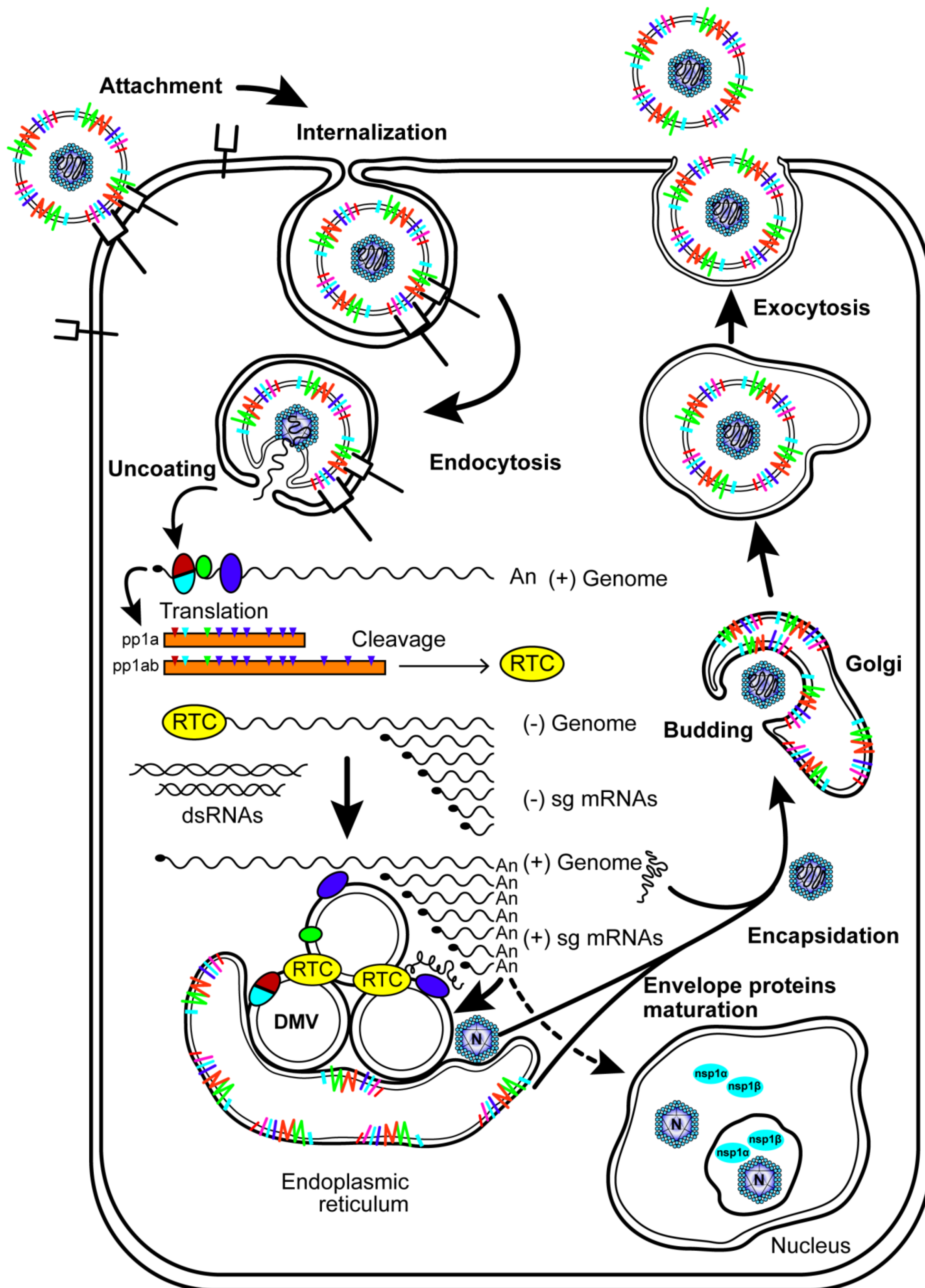

Attachment
Internalization
Endocytosis
Uncoating
An (+) Genome
Translation
pp1a
pp1ab
Cleavage
RTC
RTC
(-) Genome
dsRNAs
(-) sg mRNAs
An
(+) Genome
(+) sg mRNAs
RTC
RTC
DMV
N
Endoplasmic
reticulum
Exocytosis
Golgi
Budding
Encapsidation
Envelope proteins
maturation
nsp1α
nsp1β
N
nsp1β
nsp1α
N
Nucleus

Figure 1.3 Overview of PRRSV life cycle in macrophages.

PRRSV enters macrophages in a low pH microenvironment. The first host receptor CD169 may interact with sialic acid on the GP5-M heterodimer, which promotes the attachment and internalization of the virion in the clathrin coated vesicle. The virion subsequently enters the cell via the standard endocytotic route. The second receptor CD163 locates in the endosome compartment, binds to the GP2a-GP3-GP4 heterotrimer and participates in the uncoating of the virion. The virion fuses with the endosome envelope and releases its genome into the cytoplasm. The replication cycle of PRRSV is entirely completed in the cytoplasm. The viral genome is translated to produce two replicases pp1a and pp1ab, which comprises all functions required for viral RNA synthesis. Four viral proteases cleave pp1a and pp1ab to produce 14 nsps, which assemble into a replication and transcription complex (RTC). RTC accumulates at the virus-induced endoplasmic reticulum (ER)-derived double-membrane vesicles (DMVs), where viral RNA synthesis occurs. Both minus-strand genomic RNA and minus-strand sg RNAs are produced and serve as the templates for the synthesis of new genomic RNA and sg mRNAs. Viral dsRNA can also be produced, which triggers the induction of type I IFNs. Six sg mRNAs express eight structural proteins, including seven envelope proteins residing on the inner membranes of the ER and Golgi complex, and the nucleocapsid (N) protein encapsidates viral genome. Interestingly, N protein together with nsp1α and nsp1β can enter the nucleus/nucleolus, but the effects of the transportation are still unknown. The genome encapsidated N protein is enveloped by budding into the lumen of the smooth ER and/or Golgi complex. Virions accumulate in intracellular vesicles to finish the maturation program and then are transported to the plasma membrane for the release of progeny viruses via the exocytic pathway. This figure is modified based on previous studies (202, 203).

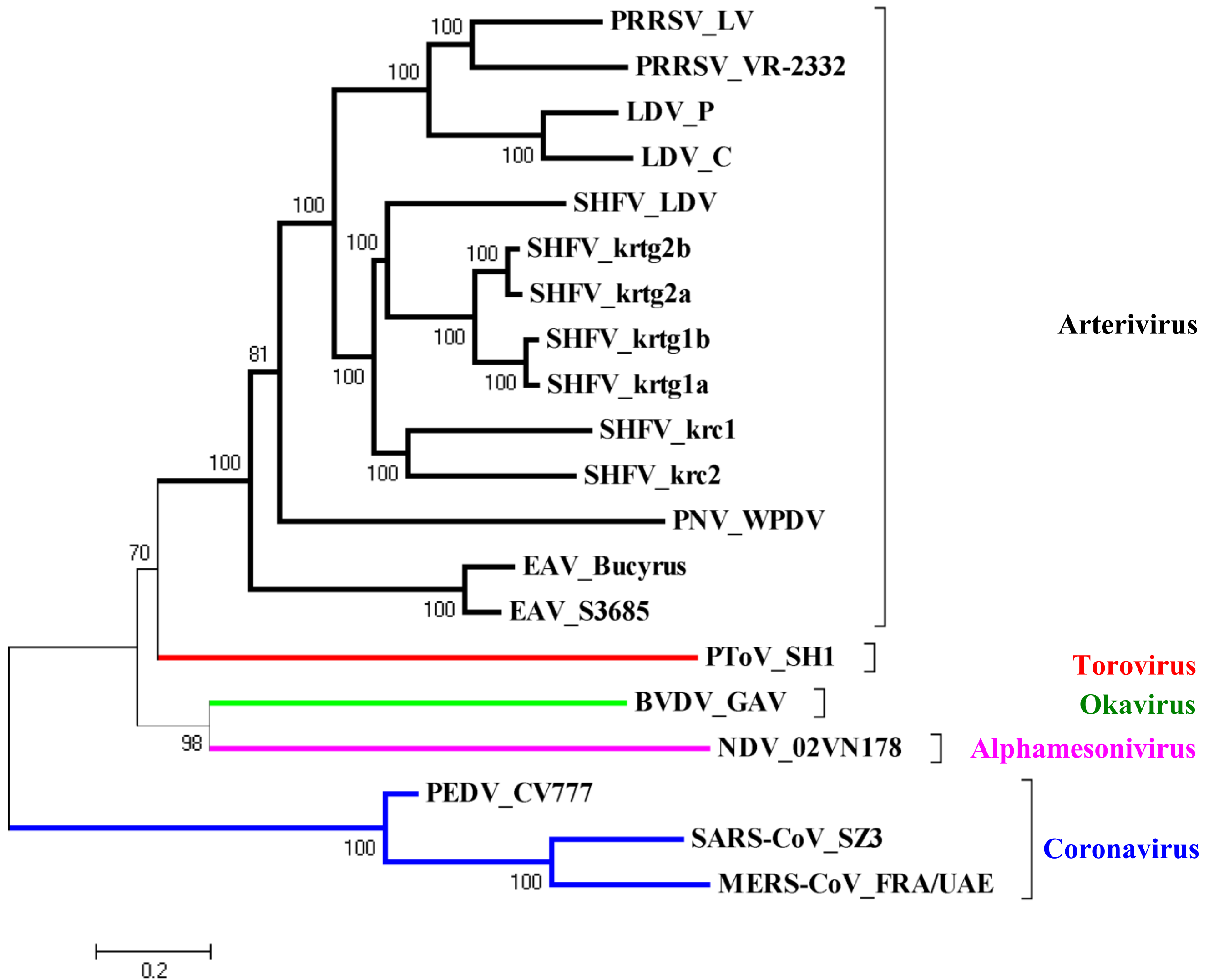
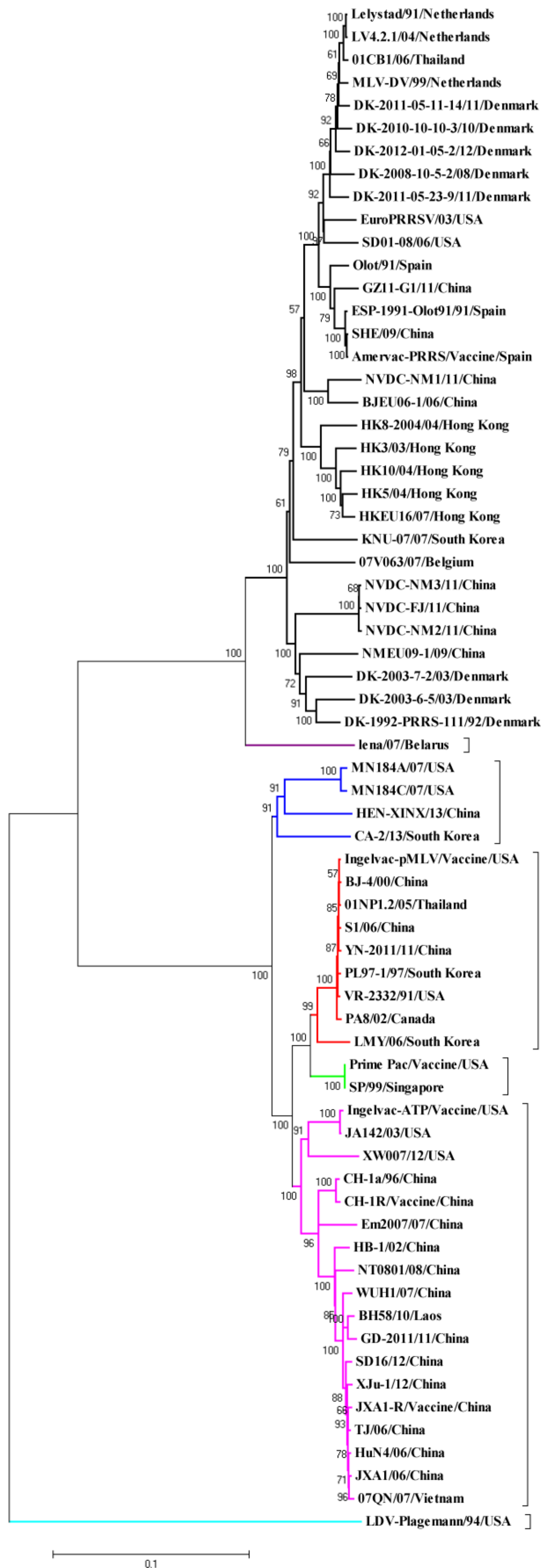


Figure 1.4 The phylogenetic tree of *Nidoviruses*.

The phylogenetic tree was constructed based on the complete genome sequences of 20 representative viruses in the order of *Nidovirales* using the neighbor-joining method and the maximum composite likelihood model. The robustness of the tree was evaluated by bootstrapping using 1000 replicates. The numbers at each branch are the bootstrap values (%) that support the grouping. The viruses that belong to different *Genuses* are shown in different colors. This phylogenetic tree indicates that the closest evolutionary related virus to PRRSV is LDV.



Genotype I Subtype 1

Genotype I Subtype 3

Genotype II Subtype 1

Genotype II Subtype 5

Genotype II Subtype 7

Genotype II Subtype 8

Figure 1.5 PRRSV isolates show high genetic diversity.

This phylogenetic tree was constructed using 66 complete genomes of PRRSV isolates, including 33 type I and 33 type II PRRSV strains, and LDV Plagemann strain, the representative virus of *arterivirus*. The viruses are indicated by the name and the reported year, followed by the country/region of origin (eg. Lelystad/91/Netherlands means the Lelystad virus was isolated in 1991 in Netherlands). The phylogenetic tree was built using the neighbor-joining method and the maximum composite likelihood model. The robustness of the tree was evaluated by bootstrapping using 1000 replicates. The numbers at each branch are the bootstrap values (%) that support the grouping. The branches of different genotypes and subtypes are marked in different colors. This tree shows that the two genotypes of PRRSV evolved independently. Each genotype of PRRSV shows high diversity and includes subtypes. The viruses are clustered into different subtypes according to previous studies (197, 198).

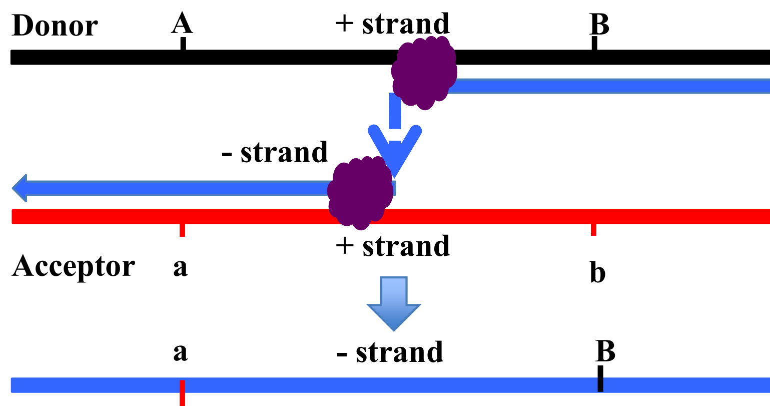


Figure 1.6 Copy-choice recombination.

During anti-sense strand synthesis, the viral RNA-dependent RNA polymerase (RdRp), along with the nascent RNA, switches positive template strands from donor to acceptor. The RdRp continues along the acceptor templates. The produced anti-sense strand contains specific sequences from both donor and acceptor strands. RdRp is shown in purple, donor strand in black, acceptor strand in red, and anti-sense strand in blue.

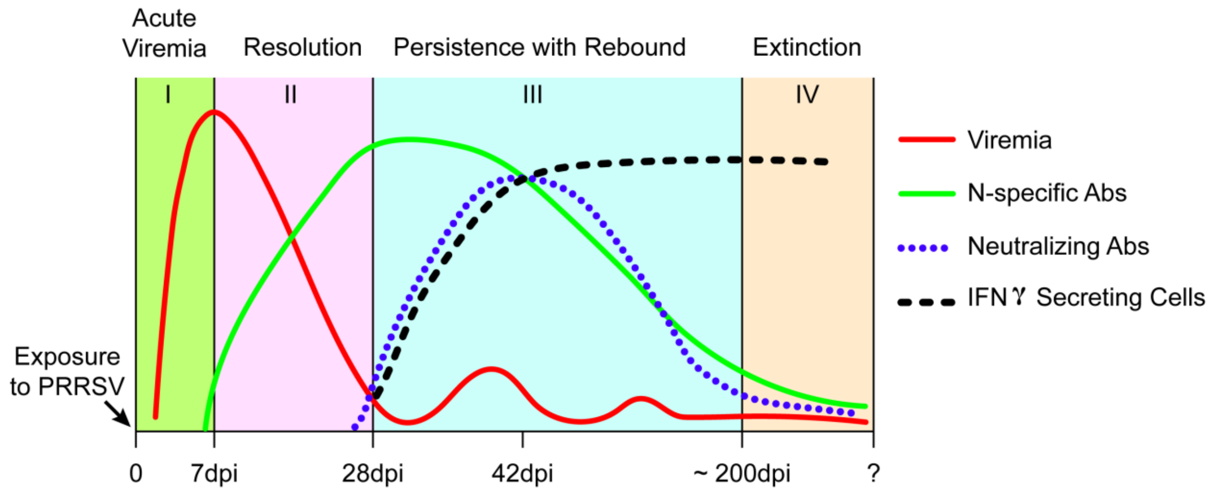


Figure 1.7 Virus replication and adaptive immune responses against PRRSV.

PRRSV reaches the first peak of viremia around 7 days post infection (dpi). Rebound viremia may show up at any time after the first peak during persistent infection. PRRSV-specific Abs targeting at N protein are detectable after 7dpi, but the early produced Abs cannot provide protection for pigs against PRRSV infection. Neutralizing Abs can provide protection, but usually appear after 28dpi. IFN-γ secreting cells may also proliferate after 28dpi. This figure is modified based on a previous study (133).

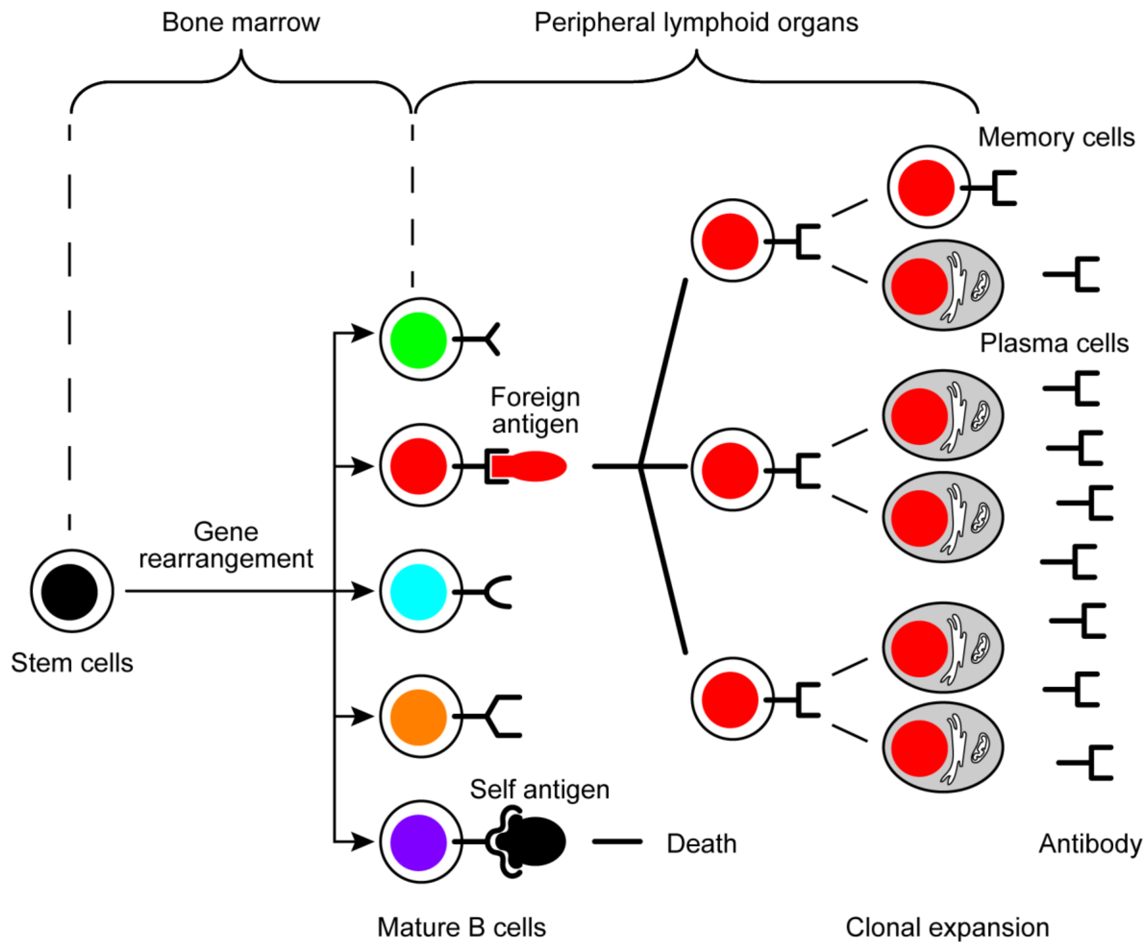
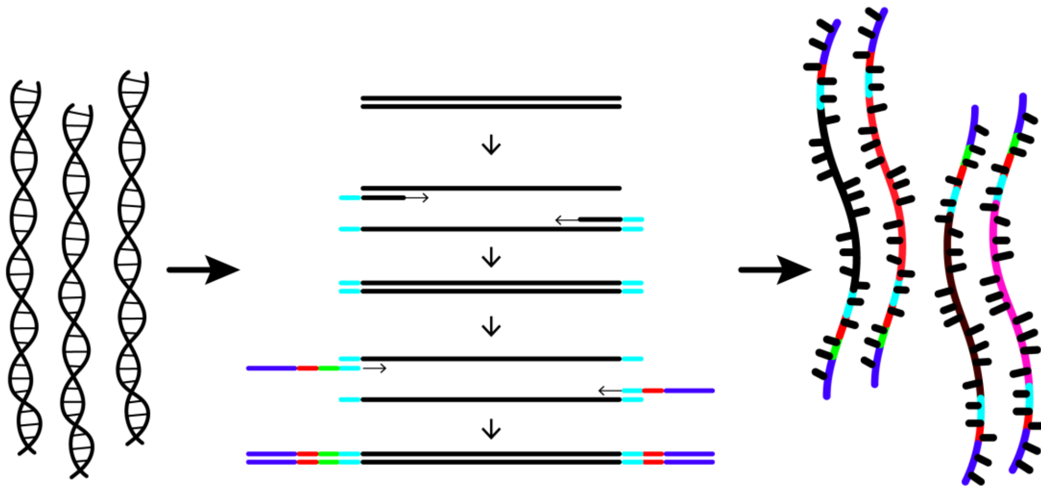


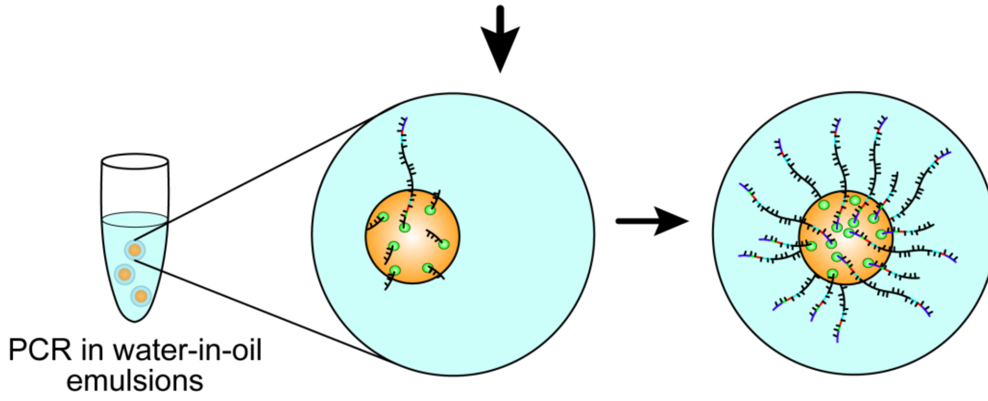
Figure 1.8 Clonal expansion theory.

In bone marrow, stem cells are developed into mature antigenetically committed B cells. Mature B cells are transported into peripheral lymphoid organs. When a foreign antigen enters the peripheral lymphoid system, any B cell displaying an antibody specific to this antigen will be activated and go through clonal expansion. Memory cells and plasma cells are produced and antigen-specific antibodies are secreted. These antibodies have same antigen binding activities.

A



B



C

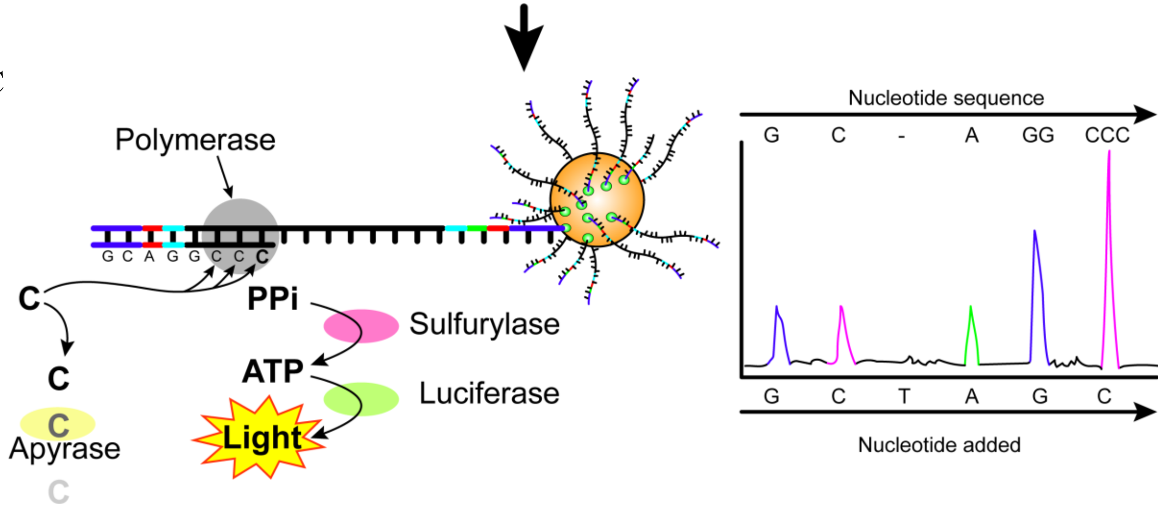


Figure 1.9 454 sequencing system.

In the first step (A), two rounds of PCR are used to prepare the library. In the first round PCR, target gene specific primers are used. In the second round PCR, universal primers with 454-adaptor sequence and unique multiplex identifier (MID) are used. All amplicons are normalized to the same concentration and mixed together to form an amplicon library. In the second step (B), the library is submitted to emulsion PCR. The PCR is completed in water-in-oil droplets. In the third step (C), isolated DNA-carrying beads are loaded into individual wells on a PicoTiter plate to perform 454 pyrosequencing. Only one type of nucleotides is flowed over the plate at a time. When the nucleotide matches to the template and is incorporated into the strand by polymerase, a pyrophosphate is released, which is utilized to produce ATP with the function of sulfurylase. ATP activates the luciferin by luciferase to emit light. The light signals are proportional to the numbers of incorporated nucleotides in the given flow. The extra nucleotides after each flow are degraded by apyrase. The nucleotide sequence is determined with the GS Amplicon Variant Analyzer software. This figure is modified based on a previous study (65, 90).

Table 1.1 Characteristics and functions of PRRSV nonstructural and structural proteins.

Proteins	Genes	No. of aa		Characteristics and functions
		EU	NA	
nsp1α	ORF1a	?	180	Zinc-finger protein. Accessory protease PCPα. Regulator of sg mRNA synthesis. Potential IFN antagonist.
nsp1β		?	203	Protease PCPβ. Potential IFN antagonist.
nsp2		1078	1196	Protease PLP2. Deubiquitinating enzyme. Potential IFN antagonist. Transmembrane protein involved in membrane modification. Forming replication complex.
nsp3		230	230	Transmembrane protein involved in membrane modification. Forming replication complex.
nsp4		203	204	Main protease SP. IFN inhibition.
nsp5		170	170	Transmembrane protein possibly involved in membrane modification.
nsp6		16	16	?
nsp7α		149	149	Recombinant nsp7 is highly immunogenic, may activate immune responses through
nsp7β		120	110	class I MHC-mediated antigen presentation pathways.
nsp8		45	45	?
nsp9	ORF1b	685	685	RNA-dependent RNA polymerase (RdRp)
nsp10		442	441	RNA helicase. Contains putative zinc-binding domain
nsp11		224	223	Uridylate-specific endoribonuclease (NendoU)
nsp12		152	153	?
GP2a	ORF2a	249	256	Minor highly glycosylated structural protein. Essential for virus infectivity. Incorporated into virions as a multimeric complex. Viral attachment protein.
E	ORF2b	70	73	Minor unglycosylated and myristoylated structural protein. Essential for virus infectivity. Incorporated into virions as a multimeric complex. Possesses ion-channel-like properties and may function as a viroporin in the envelope.
GP3	ORF3	265	254	Minor highly glycosylated structural protein. One of the most variable PRRSV proteins. Essential for virus infectivity. Highly antigenic and may involve in viral neutralization. Incorporated into virions as a multimeric complex. A subset of GP3 is secreted as a non-virion-associated soluble protein.
GP4	ORF4	183	178	Minor highly glycosylated structural protein. Essential for virus infectivity. Key glycoprotein for formation of multiplex complex incorporated into virions. Viral attachment protein and may involve in viral neutralization.
GP5	ORF5	201	200	Major glycosylated structural protein. Transmembrane protein with a variable number of potential N-glycosylation sites. The most variable structural protein involves in virus neutralization. GP5-M is crucial for virus assembly. May involve in the entry of virus into host cells and in the apoptosis phenomenon.
ORF5a	ORF5a	43	46/51	Minor unglycosylated and hydrophobic structural protein. Essential for virus viability. Incorporated into virions as a multimeric complex.
M	ORF6	173	174	Major unglycosylated structural protein. Highly conserved. GP5-M heterodimerization is crucial for virus infectivity. Play a key role in virus assembly and budding.
N	ORF7	128	123	Major unglycosylated and phosphorylated structural protein. The sole component of the viral capsid and forms dimer through covalent and non-covalent bonds. Can enter the nucleus/nucleolus. Highly immunogenic.

*The table is modified from previous studies (84, 158).

Table 1.2 Potential immune evasion strategies utilized by PRRSV.

Strategy	Mechanism	Consequences	Reference
Quasispecies variation	Mutation and recombination	Insufficient protection against heterologous variations	(187)
Minimal IFN-α production	Inhibition of dsRNA signaling pathway	Reduced the production of proinflammatory cytokines and the induction of acquired immunity	(233)
Presence of a decoy epitope in GP5	Masking the neutralizing epitope	Delayed the neutralizing antibody response	(163)
Antibody-dependent enhancement (ADE)	Enhancement of virus entry into target cells by antibody	Reduced the effectiveness of neutralizing antibody response	(40)
N-linked glycosylation of GPs	Glycan shielding of neutralizing epitopes	Delayed neutralizing antibody response and diminished viral neutralization sensitivity	(7)
Lack of surface expression of viral proteins in infected cells	Budding of viral proteins into the lumen of ER and/or Golgi compartments	Infected cells are invisible to PRRSV-specific antibodies and refractory to antibody- and complement-mediated cell lysis	(63)
Interfere antigen-presentation by DCs	Apoptosis of DCs. Downregulated CD11b/c, CD14, CD80/86, MHC I and II, inflammatory cytokines and T cells. Upregulated IL-10.	Downregulation of viral antigen presentation	(239)
Prevention of normal B cell repertoire development	Biased expansion of a subpopulation of the antibody repertoires	Abnormal antibody response to PRRSV infection	(37)
N protein nuclear localization	?	Decreased the production of neutralizing antibodies and facilitated viral persistence	(169)
Block the production of antimicrobial molecules (AMPs)	Modulation of porcine innate immunity by AMPs	Downregulated the expression of AMPs and decreased anti-PRRSV activities	(190)
Nsp1α inhibits type I IFN production	Degradation of CREB-binding protein in the nucleus and Blocking NF-κB activation in the cytoplasm	Nsp1α blocks phosphorylation of IκB and thus prevents IκB degradation to keep NF-κB silent	(111)
Nsp1β subverts host innate immunity	Antagonizing IRF3 activation	Inhibited IRF3-dependent gene expression. Inhibited the dsRNA-induced phosphorylation and nuclear translocation of IRF3	(13)
Nsp11 inhibits type I IFN induction	NendoU activity of nsp11 is associated with the inhibition of IRF3 activation	Inhibited IRF3 phosphorylation and blocked the nuclear translocation of IRF3 in stimulated cells	(250)
Regulation of ISGylation pathways	PLP2 antagonizes the immune responses by removing Ub or Ub-like molecules from cellular proteins.	Inhibited ISG15 production and conjugation	(217)
Suppression of NK cell-mediated cytotoxicity	?	Reduced the susceptibility of PRRSV-infected cells toward NK cytotoxicity	(43)

Table 1.3 Commercial PRRS vaccines.

Name	Parental virus	Description	Year	Manufacturer	Marketing area
Amervac PRRS	VP046 BIS	Type 1 MLV	\	HIPRA	Europe
Suipravac PRRS	5710	Type 1 KV	\	HIPRA	Europe
Porcilis PRRS	DV	Type 1 MLV	2000	Merck	Europe
Pyrsvac-183	All-183	Type 1 KV	2000	Syva	Spain
Progressis	P120	Type 1 KV	2000	Merial	Europe
PRRS Vaccine	VI-94+KPR-96, VI-94+KPR-97	Type 1 KV	\	FGBI-Federal Centre for Animal Health	Russia
SUIVAC PRRS-IN	VD-E1, VD-E2, VD-A1	Type 1 KV	\	Dyntec	Czech Republic, Russia
SUIVAC PRRS-INe	VD-E1, VD-E2	Type 1 KV	\	Dyntec	Czech Republic, Portugal
PRRS Vaccine	VP-046 BIS	Type 1 MLV	\	Philippines Bureau of Animal Industry	Philippines
Ingelvac PRRS MLV	VR-2332	Type 2 MLV	1994	Boehringer Ingelheim	Worldwide
Ingelvac PRRS ATP	JA-142	Type 2 MLV	2004	Boehringer Ingelheim	North America
Fostera PRRS	P129	Type 2 MLV	2012	Zoetis	Worldwide
Prime Pac PRRS	Prime Pac	Type 2 MLV	2014	Merck	United states
ImmunoPRRS	\	Avian Igs	2007	Iasa	Mexico
MJPRRS	\	Type 2 KV	2008	MJ biologics	United States
PRRS RS	\	Type 2 GP5/6 vectored vaccine	2009	Sirrah Bios	United States
CH-1R	CH-1a	Type 2 MLV	2009	Shanghai Hile	China
PRRS vaccine	CH-1a	Type 2 KV	2009	Harbin Weike	China
PRRS MLV	R98	Type 2 MLV	2010	Jiangsu Nannong	China
HP-PRRS vaccine	JXA1	Type 2 KV	2007	Zhongmu	China
HP-PRRS JXA1-R	JXA1	Type 2 MLV	2009	Guangdong Dahuanong	China
HP-PRRS HuN4-F112	HuN4	Type 2 MLV	2010	Harbin Weike	China
HP-PRRS TJM-F92	TJM	Type 2 MLV	2011	Qindao Yiban	China
SuiShot PRRS	\	KV	\	ChoongAng	South Korea
PRRS Vaccine	\	MLV	\	Kaketsuken	Japan
BSL-PS 100	\	MLV	\	Bestar	Singapore
BSK-PS 100	\	KV	\	Bestar	Singapore

\: No available information.

Table 1.4 Comparison of commercial sequencing methods.

Method	Sanger 3730xl	454 GS FLX	Illumina HiSeq 2000	SOLiDv4
Year of launch	1995	2008	2010	2010
Mechanism	Dideoxy chain termination	Pyrosequencing	Sequencing by synthesis	Ligation and two-base coding
Read length	400~900bp	400~700bp	50-250bp	85-100bp
Accuracy	99.999%	99.9%	98%	99.94%
Reads per run	N/A	1 million	Up to 3 billions	1200~1400 millions
Output data per run	1.9~84K	700M	600G	120G
Time per run	20min~3h	24 hours	10 days	7-14 days
Cost per run	$4	$7000	$23,000	$15,000
Cost per million bases	$2400	$10	$0.05~$0.15	$0.13
Advantages	High quality, long read length	Fast, long read size	High throughput	Accuracy
Disadvantages	High cost low throughput	Homopolymer errors	Short read assembly	Short read assembly

* This table is modified from a previous study (129).

Chapter 2 - Analysis of mutations within hypervariable regions of the PRRSV genome during acute infection and rebound

Abstract:

Genetic variation in both structural and non-structural genes is a key factor in the capacity of PRRSV to maintain persistence within animals, farms and metapopulations. However, the exact mechanisms of how genetic variation contributes to persistence remain unclear. As part of a study to understand the role of host genetics in disease resistance, a subpopulation of pigs were identified that were still viremic at 42 days after PRRSV infection. This study focused on the deep sequencing of nine regions of the PRRS genome in four selected pigs. Samples for sequencing included the parental virus, NVSL 97-7895 and sera collected at 4, 28 and 42 days after infection, as well as tonsils collected at 42dpi. Specific and universal primers were designed for the amplification and sequencing of hypervariable regions within nsp1, nsp2, ORF3 and ORF5. The amplicon library was constructed based on two rounds of PCR, which was submitted for 454-pyrosequencing. When compared against the NVSL sequence, virus collected at 28dpi had the most mutations. Substitutions that were either increasing or decreasing in percentage during infection were present in both non-structural and structural regions, including the appearance of $L_{852}S$ in nsp2, $D_{85}E$ in GP3, $A_{27}V$ and $N_{32}S$ in GP5 and the disappearance of $P_{96}L$ in GP3, respectively. In addition, the $N_{32}S$ substitution in GP5 created a new N-glycosylation site.

2.1 Introduction

Nucleotide variation is not constant across the PRRSV genome, but can be located to hotspots that possess sequence hypervariability. Examples of non-structural proteins include nsp1 and nsp2 (67, 82). Mutational hotspots have also been located within the structural genes, such as ORF3 and ORF5 (140, 150). Presumably, mutations in hypervariable regions play crucial roles in the escape from host defense (7, 13, 67, 91, 187, 219, 238). The best-studied example is ORF5, which includes two hypervariable regions flanking a highly conserved region bound by glycosylation sites. Presumably, altered glycosylation combined with peptide sequence hypervariability protect a conserved epitope from nAbs: an immunological escape strategy described for gp120 of HIV (240).

Virus rebound is a phenomenon first described in HIV in 1988 (205), and also in PRRSV in 2010 (177). During PRRSV infection, viremia typically reaches a peak between 4 and 11 days after infection followed by the decay and disappearance of PRRSV from the blood. However, virus replication can be detected in lymphoid tissues, including the tonsil. Rebound is the reappearance of subsequent peaks, which can occur at any time after infection. In a study directed at identifying genomic markers linked to PRRS, the experimental infection of several hundred pigs identified a subpopulation that showed virus rebound (16). The new virus may represent a quasispecies variant that has attained a new adaptive peak, either through a change in tropism or escape from adaptive immunity. The purpose of this study was to incorporate next-generation sequencing as a means to characterize viruses during the course of infection. The focus was on deep sequencing nine regions of the PRRSV genome that were previously described as exhibiting nucleotide and peptide sequence hypervariability.

2.2 Materials and Methods

2.2.1 Sample sources

The samples for this study were obtained from the PRRS Host Genetics Consortium (PHGC) (16, 136, 185). All studies involving animals and live viruses were performed after approval by the Kansas State University Institutional Biosafety and Animal Care and Use Committees. Each infection trial included approximately 200 three week-old pigs infected with a high virulence type 2 PRRSV isolate, NVSL 97-7895 (GenBank accession no. AY545985). Blood samples were collected at 0, 4, 7, 11, 14, 21, 28, 35, 42 days post-infection (dpi). PRRSV

viremia was measured using Applied Biosystems AgPath-ID™ NA & EU PRRSV One-Step Multiple qRT-PCR kit according to the manufacturer's recommendations. The results were reported as the log10 copies per 50 µl reaction. Four pigs that exhibited a rebound in viremia were subject to further study.

2.2.2 Library preparation and 454 sequencing

Samples used for the library preparation include sera obtained at 4, 28 and 42 dpi from 4 pigs, tonsils collected at 42dpi from 2 pigs, as well as the NVSL 97-7895 inoculum used for infection. Total RNA was extracted from 100 µl of tissue culture medium or serum, or 50 mg tonsil homogenized in 1 ml TRIzol® Reagent (Invitrogen) using acid guanidinium thiocyanate-phenol-chloroform extraction method (60) and eluted in 50 µl RNase-free water. cDNA was generated by reverse transcription using random hexamer primers and Transcriptor High Fidelity cDNA Synthesis Kit (Roche). The preparation of the amplicon library incorporated two rounds of PCR (65). The first round of PCR was performed using virus sequence-specific primers that possessed virus-specific sequence. A second round of amplification was performed using 454-adaptor multiplex identifier (MID) primers (Table 2.1). Primers were selected that yielded a product between 447 bp and 542 bp in length. Fifteen MIDs were used for 15 samples and each sample used the same MID for 9 amplicons from the hypervariable regions of PRRSV. A total of 135 amplicons were obtained to form the library. More detailed methods describing library preparation, 454 sequencing and data analysis are presented above in chapter 1.7.

2.3 Results

2.3.1 PRRSV rebound

The analysis of PRRSV replication in small groups of pigs indicates that virus replication achieves a peak within 7-21 days after infection, which is followed by the disappearance of detectable virus from the circulation. The last phase of infection is the gradual decay of replication in lymphoid organs followed by virus extinction. The mechanism responsible for the eventual disappearance of virus is not well understood. In this study, a group of 200 pigs were experimentally infected and viremia was followed for 42 days. Results of PRRSV PCR for 141 pigs (which possessed a complete set of data points) are shown in Figure 2.1A. In this example, all pigs were productively infected, achieving levels of viremia between 5.5 and 7.5 log

templates per 50 µl PCR reaction. The results show several peaks indicating virus rebound in the blood. Out of this population, four pigs that showed rebound were selected for further study (Fig. 2.1B). All four pigs showed rebound peaks at between 28dpi and 42dpi.

2.3.2 Mutations identified by deep sequencing

The regions selected for amplification are shown in Figure 2.2. The first round of PCR yielded all products of the expected size. As a representative example, the 9 PCR products amplified from the tissue culture fluid used for inoculation of pigs is shown in Figure 2.3. A total of 135 amplicons were submitted for 454 sequencing. The coverage for each amplicon was between 118 and 4073 reads with an average of 679 reads. A summary showing the total number of nucleotide substitutions is shown in table 2.2. The parental virus had 14 mutations compared to the GenBank sequence. When compared to the inoculum, sera collected at 28dpi had the greatest number of nucleotide changes.

To investigate whether the viral quasispecies in peripheral blood could represent the viral population in lymphoid tissues, we compared the mutations in sera and tonsils collected at 42dpi from the same pigs. We found 16 substitutions shared between serum and tonsil samples, within which, $T_{2555}C$ in nsp2, $C_{255}A$ in ORF3 and $C_{80}T$ in ORF5 were shared between pigs (Table 2.3). PRRSV could replicate in tonsil and identical mutations shared in tonsils and sera indicated that the tonsil was a source of PRRSV quasispecies in the serum. However, we also identified 8 substitutions only occurring in sera or tonsils but not shared between each other (Table 2.3). Difference of viral quasispecies in tonsils and sera was consistent with the notion that PRRSV has different replication sites and the evaluation of viral quasispecies in sera only may not represent viruses replication in other lymphoid organs such as in the tonsil.

We also found five substitutions that showed the trend of either increasing or decreasing in frequency during infection (Table 2.4). These nucleotide changes were consistent at least in two pigs at each time point and could lead to amino acid substitutions. We found the trend of decreasing variation frequency at the $C_{287}T$ mutation in ORF3 (resulted in $P_{96}L$ in GP3). The percentage of $C_{287}T$ mutation in the parental virus was 44% and from 33% to 57% in the 4dpi sera. However, the mutation was gone in the 28dpi and 42dpi serum and tonsil samples from all the pigs except the 42dpi serum from pig 6685, which had 26% $C_{287}T$ mutation in 595 reads.

Furthermore, there were four mutations, including $T_{2555}C$ ($L_{852}S$) in nsp2, $C_{255}A$ ($D_{85}E$) in ORF3, $C_{80}T$ ($A_{27}V$) and $A_{95}G$ ($N_{32}S$) in ORF5, that showed the trend of increasing variation frequency.

Remarkably, the two substitutions in ORF5 drew our attention. The $C_{80}T$ ($A_{27}V$) substitution was located in the previously described decoy epitope (163). The $A_{95}G$ ($N_{32}S$) substitution created a new N-glycosylation site (from NAN to NAS) in two pigs (Fig. 2.4). Several N-glycosylation sites in GP3 and GP5 have been reported to help PRRSV escape host immune responses by glycan shielding (7, 238). The influence of this new generated N-glycosylation site needs further investigation.

2.4 Discussion

PRRSV is a rapidly evolving RNA virus due to its high mutation rate and lack of proofreading-repair ability (96, 210). As a consequence, PRRSV circulates *in vivo* as a quasispecies with a dynamic distribution subjected to a continuous process of genetic variation, competition and selection (86). The genomic heterogeneity provides rapid adaptation of a virus population to changes in the microenvironment when the viral quasispecies is subjected to selection pressure such as host immune responses (121, 134). In this study, we dynamically analyzed the mutations in the hypervariable regions of PRRSV genome during the infection in pigs. These regions are not only the most variable segments of the PRRSV genome, but also are vital in dictating the host immune responses (7, 13, 219, 220, 238). In detail, nsp1α/β followed by nsp2 have strong to moderate inhibitory effects on beta interferon (IFN-β) promoter activation and nsp1β can inhibit both the induction of IRF3- and NF-κB-mediated genes (13). PRRSV nsp2 contains an ovarian tumor (OUT) domain, which antagonizes the type I IFN induction by interfering with NF-κB signaling pathway (219). In addition, changes of N-glycosylation sites in GP3 and GP5 can alter the sensitivity of PRRSV to nAbs and help PRRSV escape host humoral immune response through glycan shielding (7, 238).

Numbers of mutations dynamically changed in all hypervariable regions of PRRSV genome during the infection in pigs; however, 28dpi sera always have more nucleotide mutations than the other samples (table 2.2). The increased numbers of substitutions from 0dpi to 28dpi are possibly due to the accumulation of mutations during error-prone replication of PRRSV, whereas the decreased numbers of substitution from 28dpi to 42dpi are likely due to competition and selection of viral quasispecies during infection. The trend of increasing percentages of mutations

($L_{852}S$ in nsp2, $D_{85}E$ in GP3, $A_{27}V$ and $N_{32}S$ in GP5) indicates these may be favorable residues of viruses. Inversely, the trend of decreased percentages of the mutation $P_{96}L$ in GP3 may be the negative selection of PRRSV incapable of resting in the new host microenvironment. This selected subpopulation replicated rapidly and became predominant in the quasispecies (42dpi), which was consistent with the rebound viremia detected at around 42dpi as shown in Figure 2.1. Although the substitutions may be the results of immunological selection (156, 194), another possible explanation is that these mutations may be associated with the adaption of viral quasispecies to replicate in pigs (188).

PRRSV quasispecies in sera and tonsils were different, which was consistent with the result observed in HCV that viral populations in serum and liver were distinct (15). The phenomenon also indicated that lymphoid tissue tropism might affect the selection of a favorable PRRSV population capable of persisting in different lymphoid cells (184, 187). Our data imply that beside the serum, lymphoid organs should also be considered to evaluate PRRSV diversity in pigs in the future.

Intriguingly, we found a mutation creates a new N-glycosylation site in GP5. The new N-glycosylation site created by $N_{32}S$ mutation is located at the same region of N-glycosylation sites that have been identified (238). N-glycosylation sites in both GP3 and GP5 could help PRRSV evade host humoral immune responses by glycan shielding (7, 238). Previous studies showed that within quasispecies, virus variants containing mutations in envelope glycoproteins that altered recognition by nAbs would be positively selected in situations where the nAbs play an important role for virus control (259). Several mechanisms may account for the immune evasion of neutralization-resistant mutants. First, amino acid substitutions within the neutralization determinant may alter the binding affinity of nAbs to the virion (244). Second, mutations at distant sites may change the global conformation of the antigenic determinant (74, 167). Third, mutations may allow additional glycosylation sites that mask neutralizing epitopes (113, 177, 178). This new generated N-glycosylation site might have a similar function. However, the substitution was only presented in two pigs but not in all pigs. The alternative explanation is that mutations that associated with PRRSV immunological selection and immune evasion might present in regions other than these hypervariable regions. We are still working on determining the function of this new N-glycosylation site by a PRRSV reverse genetics system.

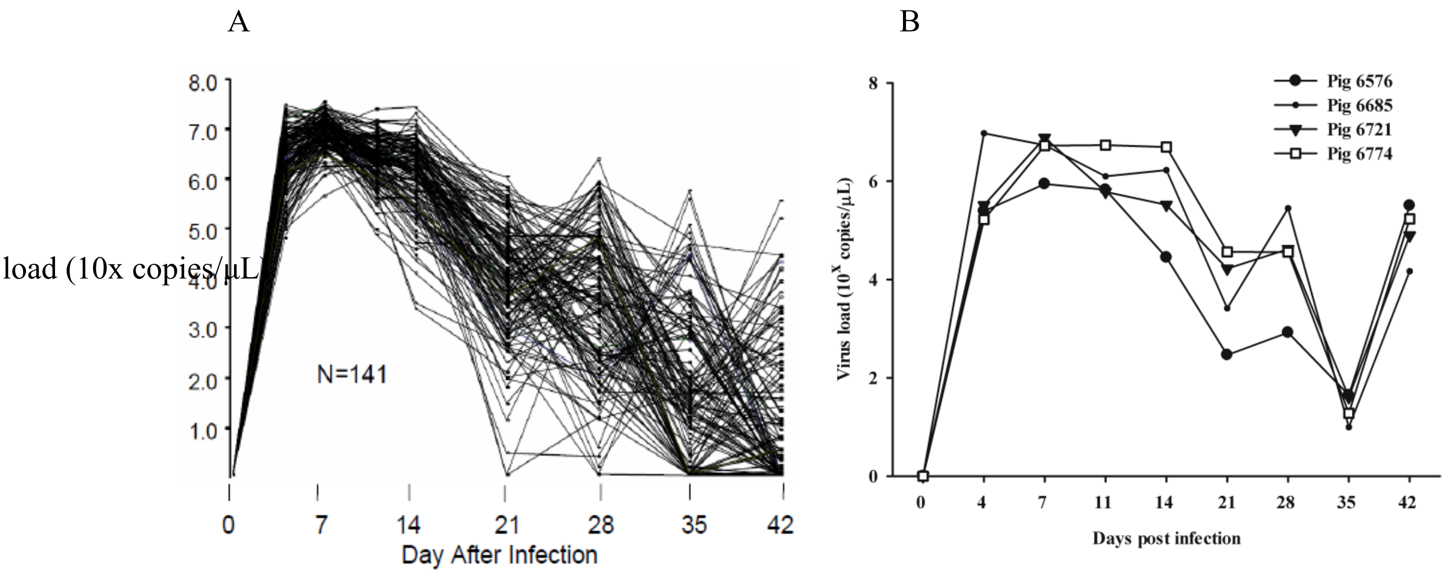


Figure 2.1 Raw phenotypic data of log-transformed viremia in pigs infected with PRRSV. (A) Viremia of 141 pigs within 42 dpi. (B) Four pigs showing rebound viremia were selected for deep sequencing.

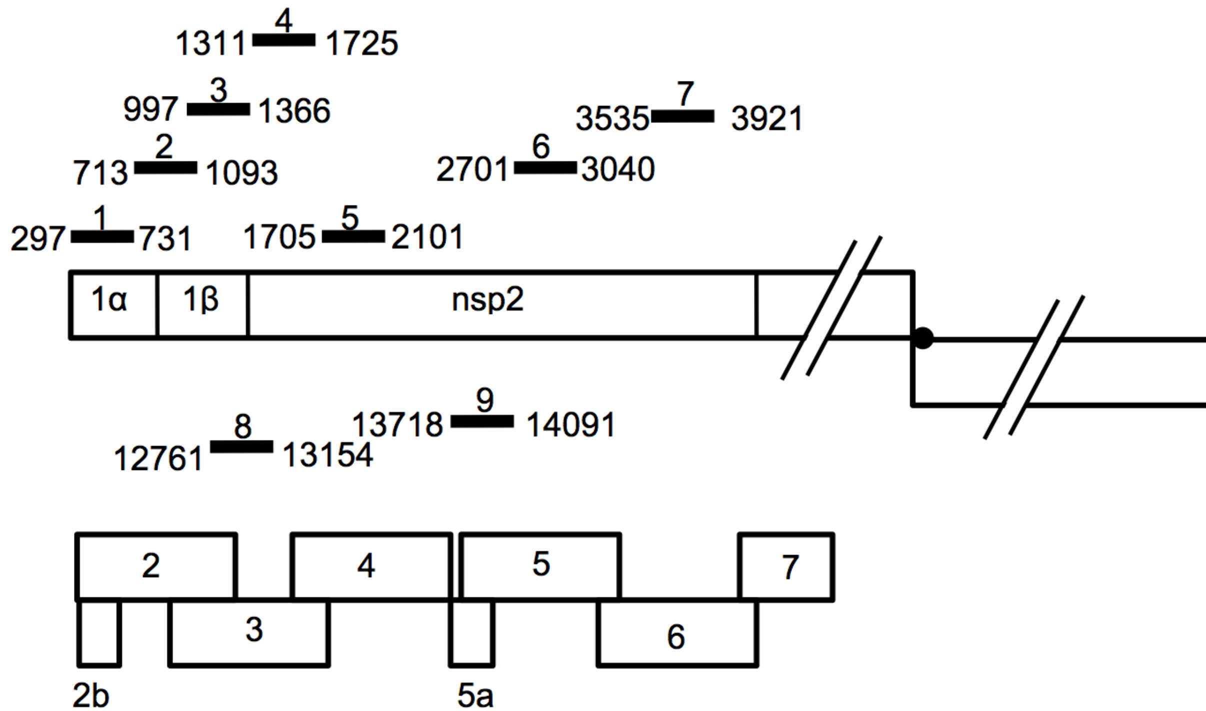


Figure 2.2 Location of regions on the PRRSV genome selected for 454 sequencing

The nine amplicons are located in nsp1α/β, nsp2, ORF3 and ORF5. The numbers show the location of the fragments on the PRRSV genome according to NVSL 97-7895 (GenBank accession number: AY545985).

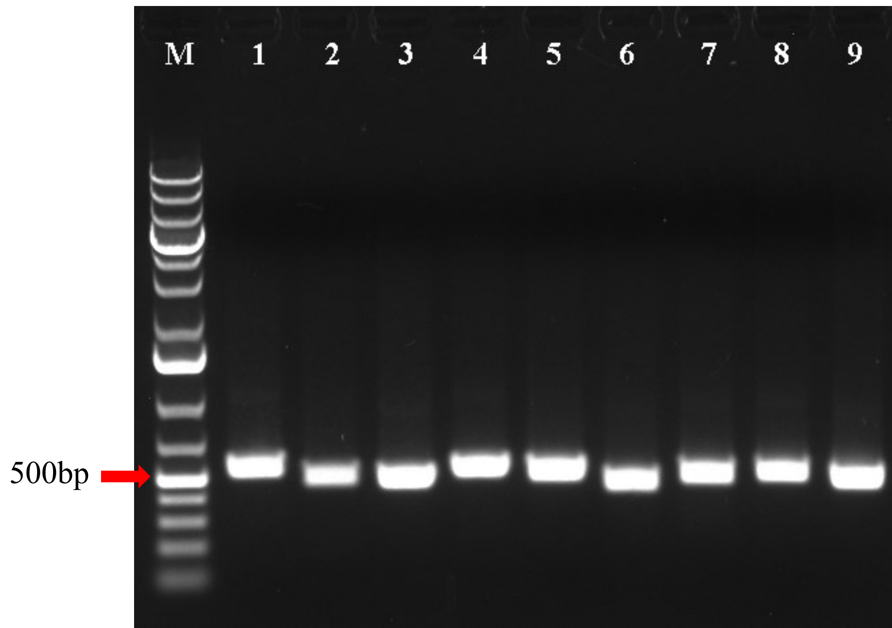


Figure 2.3 The nine amplicons from the parental virus NVSL 97-7895.

The sizes of nine amplicons are between 447 bp and 542 bp. Each amplicon from the other sera and tonsils has the same size.

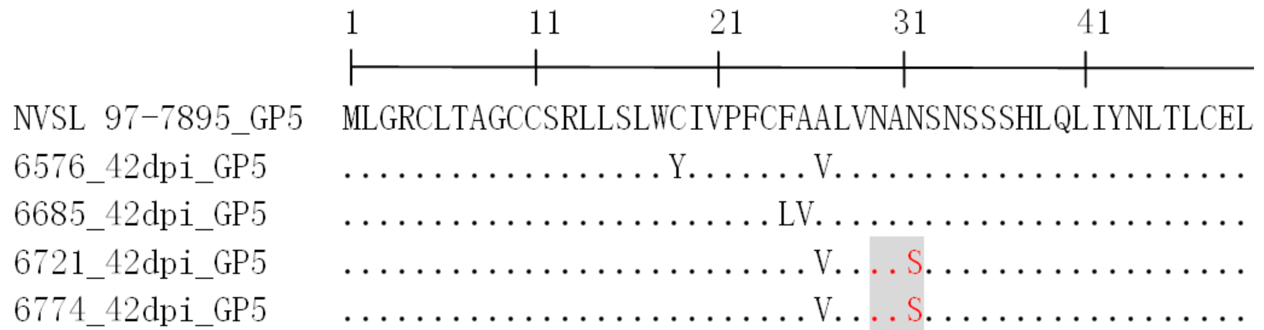


Figure 2.4 The generation of a new N-glycosylation site in GP5.

The alignment of amino acid (aa) sequences of GP5 from 42dpi sera identified the $N_{32}S$ mutation in GP5 creating a new N-glycosylation site (NAS) (marked in red).

Table 2.1 Primers used in this study.

Names	Sequences*
First Round PCR Primers	
Univ_A_nsp1α-F297	TCTCGGTTCTGCATTCGACCTCTGAATCTCCAAGTTCCTGA
Univ_B_nsp1α-R731	ATTCGCTGGCACGCACTTCATAGCACACTCAAAAGGGCAA
Univ_A_nsp1β-F713	TCTCGGTTCTGCATTCGACCCTTTTGAGTGTGCTATGGCT
Univ_B_nsp1β-R1093	ATTCGCTGGCACGCACTTACACCATGCTTGGTTTGATAGCC
Univ_A_nsp1β-F997	TCTCGGTTCTGCATTCGAACTGCTGGTGGCGCTTGTT
Univ_B_nsp1β-R1366	ATTCGCTGGCACGCACTTCGTGCTTTCCTTGCTCTCTTTC
Univ_A_nsp2-F1311	TCTCGGTTCTGCATTCGATTCCGGTTTGGCAGTCACA
Univ_B_nsp2-R1725	ATTCGCTGGCACGCACTTTCACAGAGACAGTCCAATGCTC
Univ_A_nsp2-F1705	TCTCGGTTCTGCATTCGAAGCATTGGACTGTCTCTGTGA
Univ_B_nsp2-R2101	ATTCGCTGGCACGCACTTGCCGCAACCTCTTCCG
Univ_A_nsp2-F2701	TCTCGGTTCTGCATTCGACCGCTCCACGCAGGAAGGT
Univ_B_nsp2-R3040	ATTCGCTGGCACGCACTTTGGTGCGTCAGCGTTGTTGC
Univ_A_nsp2-F3535	TCTCGGTTCTGCATTCGACTCACACGCCTGCACCTTC
Univ_B_nsp2-R3921	ATTCGCTGGCACGCACTTAGAAGAAAACAGGGAGATGGGA
Univ_A_ORF3-F12761	TCTCGGTTCTGCATTCGATGTGCTGTGGTTGCGGATTC
Univ_B_ORF3-R13154	ATTCGCTGGCACGCACTTCGTTCTCCCCGTCGTGAAC
Univ_A_ORF5-F13718	TCTCGGTTCTGCATTCGACTGAGACCATGAGGTGGGCAAC
Univ_B_ORF5-R14091	ATTCGCTGGCACGCACTTAACCCGGCGGTAGACACAGT
Second Round PCR primers	
A-KMID1-Univ-A	**CCATCTCATCCCTGCGTGTCTCCGAC***TCAG****ACACGACGACT***TCTCGGTTCTGCATTCGA
A-KMID2-Univ-A	**CCATCTCATCCCTGCGTGTCTCCGAC***TCAG****ACACGTAGTAT***TCTCGGTTCTGCATTCGA
A-KMID3-Univ-A	**CCATCTCATCCCTGCGTGTCTCCGAC***TCAG****ACACTACTCGT***TCTCGGTTCTGCATTCGA
A-KMID4-Univ-A	**CCATCTCATCCCTGCGTGTCTCCGAC***TCAG****ACGACACGTAT***TCTCGGTTCTGCATTCGA
A-KMID5-Univ-A	**CCATCTCATCCCTGCGTGTCTCCGAC***TCAG****ACGAGTAGACT***TCTCGGTTCTGCATTCGA
A-KMID6-Univ-A	**CCATCTCATCCCTGCGTGTCTCCGAC***TCAG****ACGCGTCTAGT***TCTCGGTTCTGCATTCGA
A-KMID7-Univ-A	**CCATCTCATCCCTGCGTGTCTCCGAC***TCAG****ACGTACACACT***TCTCGGTTCTGCATTCGA
A-KMID8-Univ-A	**CCATCTCATCCCTGCGTGTCTCCGAC***TCAG****ACGTACTGTGT***TCTCGGTTCTGCATTCGA
A-KMID9-Univ-A	**CCATCTCATCCCTGCGTGTCTCCGAC***TCAG****ACGTAGATCGT***TCTCGGTTCTGCATTCGA
A-KMID10-Univ-A	**CCATCTCATCCCTGCGTGTCTCCGAC***TCAG****ACTACGTCTCT***TCTCGGTTCTGCATTCGA
A-KMID11-Univ-A	**CCATCTCATCCCTGCGTGTCTCCGAC***TCAG****ACTATACGAGT***TCTCGGTTCTGCATTCGA
A-KMID12-Univ-A	**CCATCTCATCCCTGCGTGTCTCCGAC***TCAG****ACTCGCGTCGT***TCTCGGTTCTGCATTCGA
A-KMID13-Univ-A	**CCATCTCATCCCTGCGTGTCTCCGAC***TCAG****AGACTCGACGT***TCTCGGTTCTGCATTCGA
A-KMID14-Univ-A	**CCATCTCATCCCTGCGTGTCTCCGAC***TCAG****AGTACGAGAGT***TCTCGGTTCTGCATTCGA
A-KMID15-Univ-A	**CCATCTCATCCCTGCGTGTCTCCGAC***TCAG****AGTACTACTAT***TCTCGGTTCTGCATTCGA
B-K-Univ-B	**CCTATCCCCTGTGTGCCTTGGCAGTC***TCAG*ATTCGCTGGCACGCACTT

* 454 adaptor sequences are highlighted in bold. Key sequences are italic and underlined. MID sequences are bold and italic. Universal sequences are underlined. Target gene sequences are shown in regular.

Table 2.2 Total number of substitutions for all four animals.

Segment	Size	Number of substitutions			
		Day 4	Day 28	Day 42	Tonsil
nsp1α (297-731)	435	3	33	6	4
nsp1β (713-1093)	362	0	47	4	3
nsp1β (997-1366)	273	0	11	11	1
nsp2 (1311-1725)	359	2	2	6	4
nsp2 (1705-2101)	376	1	28	6	4
nsp2 (2701-3040)	340	4	5	5	3
nsp2 (3535-3921)	387	2	43	2	1
ORF 2/3 (12761-13154)	394	4	4	7	2
ORF 5a/5 (13718-14091)	374	1	32	8	4

Table 2.3 Mutations identified in serum and tonsil samples collected at 42dpi.

			Source			
			Pig 6576		Pig 6721	
Segment	Size	Position*	Serum	Tonsil	Serum	Tonsil
nsp1α (297-731)	435	C227	T (55%)	T(85%)	-	-
		C230	-	-	T(95%)	T(24%)
nsp1β (713-1093)	362	A587	-	-	G(37%)	G(60%)
		A672	G(84%)	G(74%)	-	-
nsp1β (997-1366)	273	*C871*	-	-	*T (33%)*	-
		A922	-	-	*G (34%)*	-
		G1109	-	*A (13%)*	-	-
nsp2 (1311-1725)	359	G1268	A(18%)	A(15%)	-	
		G1294	A(17%)	A(14%)	-	-
		C1357	-	*A (25%)*	-	-
nsp2 (1705-2101)	376	*T1632*	-	*C (10%)*	-	-
		C1834	T(19%)	T(12%)	-	-
		A1846	G(17%)	G(6%)	-	-
nsp2 (2701-3040)	340	**T2555**	**C(97%)**	**C(99%)**	**C(98%)**	**C(97%)**
		A2621	G(12%)	G(9%)	-	-
		G2830	-	*A (24%)*	-	-
nsp2 (3535-3921)	387	*C3427*	-	*T (13%)*	-	-
ORF3 (12761-13154)	394	**C255**	**A (100%)**	**A (100%)**	**A(100%)**	**A (100%)**
		C306	*T (17%)*	-	-	-
		C372	T(13%)	T(19%)	-	-
ORF5 (13718-14091)	374	G56	A(29%)	A(34%)	-	-
		C80	**T(70%)**	**T(35%)**	**T(42%)**	**T(62%)**
		A95	-	-	G(52%)	G(25%)
		A243	-	-	T(100%)	T(92%)

*The locations of substitutions in nsp1α, nsp1β and nsp2 are based on ORF1a gene of NVSL 97-7895. The location of mutations in ORF3 and ORF5 are based on the nucleotide positions in ORF3 and ORF5 genes of NVSL-97-7895. Substitutions shared between sera and tonsils are shown in regular except the three substitutions that shared between pigs are highlighted in bold. Eight substitutions that differed between sera and tonsils in the same pig are marked in italic.

Table 2.4 Substitutions that are either increasing or decreasing in frequency during infection.

Samples	$T_{2555}C$ (L-S) in nsp2		$C_{255}A$ (D-E) in ORF3		$C_{287}T$ (P-L) in ORF3		$C_{80}T$ (A-V) in ORF5		$A_{95}G$ (N-S) in ORF5	
	Depth	Proportion	Depth	Proportion	Depth	Proportion	Depth	Proportion	Depth	Proportion
Inoculum	1129	0	498	0.56	497	0.44	320	0	320	0
6576 4dpi serum	1055	0	1305	0.84	1301	0.37	1121	0	1118	0
6685	1175	0	629	0.84	627	0.57	548	0	545	0
6721	295	0	833	0.85	814	0.31	1235	0	1235	0
6774	1063	0.22	775	0.84	774	0.48	598	0	598	0
Mean	897	0.05	885	0.84	879	0.43	875	0	874	0
6576 28dpi serum	4073	0.10	2180	0.89	2166	0.21	1528	0.20	1945	0
6685	718	0.77	435	0.96	473	0	662	0.45	633	0.23
6721	653	0	804	0.99	852	0	680	0.7	661	0.06
6774	299	0.54	205	1.00	222	0	289	0.54	259	0.14
Mean	1435	0.35	906	0.96	928	0.05	790	0.47	874	0.11
6576 42 dpi serum	380	0.97	523	1.00	571	0	450	0.70	452	0
6685	591	0	599	1.00	595	0.26	118	0	118	0
6721	359	0.98	442	1.00	465	0	426	0.42	421	0.52
6774	280	0	421	1.00	437	0	243	0.7	242	0.62
Mean	402	0.49	496	1.00	517	0.06	309	0.45	308	0.285
6576 tonsil	368	0.99	356	1.00	384	0	354	0.35	359	0
6721 tonsil	215	0.97	289	1.00	313	0	305	0.62	305	0.25
Mean	292	0.98	322	1.00	348	0	330	0.48	332	0.125

Chapter 3 - PRRSV replication and quasispecies evolution in pigs that lack adaptive immunity

Abstract:

The replication of PRRSV was studied in a line of pigs possessing a severe combined immunodeficiency (SCID). Real-time RT-PCR revealed a unique course of infection for the SCID group: initially viremia was lower for days 0 through 11, but by Day 21 was elevated compared to normal littermates. The absence of PRRSV-specific antibody in the infected SCID group confirmed the SCID phenotype. Deep sequencing of the structural genes at days 11 and 21 identified seven amino acid substitutions in both normal and SCID pigs. The most significant change was a $W_{99}R$ substitution in GP2, which was present in the inoculum at a frequency of 35%, but by Day 21 had disappeared from all pigs regardless of immune status. Therefore, amino acid substitutions that appear during acute infection are likely the result of the adaptation of the virus to replication in pigs.

3.1 Introduction

PRRSV is considered one of the most rapidly evolving RNA viruses (160). Similar to other RNA viruses, PRRSV exists in a pig as a quasispecies of closely related sequences (91, 187). Antigenic and genetic drift are the likely mechanisms that explain the emergence of genetic variants, which can evade host immune defenses. Another consequence of genetic variation is the appearance of viruses with enhanced virulence (49, 226). Immunological selection is considered the principal force driving the genetic diversity of PRRSV (156). Several studies have documented the emergence of amino acid substitutions during PRRSV infection. For example, consistent mutations were observed in envelope glycoproteins and M protein of persistent PRRSV during experimental infection (4). Amino acid substitution could also be identified when analyzing PRRSV quasispecies during natural infection (91). However, specific experimental evidence demonstrating the quantitative influence of adaptive immunity on the evolution of PRRSV during infection of the pig is lacking.

The recent characterization of a line of pigs with severe combined immunodeficiency (SCID) creates the opportunity to study the role of adaptive immunity in PRRSV protection and pathogenesis. The SCID pig, first described by us, lack B and T cells, does not produce antibody in response to PRRSV infection and fails to reject human tumors (9, 164). In this study, we evaluated viremia and antibody responses in normal and SCID littermates during the first 21 days after infection. Deep sequencing of the structural genes was used as a means to evaluate the makeup of the qausispecies population of each group.

3.2 Materials and Methods

3.2.1 Animals and viruses

Prior to initiating experiments involving animals and viruses, all works were approved by Kansas State University's Institutional Biosafety Committee and Institutional Animal Care and Use Committee. Five SCID pigs and seven unaffected normal littermates were derived from matings between two immunocompetent heterozygous (SCID$^{+/-}$) parents. The identification of normal and SCID pigs was based on staining peripheral blood for the presence of B and T cells. For antibody staining, 100 μl aliquots of whole blood were placed in 12 mm x 75 mm polystyrene flow cytometry (FACS) tubes and incubated for 10 minutes at room temperature in

the presence of 10% normal mouse serum. Primary antibodies (50 μl) were diluted 1/20 in PBS with 2% BSA, added to whole blood, and incubated for 30 minutes at room temperature. Primary antibodies included FITC-conjugated mouse anti-porcine CD3ε (clone PPT3; Southern Biotech) for the staining of T cells and APC-conjugated mouse anti-porcine CD21 (clone B-ly4; BD Biosciences) for the identification of B cells. Red blood cells were lysed by the addition of 1x multispecies lysis solution (eBiosciences). Cells were then washed twice in PBS with 2% BSA (Fraction V; Hyclone) and immediately analyzed on an EC800 flow cytometer (Sony Biotechnology) with FCS Express 4 software (De Novo Software). Absolute cell counts were calculated directly by EC800 software (version 1.3).

Pigs were infected with a type II PRRSV isolate, KS06-72109 (GenBank # KM252867). Each pig was intramuscularly and intranasally inoculated with 2 ml $10^{5.0}$ $TCID_{50}$/ml of virus diluted in MEM. Pigs were monitored daily for clinical signs and serum samples were collected at 0, 4, 7, 11, 14 and 21 days after infection. The experiment was terminated when the SCID pigs became moribund. All samples were stored at -80 °C until further use.

3.2.2 PRRSV real-time RT-PCR

PRRS viral RNA was quantified using EZ-PRRSV™ MPX 4.0 Real Time RT-PCR Target-Specific Reagents (Tetracore®, Rockville, MD) and assay performed according to the manufacturer's instructions. For consistency, each plate contained a set of Tetracore® Quantification Standards and Controls. All PCR reactions were carried out on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA) in a 96-well format using the recommended cycling parameters. Results were reported as the log number of templates per 50 μl PCR reaction.

3.2.3 Fluorescent microsphere immunoassay (FMIA)

The PRRSV nucleocapsid (N) protein gene was cloned and expressed in *E. coli* as described in previous studies (45, 228). Recombinant N was affinity-purified under native conditions using a PrepEase His-Tagged Protein Purification Kit (USB) according to the manufacturer's instructions. The purity and concentration of each protein was assessed by SDS-PAGE and Protein Assay (Bio-Rad), respectively.

Recombinant N protein was coupled to carboxylated Luminex MagPlex® polystyrene microspheres according to the manufacturer's directions. For the assay, approximately 2500

antigen-coated beads, diluted in PBS with 10% goat serum (PBS-GS), were placed in each well of a 96-well polystyrene round bottom plate (Costar, Corning, NY). Fifty microliters of a 1:400 dilution of serum in PBS-GS was added to duplicate wells. The plate, wrapped in foil, was incubated for 30 min at room temperature with gentle shaking then placed on a magnet to hold the beads, and the buffer was aspirated from the wells. Beads were washed three times with 190 µl of PBS-GS. For the detection of Ig, 50 µl of biotin-SP-conjugated affinity-purified goat anti-swine secondary antibody (IgG, Jackson ImmunoResearch) was added and incubated for 30 min. IgM was detected with a biotin-labeled affinity-purified goat anti-swine IgM (KPL, Gaithersburg, MD). Secondary antibodies were diluted to 2 µg/ml in PBS-GS and incubated with the microspheres for 30 min. Plates were washed three times, followed by the addition of 50 µl of streptavidin-conjugated phycoerythrin (2 µg/ml in PBS-GS; SAPE). After 30 min, the plates were washed and microspheres resuspended in 100 µl of PBS-GS and analyzed on a MAGPIX instrument (Luminex) with Luminex® xPONENT 4.2 software. The results were reported as mean fluorescence intensity (MFI).

3.2.4 Preparation of the amplicon library and deep sequencing

Eight primers directed at highly conserved regions were designed to amplify ORF2-7. Total RNA was extracted from 100 µl of tissue culture medium or serum using TRIzol® Reagent (Invitrogen). cDNA was prepared by reverse transcription with random hexamer primers using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche). As described previously (65) and shown in Figure 1.8, two rounds of PCR amplification were used to prepare the library. The first round incorporated PRRSV sequence-specific primers. A second amplification was performed using 454-adaptor multiplex identifier (MID) primers (Table 3.1). Fifteen MIDs were used for 15 samples and each sample used the same MID for eight amplicons from the structural genes of PRRSV. The sizes of amplicons were between 340bp and 446bp (Fig. 3.1).A total of 120 amplicons were obtained to create the library. The detail procedures of library preparation and 454 sequencing are described in chapter 1.7.

3.3 Results

3.3.1 PRRSV viremia in normal and SCID pigs

PRRSV viremia for the seven normal and five SCID pigs is shown in Figure 3.2. The normal littermates showed a pattern of viremia typical of PRRSV infection: reaching a peak at about 11 days after infection followed by a gradual decline. In contrast, the SCID pigs showed lower amounts of virus in the blood, which were significantly reduced relative to the normal littermates at 4, 7 and 11 days after infection. At 11 days after infection, the mean viremia for the SCID group was approximately one log lower than the normal group. Instead of declining, viremia in the SCID group remained elevated, and by 21 days after infection, viremia in the SCID group was approximately one log higher than the group of normal littermates. Soon after Day 21, the experiment was terminated when the SCID pigs began to exhibit a variety of clinical signs, including weight loss and respiratory distress. Necropsy at the time of sacrifice showed that the SCID pigs succumbed as a result of an overwhelming infection caused by a variety of bacteria. Together, these data demonstrate that PRRSV infection follows a unique course in SCID pigs.

3.3.2 PRRSV-specific IgM and IgG responses

The antibody responses for the seven normal and five SCID pigs are shown in Figure 3.3. For normal pigs, IgM was first detected at the 7-day time point, reached a peak at 11days, and by 21 days began to approach background MFI values. This pattern was consistent with the primary antibody response of pigs to PRRSV infection. MFI values for all the SCID pigs remained at background levels for all time points. PRRSV-specific IgG was initially relatively high for all pigs, the result of a high non-specific level of background activity carried over by maternal antibody. For normal pigs, the initial decay in maternal antibody was followed by a steady increase in PRRSV-specific antibody. For the SCID group, the non-specific maternal antibody response continued to decay throughout the remainder of the experiment. By Day 21 the MFI values for the SCID pigs approached background for the assay. These results confirmed that PRRSV-specific humoral immunity was absent in PRRSV-infected SCID pigs.

3.3.3 Nucleotide and amino acid substitutions at 11 and 21 dpi

PRRSV infection is characterized by the emergence of genetic variants, presumably, the result of positive and negative selection. We used a deep sequencing approach to determine the number and rate of nucleotide substitutions during acute infection in SCID pigs and normal littermates. The two time points selected for investigation were 11 and 21 days after infection. Day 11 covered a period during peak viremia when virus levels were elevated in normal pigs. By Day 21, the levels of viremia were reversed; viremia in the SCID group was elevated compared to the normal littermates (see Fig. 3.2). All five pigs in the SCID group were selected for deep sequencing; whereas, for the purpose of comparison, two normal littermates were randomly chosen. A summary of the nucleotide and amino acid substitutions in all seven pigs is presented in Figure 3.4 and Table 3.2. Using the criteria described in Methods, the coverage for each amplicon was between 60 and 1988 reads with an average of 399 reads. There were no insertions or deletions and only nine point mutations were identified, seven of which resulted in changes in amino acids. All mutations except for one were the result of a purine or pyrimidine transition. The predominance of transitions was observed by Allende, et al. (4), who followed changes in PRRSV sequence over a 150 day period. A single transversion, $A_{186}T$ in ORF4 was silent. One mutation appeared within the overlapping region of ORF2 and OR2b. The mutation was silent in GP2, but resulted in an arginine to cysteine change in the E (2b) protein.

The frequencies of amino acid substitutions at 11 and 21 days for individual pigs are summarized in Table 3.2. Overall, there was no difference in the pattern of amino acid substitutions between the SCID pigs and normal littermates. A $W_{99}R$ substitution was present in the parent virus at a frequency of 35%. By 11 days, the frequency decreased, ranging from 0% (SCID pig 2-8) to 27% (SCID pig 2-5). By 21 days, the amino acid substitution was absent in all pigs. Examples of amino acids that increased in frequency can be found in the appearance of $A_{121}V$ in GP5 (pig 1-9) and $I_{95}V$ in M protein (pig 1-4), both SCID pigs. The $A_{121}V$ substitution increased from 28% at 11 days to 90% at 21 days. The $I_{95}V$ substitution increased from 66% (11 days) to 86% (21 days). At Day 21, the $I_{95}V$ substitution appeared in a second SCID pig, 2-2. When going below the 5% threshold, the $I_{95}V$ substitution was detected at a low frequency in both normal and SCID pigs and was present in the parent virus. Overall, the data show very few mutations for the KS06 PRRSV isolate over the 21 days of infection. The most significant amino acid change was the loss of the $W_{99}R$ substitution, which appeared in both normal and SCID pigs.

3.4 Discussion

PRRSV possesses a high mutation rate, the result of the absence of proofreading activity in the virus-encoded RNA-dependent RNA polymerase. Over time, error-prone replication produces a cloud of genetic variants known as a quasispecies. The role of mutation and selection in preserving the pathogenesis or fitness of PRRSV has been studied by documenting the appearance of nucleotide substitutions in viruses over time and by comparing the sequences of non-pathogenic variants (50, 161, 253). One outcome of this body of work is the notion that amino acid substitutions in structural proteins are the result of selective pressure by B and T cells (156). One important consequence is antigenic drift, which contributes to a long-term or "persistent" infection (209).

In this study, we followed amino acid substitutions in ORFs 2-7 in pigs that lack B and T cells. The SCID phenotype was confirmed by the absence of humoral immunity. The eventual disappearance of a $W_{99}R$ substitution in GP2, present in the inoculum, occurred in SCID pigs and normal littermates. This outcome demonstrated that the tryptophan substitution at position 99 was not the result of the escape from adaptive immunity. A more plausible explanation is that the presence of Arg-99 in 35% of the virus population was the result of the selection of viruses better adapted for growth in MARC-145 cells, a simian cell line. The subsequent reversion to the wild-type sequence was due to the selection of viruses that were "re-adapted" to replication in pigs. A molecular explanation can be found in the interaction of PRRSV with CD163, the receptor on the surface of macrophages. Transfection of non-permissive cell lines with truncated CD163, which lacks the scavenger receptor cysteine-rich (SRCR) domain 1 and 2 (SRCR 1 and SRCR 2) confers permissiveness to PRRSV infection (38). However, repeating the experiment with the replacement of CD163 SRCR 5 with the corresponding domain from a similar CD163-like protein, CD163-L1, does not support PRRSV infection, indicating that SRCR 5 is essential for PRRSV infection (232). On the virus side, GP2 and GP4 have been identified to directly interact with CD163; however, the exact domains responsible for binding CD163 have not been determined. The selection of viruses with a tryptophan at position 99 of GP2 may increase the affinity of the virus for pig macrophages. Similarly, the appearance of isoleucine-46, valine-81 and phenylalanine-171 in GP4 may also be driven by increased affinity for porcine CD163. None of the amino acid substitutions mapped to known B-cell epitopes (aa 41-55 and 121-135 in GP2 and aa 51-65 in GP4) (70). The E protein functions as an ion channel (123), and also interacts

with GP2a-GP3-GP4 heterotrimers (247). The disappearance of cysteine-59 in E may also contribute to the adaption of virus to macrophages.

Compared to normal littermates, PRRSV replication followed a unique course in SCID pigs (Fig. 3.2). The lower viremia at the early stage of infection suggests that fewer numbers of permissive macrophages are available for infection. Therefore, the role of T cells would be to regulate the number and/or permissiveness of macrophages for PRRSV infection. As reviewed in Cecere et al (47) and demonstrated by Patton et al (168), T cell cytokines, such as IL-10, enhance the susceptibility of macrophages to infection. In contrast, T cell cytokines, such as interferon-gamma protects macrophages from infection (186). Therefore, T cells play a positive role in viremia by increasing the number of permissive macrophages. However, by Day 21 of infection, viremia in SCIDs becomes elevated relative to the normal littermates, a failure of T cells to control virus replication.

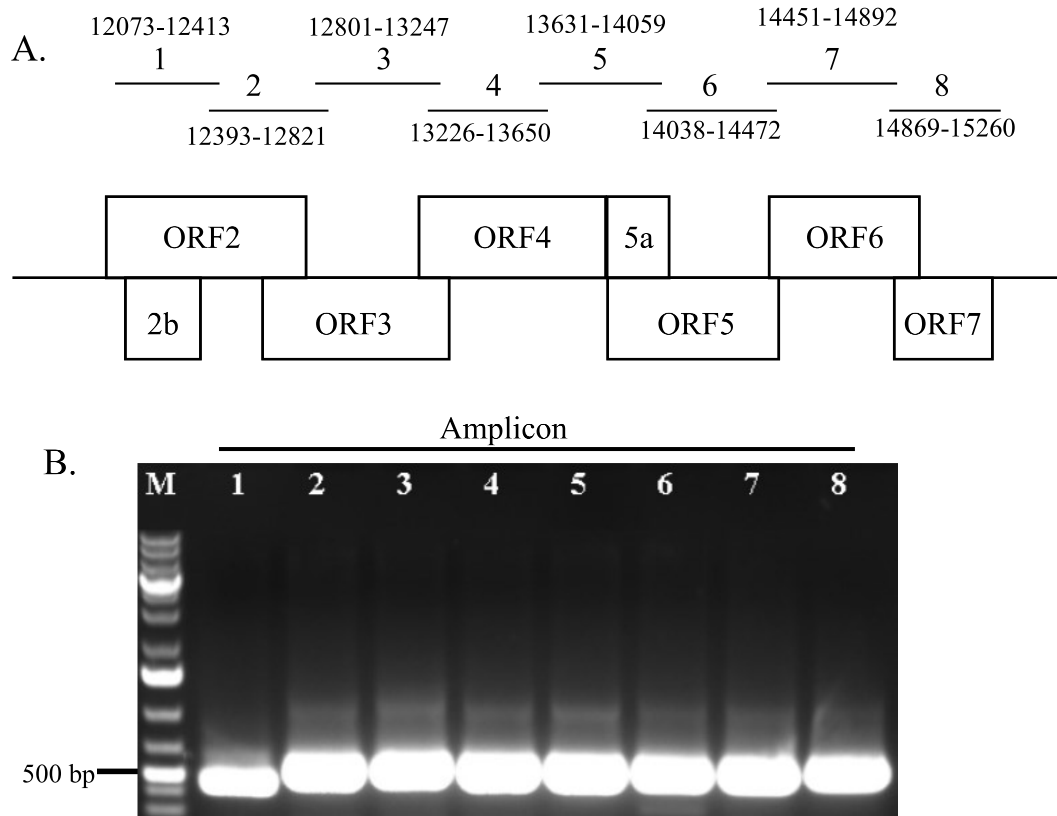


Figure 3.1 PCR amplification of the structural protein regions of PRRSV.

(A) The bars show the location of each application. The number above each bar shows the coordinates of the PRRSV genome covered by each amplicon. The lower figure in (A) shows the location of each ORF in the structural region of the genome. (B) is a representative DNA gel showing each amplicon at the end of the amplicon amplification procedure.

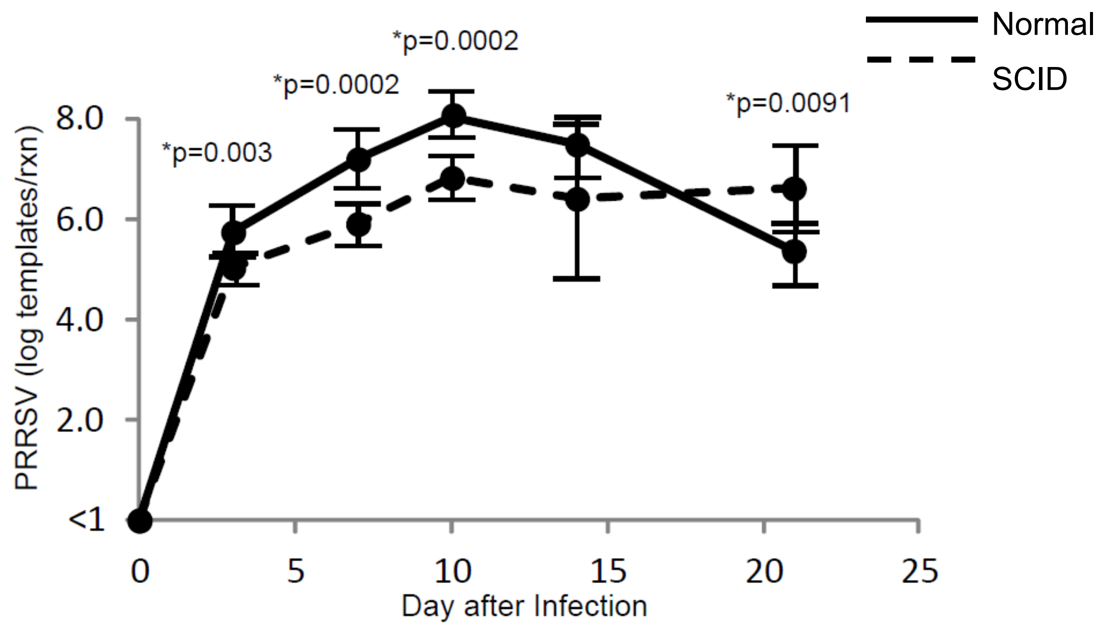


Figure 3.2 PRRSV viremia for SCID pigs and normal littermates.

Results for both panels are for 12 normal pigs (solid line) and 6 SCID pigs (dashed line). P-values are shown for those days there was a significant difference between groups. Statistics were performed using the Mann-Whitney U test.

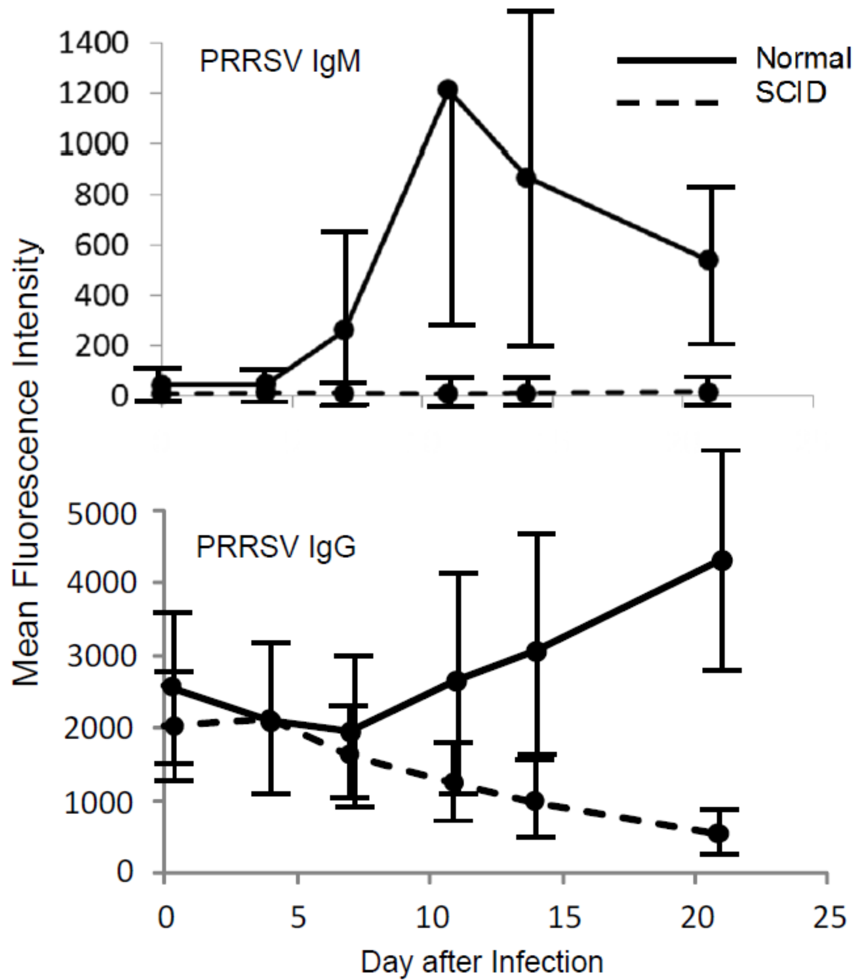


Figure 3.3 PRRSV-specific IgM and IgG responses in normal and SCID pigs.

Antibody results are shown as mean fluorescence intensity (MFI) +/- standard deviation. P-values are shown for those days when there was a significant difference between groups. Statistics were performed using the Mann-Whitney U test. Results for both panels are for 12 normal pigs (solid line) and 6 SCID pigs (dashed line).

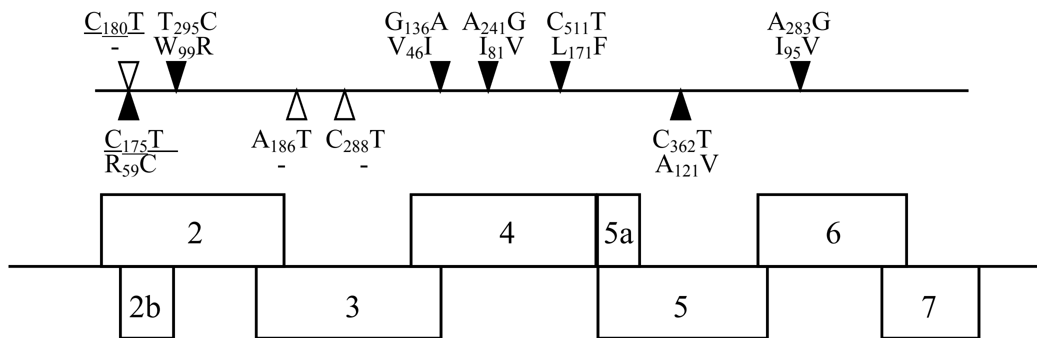


Figure 3.4 Location of mutations in the structural genes of PRRSV.

The triangles identify the location of each mutation. Open triangles identify silent mutations. The numbers identify nucleotide and amino acid position in the respective ORF/polypeptide. The underlined mutation was present in overlapping ORFs. The $C_{180}T$ was silent in ORF2, but resulted in a $R_{99}C$ change in ORF2b.

Table 3.1 Primers used in this study.

Primer	Sequence (5'-3')
ORF2-F1-KS06	TCTCGGTTCTGCATTCGAATGAAATGGGGGCTATGCAAA
ORF2-R1-KS06	ATTCGCTGGCACGCACTTCGACGCGACACCATTTCATCA
ORF2-F2-KS06	TCTCGGTTCTGCATTCGATGATGAAATGGTGTCGCGTCG
ORF2-R2-KS06	ATTCGCTGGCACGCACTTATTGCCCCTAACCAGCGGAAA
ORF3-F-KS06	TCTCGGTTCTGCATTCGATTTCCGCTGGTTAGGGGCAAT
ORF3-R-KS06	ATTCGCTGGCACGCACTTAGCCATTCTAGGTGAAACCAA
ORF4-F-KS06	TCTCGGTTCTGCATTCGATTGGTTTCACCTAGAATGGCT
ORF4-R-KS06	ATTCGCTGGCACGCACTTTGTTGGACGTAGCTGGTAAA
ORF5-F1-KS06	TCTCGGTTCTGCATTCGATTTACCAGCTACGTCCAACA
ORF5-R1-KS06	ATTCGCTGGCACGCACTTACTGTGTCAAGGAAATGACTGG
ORF5-F2-KS06	TCTCGGTTCTGCATTCGACCAGTCATTTCCTTGACACAGT
ORF5-R2-KS06	ATTCGCTGGCACGCACTTGCATATATCATTACTGGCGTGT
ORF6-F-KS06	TCTCGGTTCTGCATTCGAACACGCCAGTAATGATATATGC
ORF6-R-KS06	ATTCGCTGGCACGCACTTGCATATTTGACAAGGTTTACCACT
ORF7-F-KS06	TCTCGGTTCTGCATTCGAAGTGGTAAACCTTGTCAAATATGC
ORF7-R-KS06	ATTCGCTGGCACGCACTTTCACGCTGAAGGTGGTGCTGT
A-KMID1-Univ-A	CCATCTCATCCCTGCGTGTCTCCGACTCAGACACGACGACTTCTCGGTTCTGCATTCGA
A-KMID2-Univ-A	CCATCTCATCCCTGCGTGTCTCCGACTCAGACACGTAGTATTCTCGGTTCTGCATTCGA
A-KMID3-Univ-A	CCATCTCATCCCTGCGTGTCTCCGACTCAGACACTACTCGTTCTCGGTTCTGCATTCGA
A-KMID4-Univ-A	CCATCTCATCCCTGCGTGTCTCCGACTCAGACGACACGTATTCTCGGTTCTGCATTCGA
A-KMID5-Univ-A	CCATCTCATCCCTGCGTGTCTCCGACTCAGACGAGTAGACTTCTCGGTTCTGCATTCGA
A-KMID6-Univ-A	CCATCTCATCCCTGCGTGTCTCCGACTCAGACGCGTCTAGTTCTCGGTTCTGCATTCGA
A-KMID7-Univ-A	CCATCTCATCCCTGCGTGTCTCCGACTCAGACGTACACACTTCTCGGTTCTGCATTCGA
A-KMID8-Univ-A	CCATCTCATCCCTGCGTGTCTCCGACTCAGACGTACTGTGTTCTCGGTTCTGCATTCGA
A-KMID9-Univ-A	CCATCTCATCCCTGCGTGTCTCCGACTCAGACGTAGATCGTTCTCGGTTCTGCATTCGA
A-KMID10-Univ-A	CCATCTCATCCCTGCGTGTCTCCGACTCAGACTACGTCTCTTCTCGGTTCTGCATTCGA
A-KMID11-Univ-A	CCATCTCATCCCTGCGTGTCTCCGACTCAGACTATACGAGTTCTCGGTTCTGCATTCGA
A-KMID12-Univ-A	CCATCTCATCCCTGCGTGTCTCCGACTCAGACTCGCGTCGTTCTCGGTTCTGCATTCGA
A-KMID13-Univ-A	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGACTCGACGTTCTCGGTTCTGCATTCGA
A-KMID14-Univ-A	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGTACGAGAGTTCTCGGTTCTGCATTCGA
A-KMID15-Univ-A	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGTACTACTATTCTCGGTTCTGCATTCGA
B-K-Univ-B	CCTATCCCCTGTGTGCCTTGGCAGTCTCAGATTCGCTGGCACGCACTT

* 454 adaptor sequences are highlighted in bold. Key sequences are italic and underlined. MID sequences are bold and italic. Universal sequences are underlined. Target gene sequences are shown in regular.

Table 3.2 Amino acid substitutions in the structural proteins at 11 and 21 dpi.

		2	2b	3	4	5	5a	M	N
Parent virus		**$W_{99}R$ (35-60)**	**$R_{59}C$ (10-63)**	- (0-91)	- (0-108)	- (0-129)	- (0-129)	- (0-83)	- (0-75)
Day 11	Pig								
Normal	1-10	**$W_{99}R$ (3-477)**	- (0-502)	- (0-711)	- (0-1528)	- (0-866)	- (0-866)	- (0-638)	- (0-667)
Normal	2-1	**$W_{99}R$ (8-439)**	- (0-461)	- (0-376)	- (0-500)	- (0-529)	- (0-529)	- (0-399)	- (0-586)
SCID	1-4	**$W_{99}R$ (9-497)**	- (0-517)	- (0-845)	- (0-264)	- (0-1988)	- (0-868)	**$I_{95}V$ (66-65)**	- (0-690)
SCID	1-9	**$W_{99}R$ (10-514)**	- (0-537)	- (0-94)	**$V_{46}I$ (20-415)**	**$A_{121}V$ (28-669)**	- (0-669)	- (0-350)	- (0-541)
SCID	2-2	**$W_{99}R$ (15-471)**	**$R_{59}C$ (10-492)**	- (0-261)	**$I_{81}V$ (6-302)**	- (0-449)	- (0-449)	- (0-232)	- (0-483)
SCID	2-5	**$W_{99}R$ (27-365)**	- (0-381)	- (0-391)	- (0-311)	- (0-367)	- (0-367)	- (0-337)	- (0-454)
SCID	2-8	- (0-366)	- (0-366)	- (0-341)	- (0-281)	- (0-296)	- (0-296)	- (0-224)	- (0-614)
Day 21									
Normal	1-10	- (0-331)	- (0-331)	- (0-558)	- (0-635)	- (0-684)	- (0-684)	- (0-326)	- (0-300)
Normal	2-1	- (0-325)	- (0-325)	- (0-274)	- (0-220)	- (0-389)	- (0-389)	- (0-167)	- (0-208)
SCID	1-4	- (0-288)	- (0-288)	- (0-199)	- (0-270)	- (0-445)	- (0-445)	**$I_{95}V$ (86-538)**	- (0-359)
SCID	1-9	- (0-295)	- (0-295)	- (0-260)	- (90-398)	**$A_{121}V$ (90-483)**	- (0-483)	- (0-269)	- (0-297)
SCID	2-2	- (0-307)	- (0-307)	- (0-138)	**$L_{171}F$ (12-413)**	- (0-455)	- (0-455)	**$I_{95}V$ (17-315)**	(0-286)
SCID	2-5	- (0-298)	- (0-298)	- (0-255)	- (0-350)	- (0-447)	- (0-447)	- (0-286)	- (0-295)
SCID	2-8	- (0-245)	- (0-245)	- (0-104)	- (0-166)	- (0-229)	- (0-229)	- (0-106)	- (0-324)

*The results show the amino acid substitutions in 2 normal pigs (highlighted) and 5 SCID pigs. The cutoff for detection of a mutation was based on a minimum variation frequency value of 5%. Below each substitution is the percentage of each substitution in the population of sequences and minimum sequence coverage for the ORF.

Chapter 4 - Identification of recombination patterns between two PRRSV infectious clones

Abstract:

A reverse genetics system was developed to study the properties of recombination in PRRSV infectious clones that expressed GFP. Next generation sequencing was used as a means to map sites for recombination. Two PRRSV infectious clones, P129-EGFP-97C and P129-GFPm-d(2-6), were co-transfected into HEK-293T cells. P129-EGFP-97C is a fully functional virus that contains a non-fluorescent EGFP. P129-GFPm-d(2-6) is a defective virus that lacks ORF2-6 but contains a fluorescent protein GFPm, which possesses an EGFP cDNA sequence flanked by sequences from GFP cDNA. Successful recombination was evident by the appearance of fully functional progeny virus that expresses fluorescence. At 72h after infection, cells were sorted based on the presence of green fluorescence. Total RNA was extracted and amplified using two sets of primers to prepare an amplicon library for 454 sequencing. Deep sequencing showed that the variation frequency changed from ~37% (nucleotides that are identical to EGFP in 21nt-165nt region) to 20% ($T_{289}C$ substitution) to ~38% (456nt-651nt region) then to 100% (672nt-696nt region). The results indicated that cross-over events occurred in three conserved regions (166nt-288nt, 290nt-455nt, 652nt-671nt). In addition, we found four cross-over patterns (two single and two double cross-over) could be used to produce viable recombined viruses. The results demonstrate the utility of deep sequencing in assistance with this reverse genetics system for understanding virus recombination.

4.1 Introduction

Recombination occurs in many RNA viruses and can be of evolutionary importance. The occurrence of PRRSV recombination was first suggested by the phylogenetic analysis of field isolates (106). An up-to-date study provided evidence supporting that PRRSV recombination is a common phenomenon in the field (145). Recombination events have been identified in both type 1 and 2 PRRSV strains but not between the two genotypes (56, 83, 125, 128, 234). PRRSV could undergo homologous recombination with the frequency from <2% up to 10% *in vitro* and ~38% *in vivo* (128, 254). A recent phylogenetic analysis showed that viruses from an outbreak of novel highly pathogenic PRRS in China between 2009 and 2010 were originated from a single recombination event, indicating the potential importance of recombination for PRRS emergence (196). This result supported previous reports that recombination is sometimes a powerful mechanism for the rapid emergence of novel strains (120, 199).

Previous methods to analyze PRRSV recombination were based on PCR and sequencing to determine sites and frequency of recombination events (56, 143, 234, 254). We developed a new *in vitro* system that can be used to analyze recombination events that are nonessential and present in viable offspring (48). This reverse genetics system uses GFP- and EGFP-expressing PRRSV infectious clones to study the properties of recombination. Successful recombination is evident by producing a viable fluorescent virus from the co-transfection of a non-fluorescent viable virus with a mutation in EGFP (P129-EGFP-97C) and a fluorescent defective virus (P129-GFPm-d(2-6)) (Fig. 4.1). In this study, we took advantage of high throughput sequencing to determine the locations and frequencies of all cross-over events between EGFP and GFPm genes and to identify the cross-over patterns potentially used to produce viable recombined viruses.

4.2 Materials and Methods

4.2.1 Sample preparation

HEK-293T cells were co-transfected with two PRRSV infectious clones: P129-EGFP-97C and P129-GFPm-d(2-6). P129-EGFP-97C is a fully functional P129 virus with a non-fluorescent EGFP gene, the result of a $C_{289}T$ nucleotide substitution in the fluorophore active site of EGFP. P129-GFPm-d(2-6) is a defective virus that lacks ORFs 2-6, but contains a fluorescent GFPm gene. GFPm is a chimeric gene that contains a middle EGFP sequence flanked on each side by sequence derived from GFP (EGFP and GFP share only 83% nucleotide identity).

Successful recombination between the two infectious clones in the fluorescent gene leads to a fully functional virus that expresses fluorescence. Therefore, after 48 hours of co-transfection, the supernatant was placed on a 100% confluent monolayer of MARC-145 cells and green fluorescent plaques could be observed at 72hpi. Due to the low frequency of recombination, enrichment was done using fluorescence-activated cell sorting (FACS) for cells that expressed green fluorescence (BioRad S3 Cell Sorter). After two rounds of enrichment, roughly 80% of cells carried the fluorescent virus.

4.2.2 Library preparation and deep sequencing

Total RNA was extracted from 100 µl of the sorted cell sample using TRIzol® Reagent (Invitrogen) according to the manufacturer's protocol and eluted in 50 µl RNase-free water. cDNA was generated by reverse transcription using random hexamer primers from the Transcriptor High Fidelity cDNA Synthesis Kit (Roche). Two sets of primer pairs were used in two rounds of PCR for the preparation of the amplicon library (65). The first round of PCR was performed using EGFP sequence-specific primers and the second round of amplification was performed using 454-adaptor multiplex identifier (MID) primers (Table 4.1). Three amplicons were obtained from three overlap regions, which were 369bp, 270bp, and 336bp in length, respectively. A same MID was used for the three amplicons produced from first round PCR in the second round of PCR. The amplicon library was created by these three amplicons. The detail procedures of library preparation and 454 sequencing are described in chapter 1.7.

4.3 Results and Discussion

4.3.1 Identification of cross-over events

The numbers of reads for three amplicons were around 6000 ~ 15000. All sequences were compared to GFPm sequences. The mutations identified by 454 sequencing were shown in Table 4.2. The percentages of mutations identical to EGFP in the first variable region (from 21bp to 165bp) were around 31% to 41%, with 37% in average. The percentage of $C_{289}T$ substitution indicated that only 20% was the same as EGFP, while the other 80% was identical to GFPm. The second variable region (from 456bp to 651bp) is about 29% to 45% with the average of 38% identical to EGFP. The third variable region (672bp to 696bp) has 100% identity with EGFP. The changes of the percentages of mutations between variable regions after each conserved

region indicated that cross-over events did occur in three conserved regions: 166bp to 288bp, 290bp to 455bp, and 652bp to 671bp.

As shown in Figure 4.2, the percentage of nucleotide identity to EGFP decreased from ~37% to 20% in the 123bp-conserved region (from 166bp to 288bp), which indicated there was a cross-over occurrence. In addition, the percentage increased from 20% to ~38% in the 166bp-conserved region (from 290bp to 455bp), indicating that a second cross-over existed in this region. Remarkably, the percentage dramatically changed from 38% to 100%, which meant ~62% cross-over events proceed in only the 20bp-conserved region (from 652bp to 671bp), indicating the 20bp-conserved region is a hotspot of cross-over.

4.3.2 Potential cross-over patterns

Four kinds of recombination events could occur in our sample. As shown in Figure 4.3, there were two kinds of single cross-over recombination events (Fig. 4.3A, 4.3B), having cross-over occurring in the 20bp-conserved region (from GFPm to EGFP) and 166bp-conserved region (from GFPm to EGFP), respectively. Furthermore, there were other two types of double cross-over recombination events (Fig. 4.3C and 4.3D). One had the double cross-over occur in the 123bp-conserved region (from EGFP to GFPm) then in the 20bp-conserved region (from GFPm to EGFP), and another one occurred in the 123bp-conserved region (from EGFP to GFPm) then in the 166bp-conserved region (from GFPm to EGFP). The exact rates of these four recombination patterns could not be identified due to the limitation of the length of sequences (<400bp) obtained from the deep sequencing method we used in this study. However, the alignment analysis did provide direct evidence for cross-over events occurring in the 123bp- and 20bp-conserved regions (Fig. 4.4A, 4.4B).

In conclusion, this study used the deep sequencing method to analyze the nucleotide identity of progeny viruses to their parental viruses in the EGFP and GFPm genes. Based on thousands to ten thousands of sequences from the progeny virus quasispecies, we found that the identity percentages changed between the variable regions and conserved regions of EGFP/GFPm genes indicating the cross-over events did occur in progeny viruses in the conserved areas. There were four types of recombination patterns (two single cross-over events and two double cross-over events) utilized in the EGFP/GFPm region during the co-infection of two PRRSV infectious clones. The 20bp-conserved region shows to be a cross-over hotspot and

has the highest rate of cross-over when compared with the other two cross-over regions. Previous studies demonstrated that cross-over events occurred more frequently at the transcriptional pausing sites or polymerase-binding motifs (89, 120, 180, 246). The mechanisms responsible for this cross-over hotspot may be explored by analyzing the secondary structure of EGFP sequence. Our results indicate that deep sequencing in addition to the new in vitro reverse genetics system could be a useful tool for analyzing different kinds of recombination events that occurred during the co-infection and might contribute to elucidate the underlying mechanisms of PRRSV recombination.

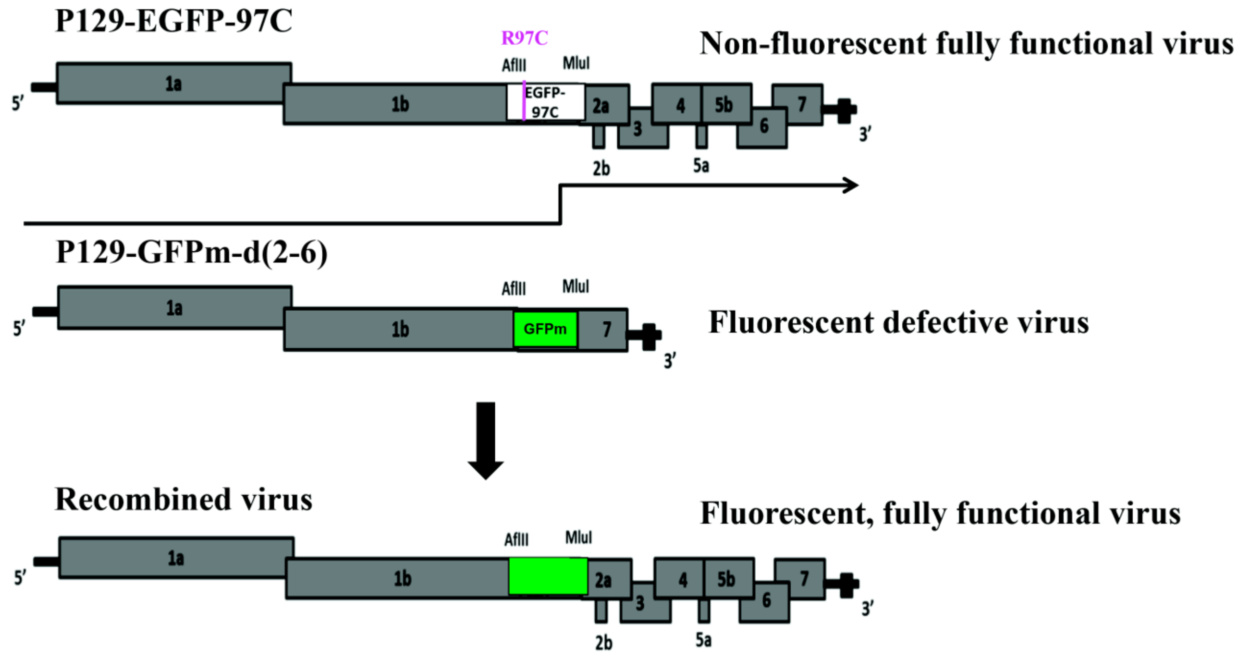


Figure 4.1 Recombination event occurred between two infectious clones.

P129-EGFP-97C is a fully functional non-fluorescent virus and P129-GFPm-d(2-6) is a fluorescent defective virus lacking ORF2-6. Recombination between these two parental viruses will produce a viable fluorescent progeny virus.

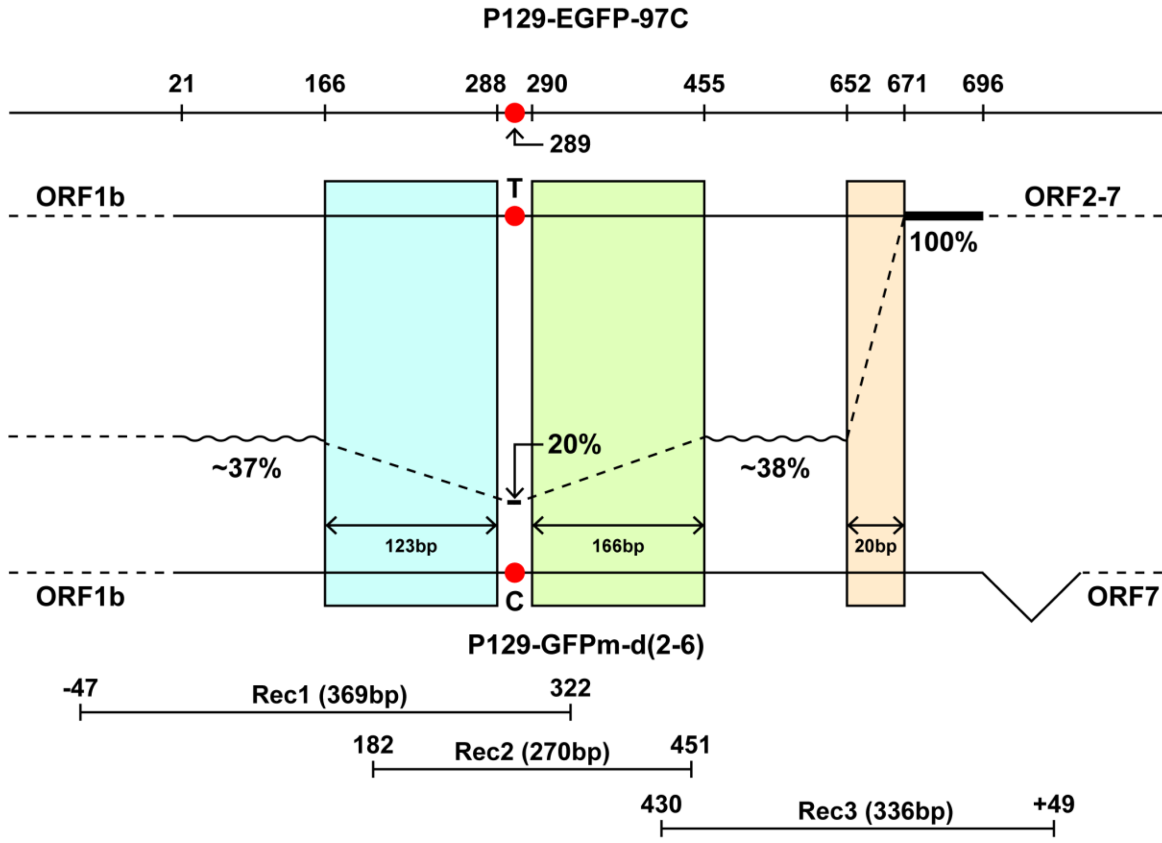


Figure 4.2 Evidence for the occurrence of cross-over events.

The percentages of nucleotide identity to EGFP decreased from 37% (21bp-165bp variable region) to 20% ($C_{289}T$ substitution), then increased to 38% (456bp-651bp variable region) and to 100% (672bp-696bp variable region). The changes indicated that the cross-over events occurred in three conserved regions: 166bp-288bp, 290bp-455bp, and 652bp-671bp.

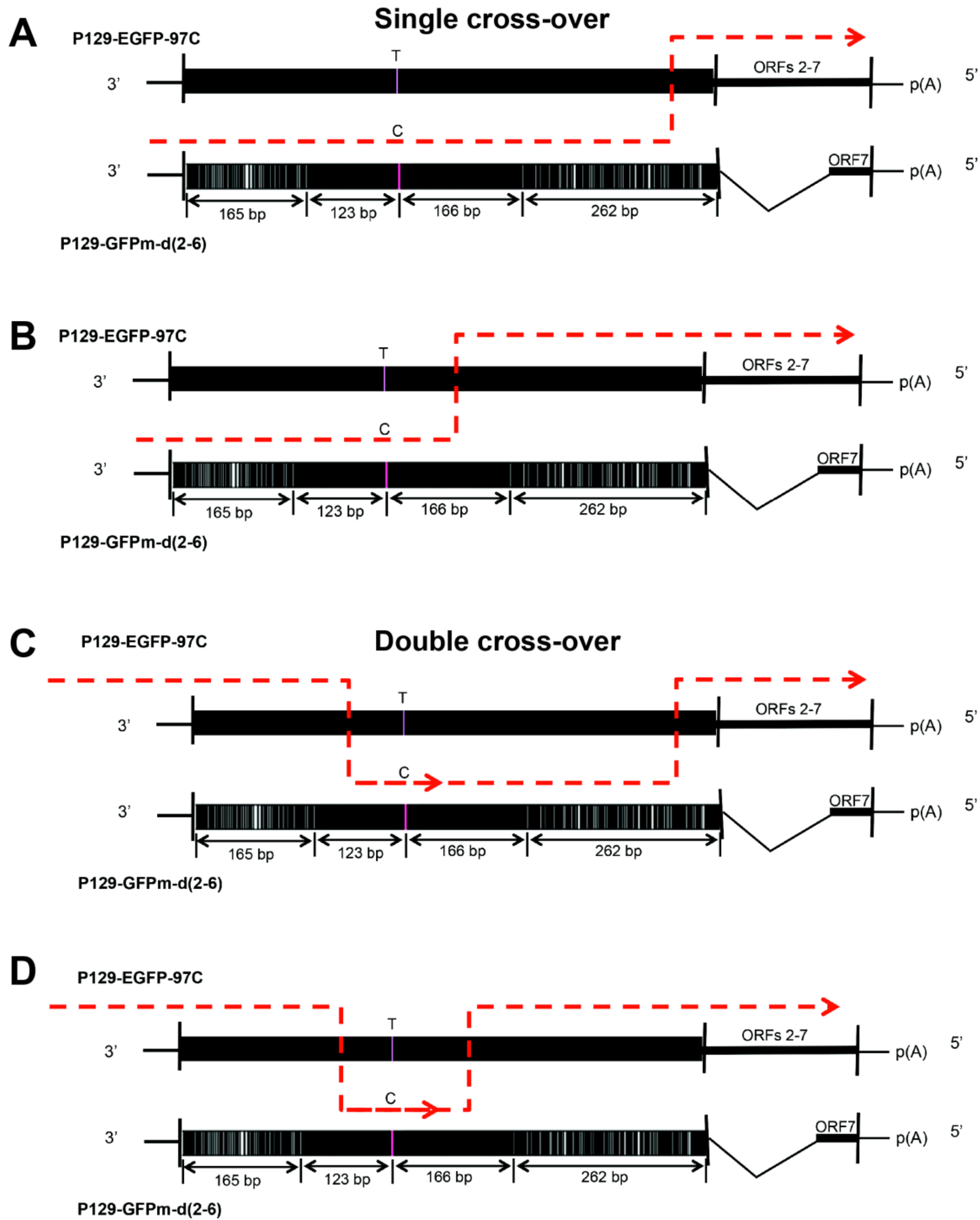


Figure 4.3 Four potential cross-over patterns for the recombination events.

Two single cross-over (4.3A and 4.3B) and two double cross-over patterns (4.3C and 4.3D) could be used to produce the viable fluorescent recombined viruses in this *in vitro* reverse genetics system.

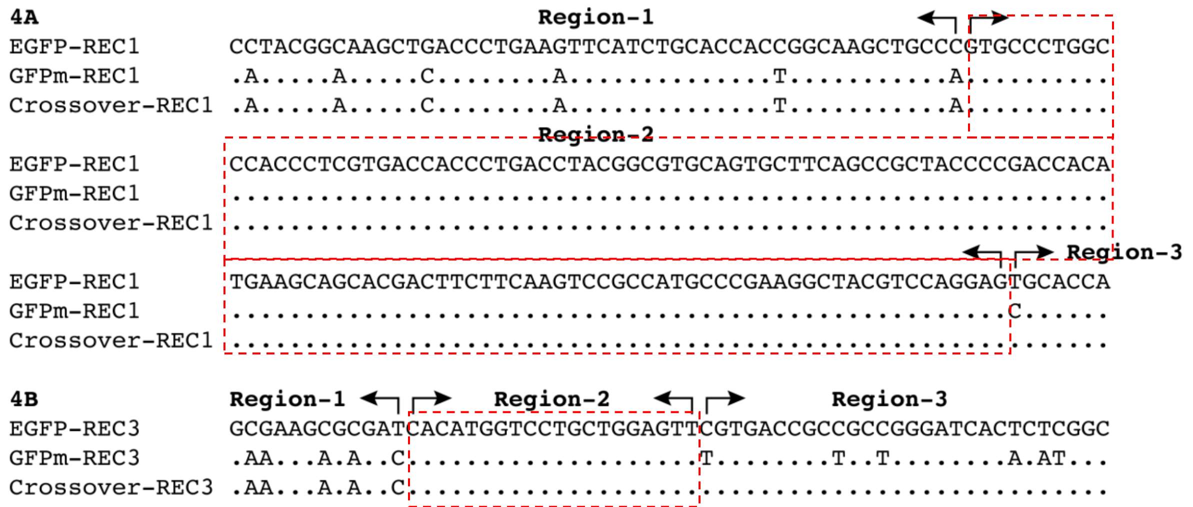


Figure 4.4 The alignment analyses identified two cross-over events.

The recombinants from cross-over events in the 123bp conserved region (4.4A) and 20bp conserved region (4.4B) were identified. The recombinants were identical to GFPm in region-1 and identical to EGFP in region-3. The conserved and cross-over regions were shown as region-2 in the red box.

Table 4.1 Primers used in this study.

PCR	Name	Sequence*(5'-3')
1st round	Univ-A-Rec-F1	**TCTCGGTTCTGCATTCGA**CCCCGTCATTGAACCAACTTT
	Univ-B-Rec-R1	**ATTCGCTGGCACGCACTT**TGTAGTTGCCGTCGTCCTTGA
	Univ-A-Rec-F2	**TCTCGGTTCTGCATTCGA**TCGTGACCACCCTGACCTAC
	Univ-B-Rec-R2	**ATTCGCTGGCACGCACTT**CGTTGTGGCTGTTGTAGTTGTA
	Univ-A-Rec-F3	**TCTCGGTTCTGCATTCGA**TACAACTACAACAGCCACAACG
	Univ-B-Rec-R3	**ATTCGCTGGCACGCACTT**TGTTCCGCTGAAACTCTGGT
2nd round	A-KMID1-Univ-A	**CCATCTCATCCCTGCGTGTCTCCGAC***TCAG*ACACGACGACT**TCTCGGTTCTGCATTCGA**
	B-K-Univ-B	**CCTATCCCCTGTGTGCCTTGGCAGTC***TCAG***ATTCGCTGGCACGCACTT**

* The universal tails are shown in bold, 454 adaptor sequences are highlighted in bold and underlined, the key sequences are shown in italic, and the multiple identifier (MID) is underlined.

Table 4.2 Mutations and their percentages in the co-infected sample.

Amplicon	Location*	Reference nucleotide (GFPm)	Variant nucleotide (EGFP)	Total Depth	Variant Frequency
>Rec1-EGFP	21	A	G	7182	37%
>Rec1-EGFP	30	T	C	7093	37%
>Rec1-EGFP	33	C	G	7086	37%
>Rec1-EGFP	39	C	G	7040	37%
>Rec1-EGFP	48	C	G	6797	35%
>Rec1-EGFP	51	G	C	6731	34%
>Rec1-EGFP	54	A	G	6716	34%
>Rec1-EGFP	60	T	C	6683	34%
>Rec1-EGFP	66	T	C	6315	31%
>Rec1-EGFP	117	A	C	6437	41%
>Rec1-EGFP	123	A	C	6425	41%
>Rec1-EGFP	129	C	G	6416	39%
>Rec1-EGFP	138	A	G	6403	39%
>Rec1-EGFP	153	T	C	6343	38%
>Rec1-EGFP	165	A	C	6300	36%
>Rec2-EGFP	**289**	**C**	**T**	**15531**	**20%**
>Rec3-EGFP	*456*	*C*	*T*	*15566*	*29%*
>Rec3-EGFP	*474*	*A*	*G*	*14345*	*37%*
>Rec3-EGFP	*480*	*T*	*C*	*14178*	*39%*
>Rec3-EGFP	*492*	*C*	*G*	*13736*	*43%*
>Rec3-EGFP	*505*	*A*	*C*	*13035*	*45%*
>Rec3-EGFP	*507*	*A*	*C*	*13023*	*45%*
>Rec3-EGFP	*514*	*A*	*G*	*12684*	*39%*
>Rec3-EGFP	*607*	*T*	*A*	*8032*	*37%*
>Rec3-EGFP	*608*	*C*	*G*	*8029*	*37%*
>Rec3-EGFP	*618*	*T*	*C*	*8407*	*36%*
>Rec3-EGFP	*633*	*T*	*C*	*8606*	*35%*
>Rec3-EGFP	*641*	*A*	*C*	*8598*	*36%*
>Rec3-EGFP	*642*	*A*	*G*	*8598*	*36%*
>Rec3-EGFP	*646*	*A*	*C*	*8711*	*35%*
>Rec3-EGFP	*648*	*A*	*C*	*8706*	*35%*
>Rec3-EGFP	*651*	*C*	*T*	*8698*	*35%*
>Rec3-EGFP	672	T	C	8616	100%
>Rec3-EGFP	681	T	C	8601	100%
>Rec3-EGFP	684	T	C	8591	100%
>Rec3-EGFP	693	A	T	8643	100%
>Rec3-EGFP	695	A	T	8648	100%
>Rec3-EGFP	696	T	C	8646	100%

* The locations of mutations are determined according to EGFP sequence. Mutations in the variable region (from 21bp to 165bp) are in regular. The $C_{289}T$ mutation is highlighted in bold and underlined. Mutations in the second variable region (from 456bp to 651bp) are marked in italic. Mutations in the third variable region (from 672bp to 696bp) are underlined.

Chapter 5 - Analysis of B-cell repertoire related to PRRSV neutralization

Abstract:

Neutralizing antibodies (nAbs) play an important role in protective immunity against PRRSV infection; however, PRRSV infection and vaccination usually induce weak and delayed nAbs. Lack of knowledge about PRRSV-specific nAbs has become a big obstacle to develop an effective PRRSV vaccine. Within the PRRS Host Genetics Consortium (PHGC) project, pigs producing a wide range of humoral immune responses from no detectable nAbs to broad nAbs have been identified. In this study, we analyzed the B cell repertoires from pigs that produced no nAbs, homologous nAbs, and broad nAbs. Swine VDJ gene segments were amplified using a single primer pair, cloned into TOPO pCR2.1 vector and submitted to high-throughput sequencing. A total of 385 VDJ sequences were obtained from mock-infected and PRRSV-infected pigs. Sequence alignment showed that the diversification of the VDJ gene was mainly due to the variation in CDRs, especially CDR3, which is the main determinant of antibody-antigen binding. Seven major V_H genes accounted for >70% of the antibody repertoires from mock-infected pigs, whereas, the percentages were <50% in PRRSV-infected pigs. In addition, one, six and two lineages were considered as the candidates associated with non-nAbs, homologous nAbs, and broad nAbs, respectively. This study provided a simple straightforward method to analyze swine immunoglobulin VDJ repertoires and identified potential lineages associated with different antibody responses.

5.1. Introduction

Neutralizing antibodies (nAbs) are important effectors of immunity against viruses (259). NAbs are considered important for protective immunity against PRRSV (133). However, the production of high titer homologous nAbs or broad nAbs are rarely observed after PRRSV infection. Therefore, current PRRSV vaccines cannot provide efficient protection, especially against heterologous isolates (66, 114, 157). The PRRS Host Genetics Consortium (PHGC) project aims to identify genetic determinants of resistance/susceptibility of commercial swine to PRRSV infection (136, 185). During the experimental infection of more than 3000 pigs with PRRSV, we have identified pigs producing a wide range of humoral immune responses, from no detectable nAbs to broad nAbs. No detectable nAbs (non-nAbs) indicate the serum has no neutralization activity against the inoculum virus and other isolates. Homologous nAbs (Homo-nAbs) only have neutralization capacity against the inoculum virus but not other heterologous isolates. Broad nAbs can neutralize not only the inoculum virus, but also a large number of unrelated isolates, including both type 1 and type 2 PRRSV. Pigs that produce non-nAbs, homologous nAbs, and broad nAbs provide an ideal opportunity to explore swine B cell repertoires associated with different antibody responses.

The genetics of swine immunoglobulin were briefly reviewed in Chapter 1.6. Antibody binding to antigen is primarily determined by the heavy chain (34, 165). Compared to mice and humans, there are relatively few genes involved in antibody production in pig. Swine utilize seven major V_H genes (V_HA, V_HB, V_HC, V_HE, V_HF, V_HY, V_HZ), two D_H segments and a single J_H gene to account for nearly entire (>90%) VDJ pre-immune repertoire (34). Furthermore, the exposure to environmental antigen does not change V_H genes that comprise the pre-immune repertoire. The same V_H genes comprise the adaptive repertoire but ~90% of them are somatically mutated (34). This unique feature provides an opportunity to analyze porcine antibody repertoire by detecting the entire VDJ repertoire, which can be recovered using a single PCR primer set.

It’s known that PRRSV subverts antibody repertoire development by proliferation of B cells bearing heavy chain hydrophobic CDR3 (37). However, the antibody repertoires associated with PRRSV neutralization have not been investigated. This study used a simple method to analyze the immunoglobulin heavy chain repertoires of samples from pigs producing different antibody responses and attempted to identify PRRSV-activated B cells and lineages potentially associated with different antibody responses.

5.2. Materials and Methods

5.2.1 Primer design and sample selection

The design of primers was based on the alignment of 46 swine Ig VDJ cDNA sequences obtained from GenBank (Table 5.1). Even though the sequences were unique, the differences were concentrated in the complementarity determining regions (CDRs), whereas the 5'UTR and framework regions (FRs) were relatively conserved (Fig. 5.1). Therefore, the entire VDJ repertoire could be analyzed by using only a single primer set with forward (5UTR-F: 5'-ATGGAGTTTCGGCTGAACT-3') and reverse (FR4-R: 5'-TGAGGACACGACGACTTCA-3') primers located within 5'UTR and FR4, respectively. The samples used in this study were derived from the PRRS Host Genetics Consortium (PHGC) PRRS-CAP Project (16, 136, 185). At 42dpi, pigs produced a wide range of antibody responses, including non-nAbs (pig no. 63), homologous nAbs (pig no. 21), and broad nAbs (pig no. 45) (Table 5.2). Lymph nodes collected at 42dpi from mock-infected pig (no. 22) as well as the above three PRRSV-infected pigs were used in the repertoire analysis. According to clonal expansion theory, antigen activated B cells undergo clonal expansion to create a population of identical B cell clones, the majority of which become plasma cells to secret identical antibodies (20, 101). Recapturing a VDJ sequence in two separate aliquots requires at least two cells of the same B-cell clone expressing this VDJ sequence (237). Therefore, two aliquots from the lymph node of each pig were utilized to determine the activated B-cell repertoires.

5.2.2 Cloning and sequencing

Lymph nodes were homogenized with TRIzol® Reagent (Invitrogen) at the concentration of 50mg/ml. Total RNA was extracted from the homogenized samples using TRIzol® Reagent (Invitrogen) and eluted in 50 µl RNase-free water. cDNA was generated by reverse transcription using random hexamer primers (Transcriptor High Fidelity cDNA Synthesis Kit, Roche). PCR was performed with the above-mentioned primer set (5UTR-F and FR4-R) amplifying entire swine VDJ repertoires (GoTaq® Green Master Mix, Promega). The annealing temperature was set at 50 °C and the final concentrations of primers were 0.25 µM. PCR products were purified (Wizard® SV Gel and PCR Clean-Up System, Promega), cloned into pCR2.1-TOPO Vector (TOPO TA Cloning® Kit, Invitrogen), and transformed into NEB 10-beta Competent *E. coli* (NEW ENGLAND BioLabs) according to recommended protocols. Positive clones were determined by blue-white selection (0.1mM IPTG and 40µg/ml Xgal) and colony PCR. Plasmid DNAs from positive clones were purified (PureYield™ Plasmid Miniprep System, Promega), the

concentrations were measured (NanoDrop ND-8000 Spectrophotometer, Thermo Scientific), and then submitted for sequencing using M13 primers (ACGT, INC.).

5.2.3 Phylogenetic analyses

A total of 385 swine VDJ sequences were obtained, including 78, 74, 104, and 129 sequences from the mock-infected pig, non-nAbs pig, homo-nAbs pig, and broad-nAbs pig, respectively. Multiplex sequence alignments were generated using CLUSTAL X version 1.83 (100). Phylogenetic analyses were conducted with MEGA4 program (221). Phylogenetic trees were constructed from aligned amino acid sequences using neighbor-joining method. The robustness of the phylogenetic constructions was evaluated by bootstrapping using 1000 replicates. The other default parameters were the same as in our previous report (55).

5.2.4 Selection of lineages potentially associated with different antibody responses

Although it is not currently possible to identify functional antibodies solely based on the sequences, a number of strategies have been developed to determine functional antibodies from antibody sequences, including population-based strategy, sequence-based strategy and evolution-based strategy (258). In this study, the following criteria were used to select lineages that might associate with PRRSV nAbs (116, 176, 248, 258). 1): Recapturing a same sequence from two separate aliquots indicates at least two identical cells expressing this VDJ sequence (237). Only sequences that could be recaptured in both aliquots were considered as the candidates. 2): Highly prevalent antibody sequences suggest a particular biological function (176). Only the abundant lineages (>3 VDJ sequences/lineage) were considered. 3): High sequence identity (>95%) indicates antibodies have similar recognition (258). Only one of the lineages with high similarity was considered. 4): Evolutionary similarity often reveals functional relationships between proteins (258). A phylogenetic tree was constructed. Only one lineage from each branch of the phylogenetic tree was considered. 5): The lineages that were clustered in the same branch with lineages identified in mock-infected pig or non-nAbs pig indicated they were likely induced by other factors not related to PRRSV, or probably encoded non-nAbs, were not considered as the candidate genes of homologous or broad nAbs. 6): About 90% of swine V_H genes are somatically mutated to comprise the adaptive repertoire (34). Similar phenomenon was observed that HIV-1 broad nAb VRC01 accumulates roughly 70 amino acids changes during the maturation process (248). Furthermore, mutations in framework regions (FRs) are generally required for broad nAbs against HIV (116). Therefore, VDJ sequences containing mutations in FRs were considered as candidate genes of broad nAbs.

5.3. Results

5.3.1 Analysis of heavy chain sequences

As shown in Figure 5.1b, the alignment of 385 VDJ sequences indicated that the diversification of VDJ genes was mainly due to the variation in the CDRs, which was consistent with previous studies (27, 34, 37). The diversification of CDRs was not only due to a high mutation rate, but also due to the variation in length. According to the variation of CDR1 and CDR2 sequences, swine V_H genes can be clustered into at least 25 types (Fig. 5.2), of which, seven major V_H genes account for the vast majority of the antibody repertoire (Fig. 5.3). CDR3 showed the highest diversity when compared with CDR1, CDR2, and framework regions (FRs). The length of CDR1 was 5 aa and CDR2 was 6-9 aa; the length of CDR3 varied from 5 to 24 aa (Table 5.3). CDR3 includes part of FR3, entire D_H and J_H regions. CDR3 extends from but doesn't include the FR3 cysteine-104, down to the 5' region of J_H, not including the invariant tryptophan that starts FR4 (37). The phylogenetic tree constructed based on 385 VDJ sequences showed that the primer set used in this study could amplify all seven major V_H genes (Fig. 5.4). In addition, we did not find any sequences shared between different pigs, which were consistent with the notion that each individual has a unique B-cell repertoire formed via VDJ rearrangement, somatic hypermutation (SHM) and gene conversion (27, 34, 76).

Seven major V_H genes, two D_H genes and a single J_H account for >90% of the preimmune repertoire (24, 34, 212). Figure 5.3 shows that for the mock-infected pig, no. 22, the seven major V_H genes (CDR1 and CDR2) still accounted for >70% of the antibody repertoire at 42 days of age. However, in PRRSV-infected pigs, the percentages were <50% mainly due to SHM in CDRs. Comparison of the B-cell repertoires between mock-infected and PRRSV-infected pigs also showed that the percentages of V_HA and V_HB genes in PRRSV-infected pigs decreased from >23% to <15% and from >13% to <4%, respectively. However, the percentage of V_HC gene increased from 10% to >16% in PRRSV-infected group, suggesting that PRRSV infection selected specific antibody lineages (37).

5.3.2 Identification of an activated B-cell compartment

The criteria for the selection of VDJ sequences from activated B cells were described in materials and methods 5.2.4 and shown in Figure 5.5. For mock-infected pig no. 22, 78 sequences were obtained from two separate aliquots containing 41 and 37 sequences, respectively. As shown in table 5.3, five lineages of VDJ sequences had identical CDR3; however, only one lineage (sequences 22-15 and 22-140) was shared between two aliquots and

all of the five lineages were less abundant with only two identical sequences. Therefore, no sequence from the mock-infected pig met the criteria. The duplicate sequences might be age-related (22).

For PRRSV-infected pigs (Table 5.3), 74 sequences (38 and 36 sequences from each aliquot) were obtained from pig no. 63, which didn't produce nAbs. Two lineages had identical sequences but only one lineage met our criteria, which was shared between two aliquots and abundant (>3 sequences) (Fig 5.6A). In pig no. 21, which produced high titers of PRRSV homologous nAbs, we had 104 sequences (52 sequences from each aliquot). Sixteen lineages had identical or highly similar CDR3 with the maximum of a lineage containing 9 identical CDR3s (Fig 5.6B1). Sequences in 10 of the 16 lineages were shared between aliquots, within which, six lineages met our criteria (Fig 5.6B1-B6). In pig no. 45, which produced broad nAbs, we got 129 sequences (73 and 56 from each aliquot). There were 14 lineages sharing identical or highly similar CDR3 with 6 lineages sharing the maximum of 3 identical CDR3s. Four of the 14 lineages were shared between aliquots, and two of them met our criteria (Fig 5.6C1-C2).

5.4. Discussion

Much of the work on the development of the swine antibody repertoire focused on studies of fetal and neonatal piglets (21-23, 25, 26, 28-33, 35, 36, 81, 146, 200, 201, 212, 214-216, 243). Only a few Ig genes account for a majority of the antibody repertoire (seven V_H genes account for >95%; two V_κ genes for ~80%; three V_λ genes for ~70%) (27, 36, 243). Furthermore, the usage of variable region gene segments remains constant during fetal and postnatal development even though 90% of the adaptive repertoire is somatically mutated (28, 34). Similar results were obtained in this study. As shown in Figure 5.3, seven major V_H genes accounted for >70% of the postnatal antibody repertoire. However, the usage of major V_H genes in PRRSV infected pigs was significantly lower (<50%) than in the mock-infected pig ($p<0.05$), mainly due to the selection of new abundant V_H genes (Table 5.3). The abundant sequences identified in PRRSV-infected pigs account for ~19% (58/307) of the VDJ repertoire, none of which is identical to the major or other known types of V_H genes (Table 5.3, Fig. 5.2). Phylogenetic analysis also showed that all the abundant sequences from PRRSV-infected pigs are not closely related with the major V_H genes (Fig. 5.4). The diversification of VDJ sequences is mainly due to high mutation rate in CDRs and the wide variation in length of CDR3.

In this study, we used a simple method to investigate swine immunoglobulin heavy chain VDJ repertoires of lymph nodes. This method could detect all major swine V_H genes (Fig. 5.4). We identified abundant sequences in all PRRSV-infected pigs that displayed different antibody

responses. Capture-recapture analysis using two separate aliquots from the same lymph node was performed to refine the activated B-cell clones. The majority (10/15) of abundant sequences from PRRSV-infected pigs could be recaptured. We believe if the depth coverage of sequencing goes higher, there will be a higher percentage of abundant sequences that can be detected in both aliquots. Assuming no other antigens stimulated immune responses in these pigs and random proliferation of B cells could not generate shared and abundant VDJ genes during PRRSV infection, these shared and abundant sequences were likely from PRRSV-activated B-cells.

Pigs that induced non-nAbs (pig no. 63) and broad nAbs (pig no. 45), only have the maximum coverage of 3 identical sequences; however, pig no. 21 that induced homologous nAbs, has the maximum coverage of 9 identical sequences, which are consistent with the no nAbs, low titer of broad nAbs, and high titer of homologous nAbs responses (Table 5.2). As shown in Fig 5.6C1, CDRs of this lineage were identical among three sequences, but four mutations that conserved in two sequences presented in FRs, which was consistent with a previous report that somatic mutations in immunoglobulin FRs were generally required for broad HIV-1 neutralization (116). In addition, several duplicate sequences could also be detected in both mock-infected and PRRSV-infected pigs (Table 5.3). The duplicate sequences were probably age-related, but not PRRSV-related as previously described (22). B cells from various lymphoid tissues of a PRRSV-infected pig sampled at the same time displayed a similar pattern suggesting widespread dissemination of the same B cell clones (22, 124). Therefore, B cell repertoires of the lymph nodes identified in this study might represent the entire B cell repertoires of PRRSV-infected pigs, which support that the shared and abundant sequences in these pigs were potentially correlated with their different antibody responses.

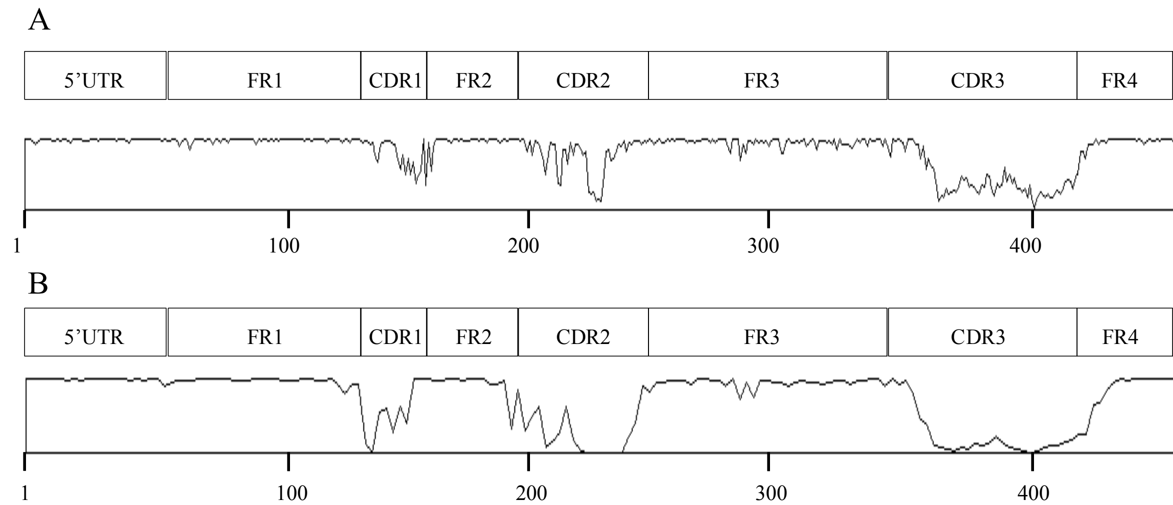


Figure 5.1 The variation of swine VDJ genes is concentrated in the CDRs.

5.1A, Comparison of 46 swine Ig heavy chain variable VDJ region mRNAs obtained from GenBank shows the variation frequency. Three complementarity determining regions (CDRs) are hypervariable while the 5’UTR and framework regions (FRs) are relatively conserved. 5.1B, Comparison of 385 swine Ig VDJ sequences shows quite similar results.

```
#VHA MEFRLNWVVL FALLQGVQGE EKLVESGGGL VQPGGSLRLS CVGSGFTFSS TYINWVRQAP GKGLEWLAAI STS--GGSTY YADSVKGRFT ISRDNSQNTA YLQMNSLRTE DTARYYCAR- --- [123]
#VHB MEFRLNWVVL FALLQGVQGE EKLVESGGGL VQPGGSLRLS CVGSGFDFSD NAFSWVRQAP GKGLEWVAAI ASSDYDGSTY YADSVKGRFT ISRDNSQNTV YLQMNSLRTE DTARYYCAI- --- [123]
#VHC MEFRLNWVVL FALLQGVQGE EKLVESGGGL VQPGGSLRLS CVGSGFTFSS YEISWVRQAP GKGLEWLAGI YSS--GGSTY YADSVKGRFT ISRDNSQNTA YLQMNSLRTE DTARYYCAR- --- [123]
#VHE MEFRLNWVVL FALLQGVQGE EKLVESGGGL VQPGGSLRLS CVGSGFTFSS YAVSWVRQAP GKGLEWLAGI DSGSYSGSTY YADSVKGRFT ISRDNSQNTA YLQMNSLRTE DTARYYCAR- --- [123]
#VHF MEFRLNWVVL FALLQGVQGQ EKLVESGGGL VQPGGSLRLS CVGSGFDFSS YGVGWVRQAP GKGLESLASI GSGSYIGSTD DADSVKGRFT ISSDNSQNTA YLQMNSLRTE DTARYYCAR- --- [123]
#VHG MEFRLNWVVL FALLQGVQGQ EKLVESGGGL VQPGGSLRLS CVGSGFDFSS YSMSWVRQAP GKGLEWVAGI YSS--GSSTY YADSVKGRFT ISSDDSQNTV YLQMNSLRTE DTARYYCAI- --- [123]
#VHH MEFRLNWVVL FALLQGVQGE VKLVESGGGL VQPGGSLRLS CVGSVFDFSS YAVSWVRQAP GKGLEWLAAI YS---GGSSY YADSVKGRFT ISKDNSQNTA YLQMNSLRTE DTARYYCATG --- [123]
#VHI MEFRLNWVVL FALLQGVQGE EKLVESGGGL VQPGGSLRLS CVGSGFTFSS YAVSWVRQAP GKGLEWLACI YSS--GSSTY YADSVKGRFT ISRDNSQNNN GLSANEQPEN RRHGPDITVQ EAQ [123]
#VHJ MEFRLNWVVL FALLQGVQGE EKLVESGGGL VQPGGSLRLS CVGSGITFSS YAVEWVRQAP GKGLEWLASI GSGSYIGSTD YADSVKGRFT ISSDDSQNTV YLQMNSLRTE DTAPITVQE- --- [123]
#VHK LEFWLN-VVL FALLQGVQGE EKLVESGGGL VQPGGSLRLS CVGSGFTFSS SPIGWVRQAP GKGLEWLASI GSGSYSGSTY YADSVNGRFT ISRDNSQNTA YLQMNSLRTE DTARYYCAR- --- [123]
#VHL ---------- ---------E VKLVESGGGL VQPGGSLRLS CIGSVFDFSS YAVSWVRQAP GKGLEWLAAI YS---GGSSY YADSVKGRFT ISKDNSQNTA YLQMN----- ---------- --- [123]
#VHM ---------- ---------- ---------- -----SLRLS CVGSGFDFSS YGVGWVRQAP GKGLEWLAGI YSG---GSTY YADSVKGRFT ISRDNSQNTV YLQMT----- ---------- --- [123]
#VHN MEFRLNWVVL FALLQGVQGE EKLVESGGGL VQPGGSLRLS CVGSGFTFSS YSMSWVRQAP GKGLEWLAGI YSS--GSSTY YADSVKGRFT ISSDNSQNMA YLQM------ ---------- --- [123]
#VHO ---------- ---------E EKLVESGGGL VQPGGSLRLS CVGSGYTFSS YPIGWVRQAP GKGLEWLAAI STS--GSSTY YADSVKGRFT ISRDNSQNTA YLQMT----- ---------- --- [123]
#VHP ---------- ---------- ---------- -----SLRLS CVGSGFDFSD YAFSWVCQAP GKGLEWLAAI STS--GSSTY YADSVKGRFT ISRDNSQNTA YLQMT----- ---------- --- [123]
#VHQ ---------- ---------E EKLVESGGGL VQPGGSLRLS CVGSGFTFSS YEISWVRQAP GKGLEWLAAI STS--GAGTV YADSVKDRFI YSRDNSQNTA YLQMN----- ---------- --- [123]
#VHR LEFRLNWVVL FALLQGFQGE VKLVESGGGL VQPGGSLRLS CVGSGYTFSS YPIGWVRQAP GKGLEWLACI YSS--GSSTY YADSVKGRFT ISKDNSQNNN SLSANDQ--- ---------- --- [123]
#VHS ---------- ---------E EKLVESGGGL VQPGGSLRLS CVGSGFTFSS YNMIWVRQAP GKGLEWLAYI TSS--GGSTY YADSVKGRFT ISSDNSQNTA YLQMT----- ---------- --- [123]
#VHT ---------- ---------E EKLVESGGGL VQPGGSLRLS CVGSGITFSS YAVSWVRQAP GKGLESLASI GSGSYIGSTD YADSVKGRFT ISSDDSQNTV YLQMN----- ---------- --- [123]
#VHU MEFRLNWVVL FALLQGVQGE EKLVESGGGL VQPGGSLRLS CVGSGFTFSS YEISWVRQAP GKGLEWLAAI GCGSYSGSTY YADSVKGRFT ISSDNSQNTA YLQMT----- ---------- --- [123]
#VHV MEFRLNWVVL FALLQGVQGE EKLVESGGGL VQPGGSLRLS CVGSGFTFSS TYINWVRQAP GKGLEWVAAI ASSDYDGSTY YADSVKGRFT ISSDNSQNTA YLQMT----- ---------- --- [123]
#VHW ---------- ---------- ---------- -----SLRLS CVGSGFTFSS TYINWVRQAP GKGLESLASI GSGSYIGSTY YADSVKGRFT ISSDDSQNMV YLQMT----- ---------- --- [123]
#VHX MEFRLNWVVL FALLQGVQGE EKLVESGGGL VQPGWSLRLS CVGSGYTFSS YGIGWVRQAP GKGLEWLAGI YSG---GSTY YADSVKGRFT ISKDNSQNTA YLQMNSLRTE DTARYYCARG --- [123]
#VHY ---------- --------ME VKLVESGGGL VQPGGSLRLS CVGSGFDFSS YEIRWVR-AP GKGLEWVAAI STS--GGSTY YADSVKGRFT ISKDNSQNTV YLQMNSLRTE DTARYYCAI- --- [123]
#VHZ MEFRLNWVVL FALLQGVQGE EKLVESGGGL VQPGGSLRLS CVGSGFTFSS YSMSWVRQAP GKGLEWLACI YSS--GSSTY YADSVKGRFT ISRDNSQNTA YLQMNSLRTE DTARYYCAKG --- [123]
```

Figure 5.2 Alignment of 25 types of swine Ig V_H genes.

V_H genes can be clustered into at least 25 types according to CDR1 and CDR2 sequences. CDR1 and CDR2 are shown in grey. GenBank accession numbers for the 25 V_H genes: V_HA (AF064686), V_HB (AF064687), V_HC (AF064688), V_HE (AF064689), V_HF (AF064690), V_HG (AY911499), V_HH (AY911500), V_HI (AF064691), V_HJ (AY911501), V_HK (AF064692), V_HL (AF321839), V_HM (AF321840), V_HN (AF321841), V_HO (AF321842), V_HP (AF321843), V_HQ (AF321844), V_HR (AF321845), V_HS (AF321846), V_HT (AF321847), V_HU (AF321848), V_HV (AF321849), V_HW (AF321850), V_HX (AY911502), V_HY (AY911503), V_HZ (AY911504).

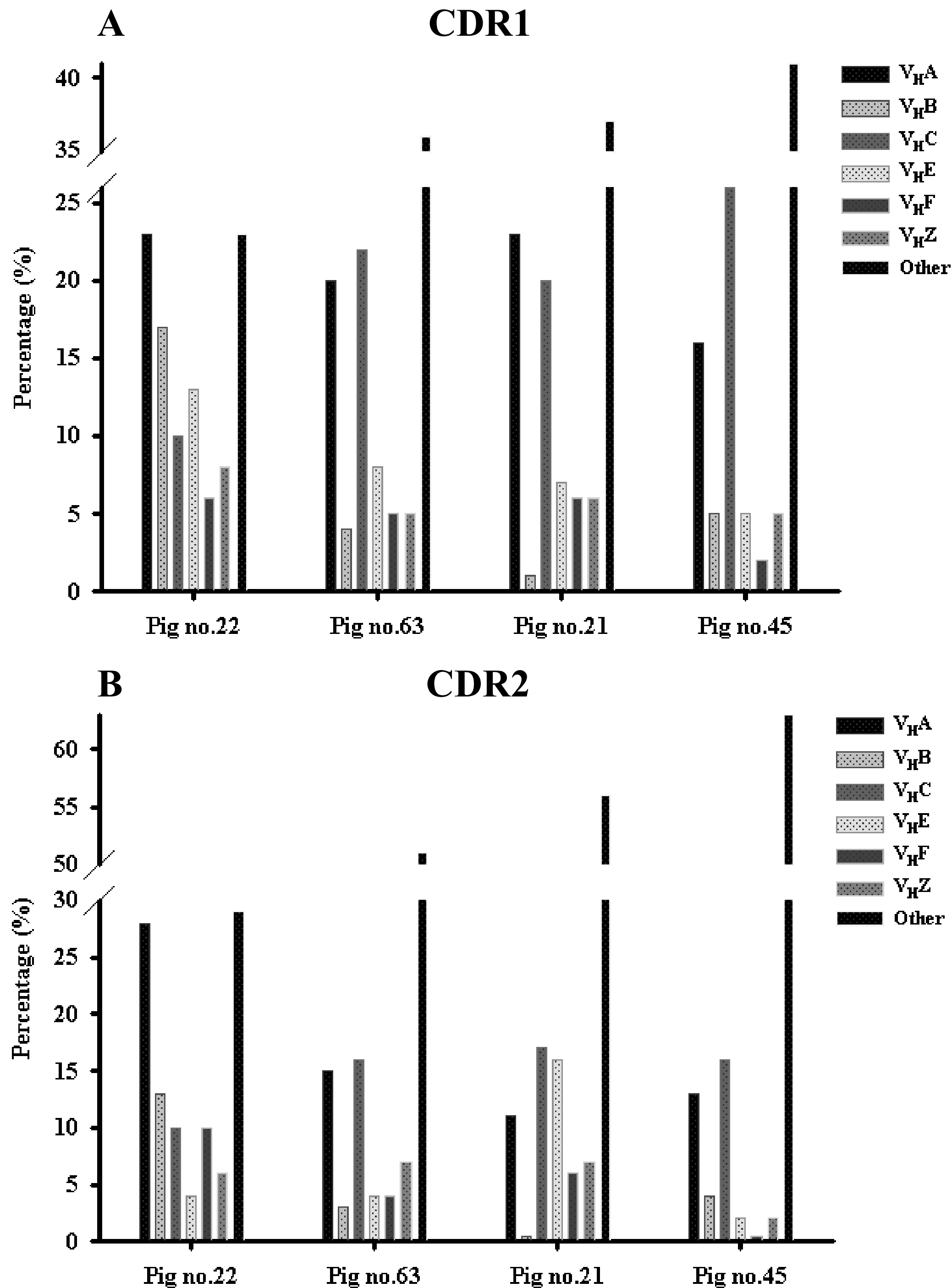


Figure 5.3 Percentages of major V_H genes comprising adaptive antibody repertoires.

(A) Percentage of CDR1 of major V_H genes in four pigs. (B) Percentage of CDR2 of major V_H genes. V_HY is not shown because CDR1 and CDR2 of VHY are identical to CDR1 of V_HC and CDR2 of V_HA, respectively. In the mock-infected pig no. 22, major V_H genes account for ~70% of the antibody repertoire, whereas in PRRSV infected pigs (no. 63, 21, and 45), major V_H genes account for <50% of the antibody repertoires.

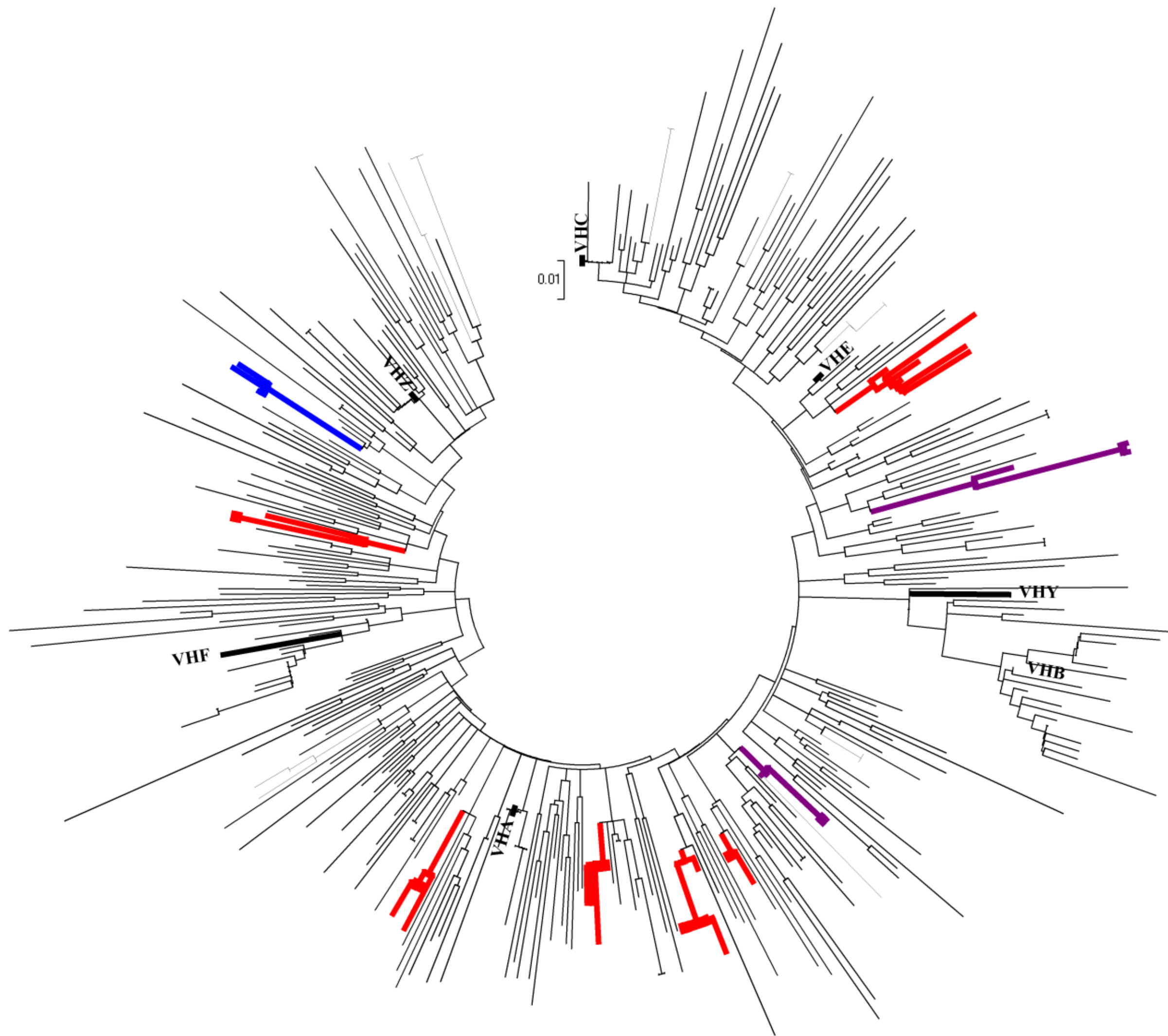


Figure 5.4 Phylogenetic analysis of swine VDJ sequences from four pigs.

A total of 385 VDJ sequences were obtained and used to construct the phylogenetic tree. The tree shows that all seven major V_H genes can be amplified using a single primer pair. The major V_H genes (V_HA, V_HB, V_HC, V_HE, V_HF, V_HY, and V_HZ) are shown in bold. A lineage of shared and abundant sequence from PRRSV-infected pig no. 63, which did not produce nAbs, is shown in blue. Six lineages of shared and abundant sequences from PRRSV-infected pig no. 21, which produced high titer of homologous nAbs, are shown in red. Two lineages of shared and abundant sequences from PRRSV-infected pig no. 45, which produced broad nAbs, are shown in purple.

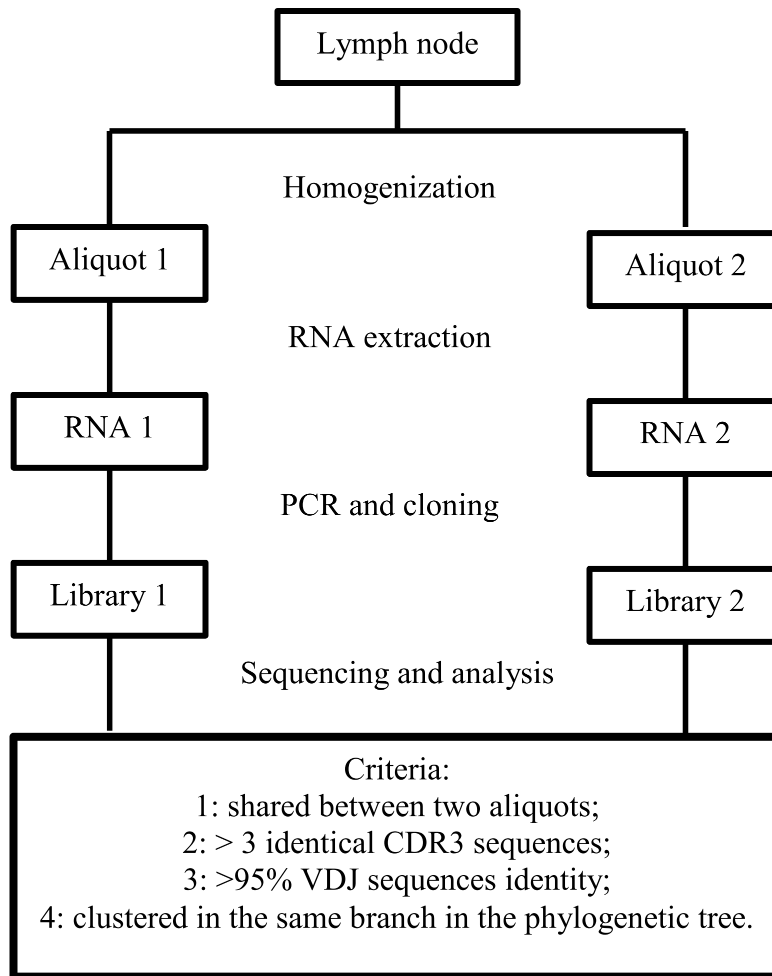


Figure 5.5 Experimental setup and selection criteria.

Lymph nodes were collected from mock- and PRRSV-infected pigs. Two separate aliquots of each homogenized lymph node were prepared and total RNAs were extracted. Two libraries were prepared and sequenced. VDJ sequences that met the above criteria were likely expressed by activated B cells.

```
A
Non-nAbs_63-26  MEFRLNWVLLFALLQGVQGEEKLVESGGGLVQPGGSLRLSCVGSGFTFSMYAVSWVRQAPGKGLEWLAGIYSSGSSTYYADSVKGRFTISRGDSQNTAYLQMNSLRREDTARYYCAGHSDGGGYGYYYFMNLWGPGVEVVVS
Non-nAbs_63-29  ......C.......................................................................................................................................
Non-nAbs_63-108 ....................G.........................................................................................................................
B1
Homo-nAbs_21-120 MEFRLNWVVLFALLQGVQGEEKLVESGGGLVQPGGSLRLSCVGSGFTFSNYEIKWVRQAPGKGLEWLASISTSGFSTYYADSVRGRFTSSRDNSQNTAYLQMNSLRTEDTARYFCASDYSGCSYGLRPHLWGPGVEVVVS
Homo-nAbs_21-27  ..................................................................................................................................-.........
Homo-nAbs_21-58  ............................................................................................................................................
Homo-nAbs_21-143 ............................................................................................................................................
Homo-nAbs_21-126 ......................V.............................................................................R.......................................
Homo-nAbs_21-147 ............................................................................................................................................
Homo-nAbs_21-122 ............................................................................................................................................
Homo-nAbs_21-116 .....................................................N..............G.YS..S........K.-......................................................
Homo-nAbs_21-152 .................................................RTY.N..............D.YS.DS........K....L...................................................
B2
Homo-nAbs_21-134 MEFRLNWVVLFALLQGVQGEVKLVESGGGLVQPGGSLRLSCVGSGFTFSGTGINWVRQAPGKGLEWLAACSGGGSNTYYADSVKGRFTISRDNSQNTAYLQMNSLRTEDTARYYCATSFSEANTISLWGPGVEVVVS
Homo-nAbs_21-108 ..............G-.........................................................................................................................
Homo-nAbs_21-38  .........................................................................................................................................
Homo-nAbs_21-42  .........................................................................................................................................
Homo-nAbs_21-51  ....................E....................................................................................................................
Homo-nAbs_21-137 ...........................................P.............................................................................................
B3
Homo-nAbs 21-62  MEFRLNWVVLFALLQGVQGEEKLVESGGGLVQPGGSLRLSCVGSGFTFSSYIVTWVRKAPGKGLEWLAHRSGSGISTYYADSVKGRFTISRDNSQNTAYLQMNSLRTEDTARYYCAKGRSGCTGNYDDWGPGVEVVVS
Homo-nAbs 21-118 ..............................................I....T.S...............I.S..Y...............................................................
Homo-nAbs 21-124 ........L..........................................T.S...............I....W.I..........A....D.............................................
Homo-nAbs 21-127 ...................................................T.S....................V...............................................................
Homo-nAbs 21-144 .........S.........................................T.S....................V.............T.................................................
Homo-nAbs 21-146 .................................................T.T.S...............I.S..FT...................S.........K................................
B4
Homo-nAbs 21-129 MEFRLNWVVLFALLQGVQGEEKLVESGGGLVQPGGSLRLSCVGSGFIFSSTYIHWVRQAPGKGLEWLAGTYTGGSNTYYADSVEGRFTISKDNSQNTAYLQMNSLRTEDTARYYCATVLVLLAIDIMDLWGPGVEVVVS
Homo-nAbs 21-64  ......C....................................................................................................................................
Homo-nAbs 21-55  ...........................................................................................................................................
Homo-nAbs 21-19  ..............................................T..RDE.S..............AVTAS..S.......K......R................................................
B5
Homo-nAbs 21-41  MEFRLNWVVLFALLQGVQGEEKLVESGGGLVQPGGSLRLSCVGSGFTFSSYEITWVRQAPGKGLEWLAGIYSSGFSTVYADSVKGRFTISRDNSQNTAYLQLNSLRTEDTARYYCAGQNDLLPGTIVDCAMNLWGPGVEVVVS
Homo-nAbs 21-103 ...............................................................................................................................................
Homo-nAbs 21-111 ....................................................................C...........R...........................................................
B6
Homo-nAbs_21-14  MEFRLNWVVLFALLQGVQGEEKLVESGGGLVQPGGSLKLSCVGSGFDFSSYNMIWVRQAPGKGLEWLAGI--TRFSGSTYYADSVKGRFTISRANSQNTAYLQMNSLRTEDTAHYYCTKNVYSYGTSCYDVVSMGLWGPGVEVVVS
Homo-nAbs_21-21  ......................................................................--..........................................................................
Homo-nAbs_21-141 .....................................R........I...TYIH..............A.GCASS..N...G......L....DD...................................................
C1
Broad-nAbS 45-3   MEFRLNWVVLFALLQSVQGEEKLVESGGGLVQPGGSLRLSCVGSGFTFSSSYINWVRQAPGKGLEWLAGIYSSGRNTYYADSVKGRFTISRDNSQNTAYLQMNSLRTEDTARYYCGRDGVYYELDLWGPGVEVVVS
Broad-nAbS 45-14  ...............G............................................................PQH.........................................................
Broad-nAbS 45-112 ...............G............................................................PQH.........................................................
C2
Broad-nAbs_45-25  MEFRLNWVVLFAPLQGVQGEEKLVESGGGLVQPGGSLRLSCVGSGYTLSSYDIGWVRQAPGRGLEWLAGLSSGGNTYYADSVKGRFTISREHPQNTAYLQMNSLRTEDTARYYCARESQFKYSVMDLWGPGVEVVVS
Broad-nAbs_45-41  ...............................................................................................................................-.........
Broad-nAbs_45-152 ............L................................F.F.GTY.N...................................................................................
```

Figure 5.6 Alignment of abundant and shared sequences likely expressed by activated B-cells.
In each pig, VDJ sequences with numbers <100 are from one aliquot and sequences with numbers >100 are from another aliquot. Three CDRs of each sequence were shown in grey.

Table 5.1 Swine Ig heavy chain VDJ region mRNAs used in this study.

No.	Name	GenBank No.	No.	Name	GenBank No.
1	N/A	SSU15194	24	pvg1a	SSU15522
2	N/A	SSU38217	25	pvg4	SSU15520
3	N/A	SSU38216	26	pvg4a	SSU15519
4	N/A	SSU38215	27	pvg2a	SSU15518
5	N/A	SSU38214	28	pvg25	SSU15517
6	N/A	SSU38213	29	pvg7a	SSU15459
7	N/A	SSU38212	30	pvg9	SSU15457
8	N/A	SSU38211	31	pvg5	SSU15456
9	N/A	SSU38210	32	pvg7	SSU15455
10	N/A	SSU38209	33	pvg19	SSU15454
11	N/A	SSU38208	34	pvg20	SSU15453
12	N/A	SSU38207	35	pvg23	SSU15452
13	N/A	SSU38206	36	pvg24	SSU15451
14	N/A	SSU38205	37	pvg17	SSU15447
15	N/A	SSU38204	38	pvg15a	SSU15446
16	N/A	SSU38203	39	PVM4A	SSU15439
17	N/A	SSU38202	40	PVM3A	SSU15438
18	N/A	SSU38201	41	PVM2A	SSU15437
19	PVAHDA	SSU15430	42	PVM1A	SSU15436
20	pvg5a	SSU15526	43	PVA2A	SSU15423
21	pvg6a	SSU15525	44	PVA5A	SSU15429
22	pvg8	SSU15524	45	PVA4A	SSU15428
23	pvg3	SSU15523	46	PVA3A	SSU15427

N/A: information not available.

Table 5.2 Virus neutralization properties of sera from the pigs used in this study.

Pig	KS62[1]	NVSL	VR-2332	P129	KS06	SD23983	AZ25	CO84	CO90	WY27	LV
	(100)[2]	(97)	(92)	(94)	(90)	(89)	(89)	(90)	(92)	(92)	(55)
No.22	ND[3]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
No.63	<16[4]	<16	<16	<16	<16	<16	ND	ND	ND	ND	<16
No.21	512	<16	<16	<16	<16	<16	ND[4]	ND	ND	ND	<16
No.45	64	32	16	32	16	16	16	32	32	16	64

[1]GenBank accession numbers for PRRSV isolates: KS62, KM035798; NVSL 97-7895, AY545985; VR-2332, AY150564; P129, AF494042; KS06, KM035803; SD23983, JX258843; AZ25, KM035800; CO84, KM03502; CO90, KM035799; WY27, KM035801; and Lelystad virus (LV), M96262.

[2]The numbers in parenthesis represent GP5 peptide sequence homology with KS62.

[3]ND, Not Determined.

[4]Neutralization titer showing inverse of the highest serum dilution with VN activity. Titer <16 indicates no detectable virus neutralization activity.

Table 5.3 Sequences with identical or high similar CDR3 from four pigs.

Sample	No.	Aliquot	Sequence	CDR1	CDR2	CDR3
Pig 22	1	1	22-15	SYGMS	NIYSTGS	ARGGGLLDG
		2	22-140	.....	....	....
	2	2	22-104	DYAFS	AIASSDYDG	AIGVDIAVPMDL
		2	22-129	.....	....	....
	3	2	22-105	STYIN	AISTSGG	ASRRYSGYVKARYAMDL
		2	22-146	.....	....	....
	4	2	22-109	SYEIS	AIGCGSYSG	AIHSYGASCYAVKYYAMDL
		2	22-135	.....	....	....
	5	2	22-117	SYSMS	CIYSSGS	ATDRYSDCYYAMDL
		2	22-122	.....	....	....
Pig 63	1	1	63-26	MYAVS	GIYSSGS	AGHSDGGGYGYYYFMNL
		1	63-29	.....	....	....
		2	63-108	.....	....	....
	2	2	63-138	RYEIS	GIYSSGG	ARFPCYRSDASCNYWEVDYYPMDL
		2	63-139	STY.N	A.ST...	....
Pig 21	1	1	21-27	NYEIK	SISTSGF	ASDYSGCSYGIRPHL
		1	21-58	.....	....	....
		2	21-116	.....	G.YS..S	....
		2	21-120	.....	....	....
		2	21-122	.....	....	....
		2	21-126	.....	....	....
		2	21-143	.....	....	....
		2	21-147	....N	....	....
		2	21-152	RTY.N	D.YS.DS	....
	2	1	21-38	GTGIN	ACSGGGS	ATSFSEANTISL
		1	21-42	.....	....	....
		1	21-51	.....	....	....
		2	21-108	.....	....	....
		2	21-134	.....	....	....
		2	21-137	.....	....	....
	3	1	21-62	SYTVS	HRSGSGI	AKGRSGCTGNYDD
		2	21-118	.....	.I.S..Y	....
		2	21-124	.....	.I....W	....
		2	21-127	.....	V	....
		2	21-144	.....	V	....
		2	21-146	.....	.I.S..F	....
	4	2	21-129	STYIH	GTYTGGS	ATVLVLLAIDIMDL
		1	21-55	.....	....	....
		1	21-64	.....	....	....
		1	21-19	RDE.S	AVTAS..	....
	5	1	21-1	SYEIN	GIYSSGS	ARGIFCSKDGVSCYYMDL
		1	21-40	.....	....	....
		1	21-48	.....	....	....
	6	1	21-14	SYNMI	GITRFSG	TKNVYSYGTSCYDVVSMGL
		1	21-21	.....	....	....
		2	21-141	.TYIH	A.GCA.SSG	....
	7	1	21-41	SYEIT	GIYSSGF	AGQNDLLPGTIVDCAMNL
		2	21-103	.....	....	....
		2	21-111	.....	..C....	....
	8	2	21-110	STVIN	GINTSDG	AAESIDDIYGADCYALGADL

		2	21-148	.....	..R....	.T...E...P........GR
		1	21-46	....S	....	.K.........S...P....
	9	1	21-6-2	VTYIN	AISSSSS	VRIYGDYRSGVRDL
		1	21-65	.....	....	....
	10	1	21-11-2	SYEIS	DIYSSGS	RGDFYGYGGSPYMDL
		1	21-35	.....	....	....
	11	1	21-25	SYGVG	SGGSGSYID	ARGRIHDYSGCYSGSPGCAMDL
		2	21-132	.....	.I......G	............R.........
	12	1	21-28	TYEIS	GIYTSGR	ARGQAPSCEWTDTMDL
		2	21-121	S.D..	....	....
	13	1	21-36	SYEIN	GIYSSGS	ARERCYLYGRSCYDMDL
		2	21-138	..GV.	V	....
	14	2	21-105	GYGVG	SIGSGSYIG	ARTFCWNYGASCYSLYYYAMDL
		2	21-131	.....	.....V...	.........A............
	15	2	21-125	SLDIH	GISRSGG	YGLYL
		2	21-139	.....	....	.....
	16	2	21-140	SYEIN	AIRSSGG	ARGRGFYIAIAIGVTVKPMDL
		2	21-155	T.AMT	V...TSS	..............A......
Pig 45	1	1	45-25	SYDIG	GLSSGG	ARESQFKYSVMDL
		1	45-41	.....	......	....
		2	45-152	GTY.N	......	....
	2	1	45-3	SSYIN	GIYSSGR	GRDGVYYELDL
		1	45-14	.....	....	....
		2	45-112	.....	....	....
	3	1	45-13	SYGIG	AIYTGS	GRRWAYNNYLDL
		1	45-23	.....	......	....
		1	45-39	.....	......	....
	4	1	45-15	SYEIS	AMETSGS	ARGYRFGIRFYQYAMDL
		1	45-22	.....	....	....
		1	45-36	.....	....	....
	5	1	45-16	SYEIS	VIYSGG	VRGYGGICYGWYDGMDV
		1	45-30	.....	......	....
		1	45-6-2	ETY.-	AIGTTGR	....
	6	1	45-20	RHEIS	GIYSSGS	AIERPRYPTLLHDLYL
		1	45-42	.....	....	....
		1	45-8-2	NTYIN	A.ASDVHDG	....
	7	1	45-21	SYEIS	GIYASGG	ARFSSYGRYGDDGMDL
		1	45-81	.....	....	....
	8	1	45-43	STYIN	AISTSGG	ANGYGASDDLPMDV
		1	45-59	.....	....	....
	9	1	45-45	TTYIN	GISTSGG	ARDLYSYGTYSYGTADYTMDL
		1	45-73	.....	....	....
	10	1	45-47	SYALS	GIDSGSYTG	ARVRRTVAIAIAIGPMDL
		1	45-50	.....	....	....
	11	1	45-75	TYEIN	GIVSSGS	AKTNCYTYGSSCYRADAMDL
		2	45-111	S....	..Y....	..................N.
	12	1	45-80	SYAVS	AVSTSGT	TRGGFGGYGASGDL
		2	45-101	....N	.I...AG	..........T...
	13	2	45-104	GYSMS	CIDSGS	CARQSYYGADYYPK
		2	45-121	S..IG	GIY.SG	....
	14	2	45-113	SDYTFS	AIPKSVLDGR	VRAMVTVPI
		2	45-124	......	.....DW..V	....

References

1. **Aaziz, R., and M. Tepfer.** 1999. Recombination in RNA viruses and in virus-resistant transgenic plants. The Journal of general virology **80 (Pt 6):**1339-1346.
2. **Albina, E., F. Madec, R. Cariolet, and J. Torrison.** 1994. Immune response and persistence of the porcine reproductive and respiratory syndrome virus in infected pigs and farm units. The Veterinary record **134:**567-573.
3. **Alexopoulos, C., S. K. Kritas, C. S. Kyriakis, E. Tzika, and S. C. Kyriakis.** 2005. Sow performance in an endemically porcine reproductive and respiratory syndrome (PRRS)-infected farm after sow vaccination with an attenuated PRRS vaccine. Veterinary microbiology **111:**151-157.
4. **Allende, R., W. W. Laegreid, G. F. Kutish, J. A. Galeota, R. W. Wills, and F. A. Osorio.** 2000. Porcine reproductive and respiratory syndrome virus: description of persistence in individual pigs upon experimental infection. Journal of virology **74:**10834-10837.
5. **An, T. Q., Z. J. Tian, C. L. Leng, J. M. Peng, and G. Z. Tong.** 2011. Highly pathogenic porcine reproductive and respiratory syndrome virus, Asia. Emerging infectious diseases **17:**1782-1784.
6. **An, T. Q., Z. J. Tian, Y. Xiao, R. Li, J. M. Peng, T. C. Wei, Y. Zhang, Y. J. Zhou, and G. Z. Tong.** 2010. Origin of highly pathogenic porcine reproductive and respiratory syndrome virus, China. Emerging infectious diseases **16:**365-367.
7. **Ansari, I. H., B. Kwon, F. A. Osorio, and A. K. Pattnaik.** 2006. Influence of N-linked glycosylation of porcine reproductive and respiratory syndrome virus GP5 on virus infectivity, antigenicity, and ability to induce neutralizing antibodies. Journal of virology **80:**3994-4004.
8. **Barfoed, A. M., M. Blixenkrone-Moller, M. H. Jensen, A. Botner, and S. Kamstrup.** 2004. DNA vaccination of pigs with open reading frame 1-7 of PRRS virus. Vaccine **22:**3628-3641.
9. **Basel, M. T., S. Balivada, A. P. Beck, M. A. Kerrigan, M. M. Pyle, J. C. Dekkers, C. R. Wyatt, R. R. Rowland, D. E. Anderson, S. H. Bossmann, and D. L. Troyer.** 2012. Human xenografts are not rejected in a naturally occurring immunodeficient porcine line: a human tumor model in pigs. BioResearch open access **1:**63-68.
10. **Bastos, R. G., O. A. Dellagostin, R. G. Barletta, A. R. Doster, E. Nelson, F. Zuckermann, and F. A. Osorio.** 2004. Immune response of pigs inoculated with Mycobacterium bovis BCG expressing a truncated form of GP5 and M protein of porcine reproductive and respiratory syndrome virus. Vaccine **22:**467-474.
11. **Bautista, E. M., and T. W. Molitor.** 1997. Cell-mediated immunity to porcine reproductive and respiratory syndrome virus in swine. Viral immunology **10:**83-94.
12. **Bautista, E. M., and T. W. Molitor.** 1999. IFN gamma inhibits porcine reproductive and respiratory syndrome virus replication in macrophages. Archives of virology **144:**1191-1200.
13. **Beura, L. K., S. N. Sarkar, B. Kwon, S. Subramaniam, C. Jones, A. K. Pattnaik, and F. A. Osorio.** 2010. Porcine reproductive and respiratory syndrome virus nonstructural protein 1beta modulates host innate immune response by antagonizing IRF3 activation. Journal of virology **84:**1574-1584.

14. **Biron, C. A., K. B. Nguyen, G. C. Pien, L. P. Cousens, and T. P. Salazar-Mather.** 1999. Natural killer cells in antiviral defense: function and regulation by innate cytokines. Annual review of immunology **17:**189-220.
15. **Blackard, J. T., G. Ma, S. Sengupta, C. M. Martin, E. A. Powell, M. T. Shata, and K. E. Sherman.** 2014. Evidence of distinct populations of hepatitis C virus in the liver and plasma of patients co-infected with HIV and HCV. Journal of medical virology **86:**1332-1341.
16. **Boddicker, N., E. H. Waide, R. R. Rowland, J. K. Lunney, D. J. Garrick, J. M. Reecy, and J. C. Dekkers.** 2012. Evidence for a major QTL associated with host response to porcine reproductive and respiratory syndrome virus challenge. Journal of animal science **90:**1733-1746.
17. **Botner, A., B. Strandbygaard, K. J. Sorensen, P. Have, K. G. Madsen, E. S. Madsen, and S. Alexandersen.** 1997. Appearance of acute PRRS-like symptoms in sow herds after vaccination with a modified live PRRS vaccine. The Veterinary record **141:**497-499.
18. **Brar, M. S., M. Shi, R. K. Hui, and F. C. Leung.** 2014. Genomic evolution of porcine reproductive and respiratory syndrome virus (PRRSV) isolates revealed by deep sequencing. PloS one **9:**e88807.
19. **Brown, D. W.** 1997. Threat to Humans from Virus Infections of Non-human Primates. Reviews in medical virology **7:**239-246.
20. **Burnet, F. M.** 1976. A modification of Jerne's theory of antibody production using the concept of clonal selection. CA: a cancer journal for clinicians **26:**119-121.
21. **Butler, J. E., D. H. Francis, J. Freeling, P. Weber, and A. M. Krieg.** 2005. Antibody repertoire development in fetal and neonatal piglets. IX. Three pathogen-associated molecular patterns act synergistically to allow germfree piglets to respond to type 2 thymus-independent and thymus-dependent antigens. Journal of immunology **175:**6772-6785.
22. **Butler, J. E., C. D. Lemke, P. Weber, M. Sinkora, and K. M. Lager.** 2007. Antibody repertoire development in fetal and neonatal piglets: XIX. Undiversified B cells with hydrophobic HCDR3s preferentially proliferate in the porcine reproductive and respiratory syndrome. Journal of immunology **178:**6320-6331.
23. **Butler, J. E., K. Santiago-Mateo, X. Z. Sun, N. Wertz, M. Sinkora, and D. H. Francis.** 2011. Antibody repertoire development in fetal and neonatal piglets. XX. B cell lymphogenesis is absent in the ileal Peyer's patches, their repertoire development is antigen dependent, and they are not required for B cell maintenance. Journal of immunology **187:**5141-5149.
24. **Butler, J. E., J. Sun, and P. Navarro.** 1996. The swine Ig heavy chain locus has a single JH and no identifiable IgD. International immunology **8:**1897-1904.
25. **Butler, J. E., J. Sun, P. Weber, S. P. Ford, Z. Rehakova, J. Sinkora, and K. Lager.** 2001. Antibody repertoire development in fetal and neonatal piglets. IV. Switch recombination, primarily in fetal thymus, occurs independent of environmental antigen and is only weakly associated with repertoire diversification. Journal of immunology **167:**3239-3249.
26. **Butler, J. E., J. Sun, P. Weber, P. Navarro, and D. Francis.** 2000. Antibody repertoire development in fetal and newborn piglets, III. Colonization of the gastrointestinal tract

selectively diversifies the preimmune repertoire in mucosal lymphoid tissues. Immunology **100:**119-130.

27. **Butler, J. E., J. Sun, N. Wertz, and M. Sinkora.** 2006. Antibody repertoire development in swine. Developmental and comparative immunology **30:**199-221.
28. **Butler, J. E., X. Sun, N. Wertz, K. M. Lager, K. Chaloner, J. Urban, Jr., D. L. Francis, P. L. Nara, and G. J. Tobin.** 2011. Antibody repertoire development in fetal and neonatal piglets XXI. Usage of most VH genes remains constant during fetal and postnatal development. Molecular immunology **49:**483-494.
29. **Butler, J. E., X. Sun, N. Wertz, A. L. Vincent, E. L. Zanella, and K. M. Lager.** 2013. Antibody repertoire development in fetal and neonatal piglets. XVI. Influenza stimulates adaptive immunity, class switch and diversification of the IgG repertoire encoded by downstream Cgamma genes. Immunology **138:**134-144.
30. **Butler, J. E., P. Weber, M. Sinkora, D. Baker, A. Schoenherr, B. Mayer, and D. Francis.** 2002. Antibody repertoire development in fetal and neonatal piglets. VIII. Colonization is required for newborn piglets to make serum antibodies to T-dependent and type 2 T-independent antigens. Journal of immunology **169:**6822-6830.
31. **Butler, J. E., P. Weber, M. Sinkora, J. Sun, S. J. Ford, and R. K. Christenson.** 2000. Antibody repertoire development in fetal and neonatal piglets. II. Characterization of heavy chain complementarity-determining region 3 diversity in the developing fetus. Journal of immunology **165:**6999-7010.
32. **Butler, J. E., P. Weber, and N. Wertz.** 2006. Antibody repertoire development in fetal and neonatal piglets. XIII. Hybrid VH genes and the preimmune repertoire revisited. Journal of immunology **177:**5459-5470.
33. **Butler, J. E., and N. Wertz.** 2006. Antibody repertoire development in fetal and neonatal piglets. XVII. IgG subclass transcription revisited with emphasis on new IgG3. Journal of immunology **177:**5480-5489.
34. **Butler, J. E., and N. Wertz.** 2012. The porcine antibody repertoire: variations on the textbook theme. Frontiers in immunology **3:**153.
35. **Butler, J. E., N. Wertz, J. Sun, H. Wang, P. Chardon, F. Piumi, and K. Wells.** 2004. Antibody repertoire development in fetal and neonatal pigs. VII. Characterization of the preimmune kappa light chain repertoire. Journal of immunology **173:**6794-6805.
36. **Butler, J. E., N. Wertz, and X. Sun.** 2013. Antibody repertoire development in fetal and neonatal piglets. XIV. Highly restricted IGKV gene usage parallels the pattern seen with IGLV and IGHV. Molecular immunology **55:**329-336.
37. **Butler, J. E., N. Wertz, P. Weber, and K. M. Lager.** 2008. Porcine reproductive and respiratory syndrome virus subverts repertoire development by proliferation of germline-encoded B cells of all isotypes bearing hydrophobic heavy chain CDR3. Journal of immunology **180:**2347-2356.
38. **Calvert, J. G., D. E. Slade, S. L. Shields, R. Jolie, R. M. Mannan, R. G. Ankenbauer, and S. K. Welch.** 2007. CD163 expression confers susceptibility to porcine reproductive and respiratory syndrome viruses. Journal of virology **81:**7371-7379.
39. **Calzada-Nova, G., W. Schnitzlein, R. Husmann, and F. A. Zuckermann.** 2010. Characterization of the cytokine and maturation responses of pure populations of porcine plasmacytoid dendritic cells to porcine viruses and toll-like receptor agonists. Veterinary immunology and immunopathology **135:**20-33.

40. **Cancel-Tirado, S. M., R. B. Evans, and K. J. Yoon.** 2004. Monoclonal antibody analysis of porcine reproductive and respiratory syndrome virus epitopes associated with antibody-dependent enhancement and neutralization of virus infection. Veterinary immunology and immunopathology **102:**249-262.
41. **Cano, J. P., S. A. Dee, M. P. Murtaugh, and C. Pijoan.** 2007. Impact of a modified-live porcine reproductive and respiratory syndrome virus vaccine intervention on a population of pigs infected with a heterologous isolate. Vaccine **25:**4382-4391.
42. **Cano, J. P., S. A. Dee, M. P. Murtaugh, C. A. Trincado, and C. B. Pijoan.** 2007. Effect of vaccination with a modified-live porcine reproductive and respiratory syndrome virus vaccine on dynamics of homologous viral infection in pigs. American journal of veterinary research **68:**565-571.
43. **Cao, J., K. Grauwet, B. Vermeulen, B. Devriendt, P. Jiang, H. Favoreel, and H. Nauwynck.** 2013. Suppression of NK cell-mediated cytotoxicity against PRRSV-infected porcine alveolar macrophages in vitro. Veterinary microbiology **164:**261-269.
44. **Carman, S., S. E. Sanford, and S. Dea.** 1995. Assessment of seropositivity to porcine reproductive and respiratory syndrome (PRRS) virus in swine herds in Ontario--1978 to 1982. The Canadian veterinary journal. La revue veterinaire canadienne **36:**776-777.
45. **Catanzariti, A. M., T. A. Soboleva, D. A. Jans, P. G. Board, and R. T. Baker.** 2004. An efficient system for high-level expression and easy purification of authentic recombinant proteins. Protein science : a publication of the Protein Society **13:**1331-1339.
46. **Cavanagh, D.** 1997. Nidovirales: a new order comprising Coronaviridae and Arteriviridae. Archives of virology **142:**629-633.
47. **Cecere, T. E., S. M. Todd, and T. Leroith.** 2012. Regulatory T cells in arterivirus and coronavirus infections: do they protect against disease or enhance it? Viruses **4:**833-846.
48. **Chand, R.** 2013. Study of Recombination in PRRSV using a novel in-vitro system. Kansas State University, Manhattan, KS.
49. **Chand, R. J., B. R. Trible, and R. R. Rowland.** 2012. Pathogenesis of porcine reproductive and respiratory syndrome virus. Current opinion in virology **2:**256-263.
50. **Chang, C. C., K. J. Yoon, J. J. Zimmerman, K. M. Harmon, P. M. Dixon, C. M. Dvorak, and M. P. Murtaugh.** 2002. Evolution of porcine reproductive and respiratory syndrome virus during sequential passages in pigs. Journal of virology **76:**4750-4763.
51. **Charerntantanakul, W.** 2009. Adjuvants for porcine reproductive and respiratory syndrome virus vaccines. Veterinary immunology and immunopathology **129:**1-13.
52. **Charerntantanakul, W.** 2012. Porcine reproductive and respiratory syndrome virus vaccines: Immunogenicity, efficacy and safety aspects. World journal of virology **1:**23-30.
53. **Charerntantanakul, W., R. Platt, W. Johnson, M. Roof, E. Vaughn, and J. A. Roth.** 2006. Immune responses and protection by vaccine and various vaccine adjuvant candidates to virulent porcine reproductive and respiratory syndrome virus. Veterinary immunology and immunopathology **109:**99-115.
54. **Chen, C., J. Li, Y. Bi, L. Yang, S. Meng, Y. Zhou, X. Jia, S. Meng, L. Sun, and W. Liu.** 2013. Synthetic B- and T-cell epitope peptides of porcine reproductive and respiratory syndrome virus with Gp96 as adjuvant induced humoral and cell-mediated immunity. Vaccine **31:**1838-1847.

55. **Chen, N., Z. Cao, X. Yu, X. Deng, T. Zhao, L. Wang, Q. Liu, X. Li, and K. Tian.** 2011. Emergence of novel European genotype porcine reproductive and respiratory syndrome virus in mainland China. The Journal of general virology **92:**880-892.
56. **Chen, N., X. Yu, L. Wang, J. Wu, Z. Zhou, J. Ni, X. Li, X. Zhai, and K. Tian.** 2013. Two natural recombinant highly pathogenic porcine reproductive and respiratory syndrome viruses with different pathogenicities. Virus genes **46:**473-478.
57. **Chen, N. H., X. Z. Chen, D. M. Hu, X. L. Yu, L. L. Wang, W. Han, J. J. Wu, Z. Cao, C. B. Wang, Q. Zhang, B. Y. Wang, and K. G. Tian.** 2009. Rapid differential detection of classical and highly pathogenic North American Porcine Reproductive and Respiratory Syndrome virus in China by a duplex real-time RT-PCR. Journal of virological methods **161:**192-198.
58. **Chen, X., and J. Liu.** 2011. Generation and immunogenicity of transgenic potato expressing the GP5 protein of porcine reproductive and respiratory syndrome virus. Journal of virological methods **173:**153-158.
59. **Chen, Z., S. Lawson, Z. Sun, X. Zhou, X. Guan, J. Christopher-Hennings, E. A. Nelson, and Y. Fang.** 2010. Identification of two auto-cleavage products of nonstructural protein 1 (nsp1) in porcine reproductive and respiratory syndrome virus infected cells: nsp1 function as interferon antagonist. Virology **398:**87-97.
60. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analytical biochemistry **162:**156-159.
61. **Collins, J. E., D. A. Benfield, W. T. Christianson, L. Harris, J. C. Hennings, D. P. Shaw, S. M. Goyal, S. McCullough, R. B. Morrison, H. S. Joo, and 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. Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc **4:**117-126.
62. **Corzo, C. A., E. Mondaca, S. Wayne, M. Torremorell, S. Dee, P. Davies, and R. B. Morrison.** 2010. Control and elimination of porcine reproductive and respiratory syndrome virus. Virus research **154:**185-192.
63. **Costers, S., P. L. Delputte, and H. J. Nauwynck.** 2006. Porcine reproductive and respiratory syndrome virus-infected alveolar macrophages contain no detectable levels of viral proteins in their plasma membrane and are protected against antibody-dependent, complement-mediated cell lysis. The Journal of general virology **87:**2341-2351.
64. **Cruz, J. L., S. Zuniga, M. Becares, I. Sola, J. E. Ceriani, S. Juanola, J. Plana, and L. Enjuanes.** 2010. Vectored vaccines to protect against PRRSV. Virus research **154:**150-160.
65. **Daigle, D., B. B. Simen, and P. Pochart.** 2011. High-throughput sequencing of PCR products tagged with universal primers using 454 life sciences systems. Current protocols in molecular biology / edited by Frederick M. Ausubel ... [et al.] **Chapter 7:**Unit7 5.
66. **Darwich, L., I. Diaz, and E. Mateu.** 2010. Certainties, doubts and hypotheses in porcine reproductive and respiratory syndrome virus immunobiology. Virus research **154:**123-132.
67. **Darwich, L., M. Gimeno, M. Sibila, I. Diaz, E. de la Torre, S. Dotti, L. Kuzemtseva, M. Martin, J. Pujols, and E. Mateu.** 2011. Genetic and immunobiological diversities of

porcine reproductive and respiratory syndrome genotype I strains. Veterinary microbiology **150:**49-62.

68. **Das, P. B., P. X. Dinh, I. H. Ansari, M. de Lima, F. A. Osorio, and A. K. Pattnaik.** 2010. The minor envelope glycoproteins GP2a and GP4 of porcine reproductive and respiratory syndrome virus interact with the receptor CD163. Journal of virology **84:**1731-1740.
69. **de Lima, M., I. H. Ansari, P. B. Das, B. J. Ku, F. J. Martinez-Lobo, A. K. Pattnaik, and F. A. Osorio.** 2009. GP3 is a structural component of the PRRSV type II (US) virion. Virology **390:**31-36.
70. **de Lima, M., A. K. Pattnaik, E. F. Flores, and F. A. Osorio.** 2006. Serologic marker candidates identified among B-cell linear epitopes of Nsp2 and structural proteins of a North American strain of porcine reproductive and respiratory syndrome virus. Virology **353:**410-421.
71. **Delputte, P. L., S. Costers, and H. J. Nauwynck.** 2005. Analysis of porcine reproductive and respiratory syndrome virus attachment and internalization: distinctive roles for heparan sulphate and sialoadhesin. The Journal of general virology **86:**1441-1445.
72. **den Boon, J. A., E. J. Snijder, E. D. Chirnside, A. A. de Vries, M. C. Horzinek, and W. J. Spaan.** 1991. Equine arteritis virus is not a togavirus but belongs to the coronaviruslike superfamily. Journal of virology **65:**2910-2920.
73. **Dewey, C., G. Charbonneau, S. Carman, A. Hamel, G. Nayar, R. Friendship, K. Eernisse, and S. Swenson.** 2000. Lelystad-like strain of porcine reproductive and respiratory syndrome virus (PRRSV) identified in Canadian swine. The Canadian veterinary journal. La revue veterinaire canadienne **41:**493-494.
74. **Diamond, D. C., B. A. Jameson, J. Bonin, M. Kohara, S. Abe, H. Itoh, T. Komatsu, M. Arita, S. Kuge, A. Nomoto, and et al.** 1985. Antigenic variation and resistance to neutralization in poliovirus type 1. Science **229:**1090-1093.
75. **Diaz, I., L. Darwich, G. Pappaterra, J. Pujols, and E. Mateu.** 2006. Different European-type vaccines against porcine reproductive and respiratory syndrome virus have different immunological properties and confer different protection to pigs. Virology **351:**249-259.
76. **Diaz, M., and M. F. Flajnik.** 1998. Evolution of somatic hypermutation and gene conversion in adaptive immunity. Immunological reviews **162:**13-24.
77. **Dokland, T.** 2010. The structural biology of PRRSV. Virus research **154:**86-97.
78. **Domingo, E., and J. J. Holland.** 1997. RNA virus mutations and fitness for survival. Annual review of microbiology **51:**151-178.
79. **Domingo, E., J. Sheldon, and C. Perales.** 2012. Viral quasispecies evolution. Microbiology and molecular biology reviews : MMBR **76:**159-216.
80. **Drexler, C. S., M. H. Witvliet, M. Raes, M. van de Laar, A. A. Eggen, and E. L. Thacker.** 2010. Efficacy of combined porcine reproductive and respiratory syndrome virus and Mycoplasma hyopneumoniae vaccination in piglets. The Veterinary record **166:**70-74.
81. **Eguchi-Ogawa, T., N. Wertz, X. Z. Sun, F. Piumi, H. Uenishi, K. Wells, P. Chardon, G. J. Tobin, and J. E. Butler.** 2010. Antibody repertoire development in fetal and neonatal piglets. XI. The relationship of variable heavy chain gene usage and the

genomic organization of the variable heavy chain locus. Journal of immunology **184:**3734-3742.

82. **Fang, Y., D. Y. Kim, S. Ropp, P. Steen, J. Christopher-Hennings, E. A. Nelson, and R. R. Rowland.** 2004. Heterogeneity in Nsp2 of European-like porcine reproductive and respiratory syndrome viruses isolated in the United States. Virus research **100:**229-235.
83. **Fang, Y., P. Schneider, W. P. Zhang, K. S. Faaberg, E. A. Nelson, and R. R. Rowland.** 2007. Diversity and evolution of a newly emerged North American Type 1 porcine arterivirus: analysis of isolates collected between 1999 and 2004. Archives of virology **152:**1009-1017.
84. **Fang, Y., and E. J. Snijder.** 2010. The PRRSV replicase: exploring the multifunctionality of an intriguing set of nonstructural proteins. Virus research **154:**61-76.
85. **Fang, Y., E. E. Treffers, Y. Li, A. Tas, Z. Sun, Y. van der Meer, A. H. de Ru, P. A. van Veelen, J. F. Atkins, E. J. Snijder, and A. E. Firth.** 2012. Efficient -2 frameshifting by mammalian ribosomes to synthesize an additional arterivirus protein. Proceedings of the National Academy of Sciences of the United States of America **109:**E2920-2928.
86. **Farci, P.** 2011. New insights into the HCV quasispecies and compartmentalization. Seminars in liver disease **31:**356-374.
87. **Firth, A. E., J. C. Zevenhoven-Dobbe, N. M. Wills, Y. Y. Go, U. B. Balasuriya, J. F. Atkins, E. J. Snijder, and C. C. Posthuma.** 2011. Discovery of a small arterivirus gene that overlaps the GP5 coding sequence and is important for virus production. The Journal of general virology **92:**1097-1106.
88. **Forsberg, R.** 2005. Divergence time of porcine reproductive and respiratory syndrome virus subtypes. Molecular biology and evolution **22:**2131-2134.
89. **Galetto, R., V. Giacomoni, M. Veron, and M. Negroni.** 2006. Dissection of a circumscribed recombination hot spot in HIV-1 after a single infectious cycle. The Journal of biological chemistry **281:**2711-2720.
90. **Gega, A., and M. J. Kozal.** 2011. New technology to detect low-level drug-resistant HIV variants. Future Virol **6:**17-26.
91. **Goldberg, T. L., J. F. Lowe, S. M. Milburn, and L. D. Firkins.** 2003. Quasispecies variation of porcine reproductive and respiratory syndrome virus during natural infection. Virology **317:**197-207.
92. **Gorbalenya, A. E., L. Enjuanes, J. Ziebuhr, and E. J. Snijder.** 2006. Nidovirales: evolving the largest RNA virus genome. Virus research **117:**17-37.
93. **Groenen, M. A., A. L. Archibald, H. Uenishi, C. K. Tuggle, Y. Takeuchi, M. F. Rothschild, C. Rogel-Gaillard, C. Park, D. Milan, H. J. Megens, S. Li, D. M. Larkin, H. Kim, L. A. Frantz, M. Caccamo, H. Ahn, B. L. Aken, A. Anselmo, C. Anthon, L. Auvil, B. Badaoui, C. W. Beattie, C. Bendixen, D. Berman, F. Blecha, J. Blomberg, L. Bolund, M. Bosse, S. Botti, Z. Bujie, M. Bystrom, B. Capitanu, D. Carvalho-Silva, P. Chardon, C. Chen, R. Cheng, S. H. Choi, W. Chow, R. C. Clark, C. Clee, R. P. Crooijmans, H. D. Dawson, P. Dehais, F. De Sapio, B. Dibbits, N. Drou, Z. Q. Du, K. Eversole, J. Fadista, S. Fairley, T. Faraut, G. J. Faulkner, K. E. Fowler, M. Fredholm, E. Fritz, J. G. Gilbert, E. Giuffra, J. Gorodkin, D. K. Griffin, J. L. Harrow, A. Hayward, K. Howe, Z. L. Hu, S. J. Humphray, T. Hunt, H. Hornshoj, J. T. Jeon, P. Jern, M. Jones, J. Jurka, H. Kanamori, R. Kapetanovic, J. Kim, J. H.

Kim, K. W. Kim, T. H. Kim, G. Larson, K. Lee, K. T. Lee, R. Leggett, H. A. Lewin, Y. Li, W. Liu, J. E. Loveland, Y. Lu, J. K. Lunney, J. Ma, O. Madsen, K. Mann, L. Matthews, S. McLaren, T. Morozumi, M. P. Murtaugh, J. Narayan, D. T. Nguyen, P. Ni, S. J. Oh, S. Onteru, F. Panitz, E. W. Park, et al. 2012. Analyses of pig genomes provide insight into porcine demography and evolution. Nature **491:**393-398.

94. **Halbur, P. G., and E. Bush.** 1997. Update on abortion storms and sow mortality. Swine Health Prod **5:**73.
95. **Han, J., Y. Wang, and K. S. Faaberg.** 2006. Complete genome analysis of RFLP 184 isolates of porcine reproductive and respiratory syndrome virus. Virus research **122:**175-182.
96. **Hanada, K., Y. Suzuki, T. Nakane, O. Hirose, and T. Gojobori.** 2005. The origin and evolution of porcine reproductive and respiratory syndrome viruses. Molecular biology and evolution **22:**1024-1031.
97. **Hoebe, K., E. Janssen, and B. Beutler.** 2004. The interface between innate and adaptive immunity. Nature immunology **5:**971-974.
98. **Holtkamp, D. J., J. B. Kliebenstein, E. J. Neumann, J. J. Zimmerman, H. F. Rotto, T. K. Yoder, C. Wang, P. E. Yeske, C. L. Mowrer, and C. A. Haley.** 2013. Assessment of the economic impact of porcine reproductive and respiratory syndrome virus on United States pork producers. J Swine Health Prod **21:**72-84.
99. **Huang, Y. W., and X. J. Meng.** 2010. Novel strategies and approaches to develop the next generation of vaccines against porcine reproductive and respiratory syndrome virus (PRRSV). Virus research **154:**141-149.
100. **Jeanmougin, F., J. D. Thompson, M. Gouy, D. G. Higgins, and T. J. Gibson.** 1998. Multiple sequence alignment with Clustal X. Trends in biochemical sciences **23:**403-405.
101. **Jerne, N. K.** 1974. Clonal selection in a lymphocyte network. Society of General Physiologists series **29:**39-48.
102. **Jiang, W., P. Jiang, X. Wang, Y. Li, Y. Du, and X. Wang.** 2008. Enhanced immune responses of mice inoculated recombinant adenoviruses expressing GP5 by fusion with GP3 and/or GP4 of PRRS virus. Virus research **136:**50-57.
103. **Johnson, C. R., T. F. Griggs, J. Gnanandarajah, and M. P. Murtaugh.** 2011. Novel structural protein in porcine reproductive and respiratory syndrome virus encoded by an alternative ORF5 present in all arteriviruses. The Journal of general virology **92:**1107-1116.
104. **Jusa, E. R., Y. Inaba, M. Kouno, and O. Hirose.** 1997. Effect of heparin on infection of cells by porcine reproductive and respiratory syndrome virus. American journal of veterinary research **58:**488-491.
105. **Kalie, E., D. A. Jaitin, Y. Podoplelova, J. Piehler, and G. Schreiber.** 2008. The stability of the ternary interferon-receptor complex rather than the affinity to the individual subunits dictates differential biological activities. The Journal of biological chemistry **283:**32925-32936.
106. **Kapur, V., M. R. Elam, T. M. Pawlovich, and M. P. Murtaugh.** 1996. Genetic variation in porcine reproductive and respiratory syndrome virus isolates in the midwestern United States. The Journal of general virology **77 (Pt 6):**1271-1276.
107. **Keffaber, K. K.** 1989. Reprodutive failure of unknown etiology. Am Assoc Swine Pract Newsl **1:**1-10.

108. **Key, K. F., G. Haqshenas, D. K. Guenette, S. L. Swenson, T. E. Toth, and X. J. Meng.** 2001. Genetic variation and phylogenetic analyses of the ORF5 gene of acute porcine reproductive and respiratory syndrome virus isolates. Veterinary microbiology **83:**249-263.
109. **Khatchikian, D., M. Orlich, and R. Rott.** 1989. Increased viral pathogenicity after insertion of a 28S ribosomal RNA sequence into the haemagglutinin gene of an influenza virus. Nature **340:**156-157.
110. **Kim, H. S., J. Kwang, I. J. Yoon, H. S. Joo, and M. L. Frey.** 1993. Enhanced replication of porcine reproductive and respiratory syndrome (PRRS) virus in a homogeneous subpopulation of MA-104 cell line. Archives of virology **133:**477-483.
111. **Kim, O., Y. Sun, F. W. Lai, C. Song, and D. Yoo.** 2010. Modulation of type I interferon induction by porcine reproductive and respiratory syndrome virus and degradation of CREB-binding protein by non-structural protein 1 in MARC-145 and HeLa cells. Virology **402:**315-326.
112. **Kim, W. I., and K. J. Yoon.** 2008. Molecular assessment of the role of envelope-associated structural proteins in cross neutralization among different PRRS viruses. Virus genes **37:**380-391.
113. **Kimata, J. T., L. Kuller, D. B. Anderson, P. Dailey, and J. Overbaugh.** 1999. Emerging cytopathic and antigenic simian immunodeficiency virus variants influence AIDS progression. Nature medicine **5:**535-541.
114. **Kimman, T. G., L. A. Cornelissen, R. J. Moormann, J. M. Rebel, and N. Stockhofe-Zurwieden.** 2009. Challenges for porcine reproductive and respiratory syndrome virus (PRRSV) vaccinology. Vaccine **27:**3704-3718.
115. **Kindt, T., R. Goldsby, and B. Osborne.** 2007. Kuby Immunology, Sixth ed.
116. **Klein, F., R. Diskin, J. F. Scheid, C. Gaebler, H. Mouquet, I. S. Georgiev, M. Pancera, T. Zhou, R. B. Incesu, B. Z. Fu, P. N. Gnanapragasam, T. Y. Oliveira, M. S. Seaman, P. D. Kwong, P. J. Bjorkman, and M. C. Nussenzweig.** 2013. Somatic mutations of the immunoglobulin framework are generally required for broad and potent HIV-1 neutralization. Cell **153:**126-138.
117. **Klotman, M. E., and T. L. Chang.** 2006. Defensins in innate antiviral immunity. Nature reviews. Immunology **6:**447-456.
118. **Kritas, S. K., C. Alexopoulos, C. S. Kyriakis, E. Tzika, and S. C. Kyriakis.** 2007. Performance of fattening pigs in a farm infected with both porcine reproductive and respiratory syndrome (PRRS) virus and porcine circovirus type 2 following sow and piglet vaccination with an attenuated PRRS vaccine. Journal of veterinary medicine. A, Physiology, pathology, clinical medicine **54:**287-291.
119. **Labarque, G. G., H. J. Nauwynck, K. Van Reeth, and M. B. Pensaert.** 2000. Effect of cellular changes and onset of humoral immunity on the replication of porcine reproductive and respiratory syndrome virus in the lungs of pigs. The Journal of general virology **81:**1327-1334.
120. **Lai, M. M.** 1992. RNA recombination in animal and plant viruses. Microbiological reviews **56:**61-79.
121. **Lauring, A. S., and R. Andino.** 2010. Quasispecies theory and the behavior of RNA viruses. PLoS pathogens **6:**e1001005.
122. **Lee, C., H. Kim, B. Kang, M. Yeom, S. Han, H. Moon, S. Park, H. Kim, D. Song, and B. Park.** 2010. Prevalence and phylogenetic analysis of the isolated type I porcine

reproductive and respiratory syndrome virus from 2007 to 2008 in Korea. Virus genes **40:**225-230.

123. **Lee, C., and D. Yoo.** 2006. The small envelope protein of porcine reproductive and respiratory syndrome virus possesses ion channel protein-like properties. Virology **355:**30-43.

124. **Lemke, C. D., J. S. Haynes, R. Spaete, D. Adolphson, A. Vorwald, K. Lager, and J. E. Butler.** 2004. Lymphoid hyperplasia resulting in immune dysregulation is caused by porcine reproductive and respiratory syndrome virus infection in neonatal pigs. Journal of immunology **172:**1916-1925.

125. **Li, B., L. Fang, Z. Xu, S. Liu, J. Gao, Y. Jiang, H. Chen, and S. Xiao.** 2009. Recombination in vaccine and circulating strains of porcine reproductive and respiratory syndrome viruses. Emerging infectious diseases **15:**2032-2035.

126. **Li, Y., E. E. Treffers, S. Napthine, A. Tas, L. Zhu, Z. Sun, S. Bell, B. L. Mark, P. A. van Veelen, M. J. van Hemert, A. E. Firth, I. Brierley, E. J. Snijder, and Y. Fang.** 2014. Transactivation of programmed ribosomal frameshifting by a viral protein. Proceedings of the National Academy of Sciences of the United States of America.

127. **Li, Y., L. Zhou, J. Zhang, X. Ge, R. Zhou, H. Zheng, G. Geng, X. Guo, and H. Yang.** 2014. Nsp9 and nsp10 contribute to the fatal virulence of highly pathogenic porcine reproductive and respiratory syndrome virus emerging in china. PLoS pathogens **10:**e1004216.

128. **Liu, D., R. Zhou, J. Zhang, L. Zhou, Q. Jiang, X. Guo, X. Ge, and H. Yang.** 2011. Recombination analyses between two strains of porcine reproductive and respiratory syndrome virus in vivo. Virus research **155:**473-486.

129. **Liu, L., Y. Li, S. Li, N. Hu, Y. He, R. Pong, D. Lin, L. Lu, and M. Law.** 2012. Comparison of next-generation sequencing systems. Journal of biomedicine & biotechnology **2012:**251364.

130. **Loemba, H. D., S. Mounir, H. Mardassi, D. Archambault, and S. Dea.** 1996. Kinetics of humoral immune response to the major structural proteins of the porcine reproductive and respiratory syndrome virus. Archives of virology **141:**751-761.

131. **Lopez Fuertes, L., N. Domenech, B. Alvarez, A. Ezquerra, J. Dominguez, J. M. Castro, and F. Alonso.** 1999. Analysis of cellular immune response in pigs recovered from porcine respiratory and reproductive syndrome infection. Virus research **64:**33-42.

132. **Lopez, O. J., M. F. Oliveira, E. A. Garcia, B. J. Kwon, A. Doster, and F. A. Osorio.** 2007. Protection against porcine reproductive and respiratory syndrome virus (PRRSV) infection through passive transfer of PRRSV-neutralizing antibodies is dose dependent. Clinical and vaccine immunology : CVI **14:**269-275.

133. **Lopez, O. J., and F. A. Osorio.** 2004. Role of neutralizing antibodies in PRRSV protective immunity. Veterinary immunology and immunopathology **102:**155-163.

134. **Lopez-Bueno, A., M. G. Mateu, and J. M. Almendral.** 2003. High mutant frequency in populations of a DNA virus allows evasion from antibody therapy in an immunodeficient host. Journal of virology **77:**2701-2708.

135. **Loving, C. L., S. L. Brockmeier, and R. E. Sacco.** 2007. Differential type I interferon activation and susceptibility of dendritic cell populations to porcine arterivirus. Immunology **120:**217-229.

136. **Lunney, J. K., J. P. Steibel, J. M. Reecy, E. Fritz, M. F. Rothschild, M. Kerrigan, B. Trible, and R. R. Rowland.** 2011. Probing genetic control of swine responses to PRRSV

infection: current progress of the PRRS host genetics consortium. BMC proceedings **5 Suppl 4:**S30.

137. **Malim, M. H., and M. Emerman.** 2001. HIV-1 sequence variation: drift, shift, and attenuation. Cell **104:**469-472.

138. **Mardassi, H., P. Gonin, C. A. Gagnon, B. Massie, and S. Dea.** 1998. A subset of porcine reproductive and respiratory syndrome virus GP3 glycoprotein is released into the culture medium of cells as a non-virion-associated and membrane-free (soluble) form. Journal of virology **72:**6298-6306.

139. **Mardassi, H., B. Massie, and S. Dea.** 1996. Intracellular synthesis, processing, and transport of proteins encoded by ORFs 5 to 7 of porcine reproductive and respiratory syndrome virus. Virology **221:**98-112.

140. **Mardassi, H., S. Mounir, and S. Dea.** 1995. Structural gene analysis of a Quebec reference strain or porcine reproductive and respiratory syndrome virus (PRRSV). Advances in experimental medicine and biology **380:**277-281.

141. **Market, E., and F. N. Papavasiliou.** 2003. V(D)J recombination and the evolution of the adaptive immune system. PLoS biology **1:**E16.

142. **Martelli, P., P. Cordioli, L. G. Alborali, S. Gozio, E. De Angelis, L. Ferrari, G. Lombardi, and P. Borghetti.** 2007. Protection and immune response in pigs intradermally vaccinated against porcine reproductive and respiratory syndrome (PRRS) and subsequently exposed to a heterologous European (Italian cluster) field strain. Vaccine **25:**3400-3408.

143. **Martin, D. P.** 2009. Recombination detection and analysis using RDP3. Methods in molecular biology **537:**185-205.

144. **Martin-Valls, G. E., L. K. Kvisgaard, M. Tello, L. Darwich, M. Cortey, A. J. Burgara-Estrella, J. Hernandez, L. E. Larsen, and E. Mateu.** 2014. Analysis of ORF5 and full-length genome sequences of porcine reproductive and respiratory syndrome virus isolates of genotypes 1 and 2 retrieved worldwide provides evidence that recombination is a common phenomenon and may produce mosaic isolates. Journal of virology **88:**3170-3181.

145. **Martin-Valls, G. E., L. K. Kvisgaard, M. Tello, L. Darwich, M. Cortey, A. J. Burgara-Estrella, J. Hernandez, L. E. Larsen, and E. Mateu.** 2013. Analysis of ORF5 and full-length genome sequences of porcine reproductive and respiratory syndrome virus isolates of genotypes 1 and 2 retrieved worldwide provides evidences supporting that recombination is a common phenomenon and may produce mosaic isolates. Journal of virology.

146. **McAleer, J., P. Weber, J. Sun, and J. E. Butler.** 2005. Antibody repertoire development in fetal and neonatal piglets. XI. The thymic B-cell repertoire develops independently from that in blood and mesenteric lymph nodes. Immunology **114:**171-183.

147. **Meier, w., J. Wheeler, R. J. Husmann, F. Osorio, and F. Zuckermann.** 2000. Characteristics of the immune response of pigs to PRRS virus. Vet Res **31:**41.

148. **Meier, W. A., J. Galeota, F. A. Osorio, R. J. Husmann, W. M. Schnitzlein, and F. A. Zuckermann.** 2003. Gradual development of the interferon-gamma response of swine to porcine reproductive and respiratory syndrome virus infection or vaccination. Virology **309:**18-31.

149. **Meng, X. J., P. S. Paul, P. G. Halbur, and M. A. Lum.** 1995. Phylogenetic analyses of the putative M (ORF 6) and N (ORF 7) genes of porcine reproductive and respiratory syndrome virus (PRRSV): implication for the existence of two genotypes of PRRSV in the U.S.A. and Europe. Archives of virology **140:**745-755.
150. **Meng, X. J., P. S. Paul, P. G. Halbur, and I. Morozov.** 1995. Sequence comparison of open reading frames 2 to 5 of low and high virulence United States isolates of porcine reproductive and respiratory syndrome virus. The Journal of general virology **76 (Pt 12):**3181-3188.
151. **Mengeling, W. L., K. M. Lager, A. C. Vorwald, and D. F. Clouser.** 2003. Comparative safety and efficacy of attenuated single-strain and multi-strain vaccines for porcine reproductive and respiratory syndrome. Veterinary microbiology **93:**25-38.
152. **Mengeling, W. L., K. M. Lager, A. C. Vorwald, and K. J. Koehler.** 2003. Strain specificity of the immune response of pigs following vaccination with various strains of porcine reproductive and respiratory syndrome virus. Veterinary microbiology **93:**13-24.
153. **Milne, I., G. Stephen, M. Bayer, P. J. Cock, L. Pritchard, L. Cardle, P. D. Shaw, and D. Marshall.** 2013. Using Tablet for visual exploration of second-generation sequencing data. Briefings in bioinformatics **14:**193-202.
154. **Murtaugh, M. P.** 2014. Advances in swine immunology help move vaccine technology forward. Veterinary immunology and immunopathology **159:**202-207.
155. **Murtaugh, M. P., and M. Genzow.** 2011. Immunological solutions for treatment and prevention of porcine reproductive and respiratory syndrome (PRRS). Vaccine **29:**8192-8204.
156. **Murtaugh, M. P., T. Stadejek, J. E. Abrahante, T. T. Lam, and F. C. Leung.** 2010. The ever-expanding diversity of porcine reproductive and respiratory syndrome virus. Virus research **154:**18-30.
157. **Murtaugh, M. P., Z. Xiao, and F. Zuckermann.** 2002. Immunological responses of swine to porcine reproductive and respiratory syndrome virus infection. Viral immunology **15:**533-547.
158. **Music, N., and C. A. Gagnon.** 2010. The role of porcine reproductive and respiratory syndrome (PRRS) virus structural and non-structural proteins in virus pathogenesis. Animal health research reviews / Conference of Research Workers in Animal Diseases **11:**135-163.
159. **Nielsen, H. S., M. B. Oleksiewicz, R. Forsberg, T. Stadejek, A. Botner, and T. Storgaard.** 2001. Reversion of a live porcine reproductive and respiratory syndrome virus vaccine investigated by parallel mutations. The Journal of general virology **82:**1263-1272.
160. **Normile, D.** 2007. Virology. China, Vietnam grapple with 'rapidly evolving' pig virus. Science **317:**1017.
161. **Opriessnig, T., P. G. Halbur, K. J. Yoon, R. M. Pogranichniy, K. M. Harmon, R. Evans, K. F. Key, F. J. Pallares, P. Thomas, and X. J. Meng.** 2002. Comparison of molecular and biological characteristics of a modified live porcine reproductive and respiratory syndrome virus (PRRSV) vaccine (ingelvac PRRS MLV), the parent strain of the vaccine (ATCC VR2332), ATCC VR2385, and two recent field isolates of PRRSV. Journal of virology **76:**11837-11844.
162. **Osorio, F. A., J. A. Galeota, E. Nelson, B. Brodersen, A. Doster, R. Wills, F. Zuckermann, and W. W. Laegreid.** 2002. Passive transfer of virus-specific antibodies

confers protection against reproductive failure induced by a virulent strain of porcine reproductive and respiratory syndrome virus and establishes sterilizing immunity. Virology **302:**9-20.

163. **Ostrowski, M., J. A. Galeota, A. M. Jar, K. B. Platt, F. A. Osorio, and O. J. Lopez.** 2002. Identification of neutralizing and nonneutralizing epitopes in the porcine reproductive and respiratory syndrome virus GP5 ectodomain. Journal of virology **76:**4241-4250.
164. **Ozuna, A. G., R. R. Rowland, J. C. Nietfeld, M. A. Kerrigan, J. C. Dekkers, and C. R. Wyatt.** 2013. Preliminary findings of a previously unrecognized porcine primary immunodeficiency disorder. Veterinary pathology **50:**144-146.
165. **Padlan, E. A.** 1994. Anatomy of the antibody molecule. Molecular immunology **31:**169-217.
166. **Papatsiros, V. G., C. Alexopoulos, S. K. Kritas, G. Koptopoulos, H. J. Nauwynck, M. B. Pensaert, and S. C. Kyriakis.** 2006. Long-term administration of a commercial porcine reproductive and respiratory syndrome virus (PRRSV)-inactivated vaccine in PRRSV-endemically infected sows. Journal of veterinary medicine. B, Infectious diseases and veterinary public health **53:**266-272.
167. **Parry, N., G. Fox, D. Rowlands, F. Brown, E. Fry, R. Acharya, D. Logan, and D. Stuart.** 1990. Structural and serological evidence for a novel mechanism of antigenic variation in foot-and-mouth disease virus. Nature **347:**569-572.
168. **Patton, J. B., R. R. Rowland, D. Yoo, and K. O. Chang.** 2009. Modulation of CD163 receptor expression and replication of porcine reproductive and respiratory syndrome virus in porcine macrophages. Virus research **140:**161-171.
169. **Pei, Y., D. C. Hodgins, C. Lee, J. G. Calvert, S. K. Welch, R. Jolie, M. Keith, and D. Yoo.** 2008. Functional mapping of the porcine reproductive and respiratory syndrome virus capsid protein nuclear localization signal and its pathogenic association. Virus research **135:**107-114.
170. **Plagemann, P. G.** 2003. Porcine reproductive and respiratory syndrome virus: origin hypothesis. Emerging infectious diseases **9:**903-908.
171. **Plagemann, P. G., R. R. Rowland, and K. S. Faaberg.** 2002. The primary neutralization epitope of porcine respiratory and reproductive syndrome virus strain VR-2332 is located in the middle of the GP5 ectodomain. Archives of virology **147:**2327-2347.
172. **Plana Duran, J., I. Climent, J. Sarraseca, A. Urniza, E. Cortes, C. Vela, and J. I. Casal.** 1997. Baculovirus expression of proteins of porcine reproductive and respiratory syndrome virus strain Olot/91. Involvement of ORF3 and ORF5 proteins in protection. Virus genes **14:**19-29.
173. **Prather, R. S., R. R. Rowland, C. Ewen, B. Trible, M. Kerrigan, B. Bawa, J. M. Teson, J. Mao, K. Lee, M. S. Samuel, K. M. Whitworth, C. N. Murphy, T. Egen, and J. A. Green.** 2013. An intact sialoadhesin (Sn/SIGLEC1/CD169) is not required for attachment/internalization of the porcine reproductive and respiratory syndrome virus. Journal of virology **87:**9538-9546.
174. **Prieto, C., F. J. Martinez-Lobo, F. Diez-Fuertes, P. Aguilar-Calvo, I. Simarro, and J. M. Castro.** 2011. Immunisation of pigs with a major envelope protein sub-unit vaccine against porcine reproductive and respiratory syndrome virus (PRRSV) results in

enhanced clinical disease following experimental challenge. Veterinary journal **189:**323-329.

175. **Qiu, H. J., Z. J. Tian, G. Z. Tong, Y. J. Zhou, J. Q. Ni, Y. Z. Luo, and X. H. Cai.** 2005. Protective immunity induced by a recombinant pseudorabies virus expressing the GP5 of porcine reproductive and respiratory syndrome virus in piglets. Veterinary immunology and immunopathology **106:**309-319.
176. **Reddy, S. T., X. Ge, A. E. Miklos, R. A. Hughes, S. H. Kang, K. H. Hoi, C. Chrysostomou, S. P. Hunicke-Smith, B. L. Iverson, P. W. Tucker, A. D. Ellington, and G. Georgiou.** 2010. Monoclonal antibodies isolated without screening by analyzing the variable-gene repertoire of plasma cells. Nature biotechnology **28:**965-969.
177. **Reiner, G., H. Willems, S. Pesch, and V. F. Ohlinger.** 2010. Variation in resistance to the porcine reproductive and respiratory syndrome virus (PRRSV) in Pietrain and Miniature pigs. Journal of animal breeding and genetics = Zeitschrift fur Tierzuchtung und Zuchtungsbiologie **127:**100-106.
178. **Reitter, J. N., and R. C. Desrosiers.** 1998. Identification of replication-competent strains of simian immunodeficiency virus lacking multiple attachment sites for N-linked carbohydrates in variable regions 1 and 2 of the surface envelope protein. Journal of virology **72:**5399-5407.
179. **Renukaradhya, G. J., V. Dwivedi, C. Manickam, B. Binjawadagi, and D. Benfield.** 2012. Mucosal vaccines to prevent porcine reproductive and respiratory syndrome: a new perspective. Animal health research reviews / Conference of Research Workers in Animal Diseases **13:**21-37.
180. **Romanova, L. I., V. M. Blinov, E. A. Tolskaya, E. G. Viktorova, M. S. Kolesnikova, E. A. Guseva, and V. I. Agol.** 1986. The primary structure of crossover regions of intertypic poliovirus recombinants: a model of recombination between RNA genomes. Virology **155:**202-213.
181. **Ropp, S. L., C. E. Wees, Y. Fang, E. A. Nelson, K. D. Rossow, M. Bien, B. Arndt, S. Preszler, P. Steen, J. Christopher-Hennings, J. E. Collins, D. A. Benfield, and K. S. Faaberg.** 2004. Characterization of emerging European-like porcine reproductive and respiratory syndrome virus isolates in the United States. Journal of virology **78:**3684-3703.
182. **Rossow, K. D.** 1998. Porcine reproductive and respiratory syndrome. Veterinary pathology **35:**1-20.
183. **Rowland, R. R., R. Kervin, C. Kuckleburg, A. Sperlich, and D. A. Benfield.** 1999. The localization of porcine reproductive and respiratory syndrome virus nucleocapsid protein to the nucleolus of infected cells and identification of a potential nucleolar localization signal sequence. Virus research **64:**1-12.
184. **Rowland, R. R., S. Lawson, K. Rossow, and D. A. Benfield.** 2003. Lymphoid tissue tropism of porcine reproductive and respiratory syndrome virus replication during persistent infection of pigs originally exposed to virus in utero. Veterinary microbiology **96:**219-235.
185. **Rowland, R. R., J. Lunney, and J. Dekkers.** 2012. Control of porcine reproductive and respiratory syndrome (PRRS) through genetic improvements in disease resistance and tolerance. Frontiers in genetics **3:**260.
186. **Rowland, R. R., B. Robinson, J. Stefanick, T. S. Kim, L. Guanghua, S. R. Lawson, and D. A. Benfield.** 2001. Inhibition of porcine reproductive and respiratory syndrome

virus by interferon-gamma and recovery of virus replication with 2-aminopurine. Archives of virology **146:**539-555.

187. **Rowland, R. R., M. Steffen, T. Ackerman, and D. A. Benfield.** 1999. The evolution of porcine reproductive and respiratory syndrome virus: quasispecies and emergence of a virus subpopulation during infection of pigs with VR-2332. Virology **259:**262-266.
188. **Sakabe, S., M. Ozawa, R. Takano, K. Iwastuki-Horimoto, and Y. Kawaoka.** 2011. Mutations in PA, NP, and HA of a pandemic (H1N1) 2009 influenza virus contribute to its adaptation to mice. Virus research **158:**124-129.
189. **Sang, Y., R. R. Rowland, and F. Blecha.** 2011. Interaction between innate immunity and porcine reproductive and respiratory syndrome virus. Animal health research reviews / Conference of Research Workers in Animal Diseases **12:**149-167.
190. **Sang, Y., P. Ruchala, R. I. Lehrer, C. R. Ross, R. R. Rowland, and F. Blecha.** 2009. Antimicrobial host defense peptides in an arteriviral infection: differential peptide expression and virus inactivation. Viral immunology **22:**235-242.
191. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proceedings of the National Academy of Sciences of the United States of America **74:**5463-5467.
192. **Scortti, M., C. Prieto, E. Alvarez, I. Simarro, and J. M. Castro.** 2007. Failure of an inactivated vaccine against porcine reproductive and respiratory syndrome to protect gilts against a heterologous challenge with PRRSV. The Veterinary record **161:**809-813.
193. **Scortti, M., C. Prieto, I. Simarro, and J. M. Castro.** 2006. Reproductive performance of gilts following vaccination and subsequent heterologous challenge with European strains of porcine reproductive and respiratory syndrome virus. Theriogenology **66:**1884-1893.
194. **Shao, W., M. Kearney, F. Maldarelli, J. W. Mellors, R. M. Stephens, J. D. Lifson, V. N. KewalRamani, Z. Ambrose, J. M. Coffin, and S. E. Palmer.** 2009. RT-SHIV subpopulation dynamics in infected macaques during anti-HIV therapy. Retrovirology **6:**101.
195. **Shen, G., N. Jin, M. Ma, K. Jin, M. Zheng, T. Zhuang, H. Lu, G. Zhu, H. Jin, M. Jin, X. Huo, X. Qin, R. Yin, C. Li, H. Li, Y. Li, Z. Han, Y. Chen, and M. Jin.** 2007. Immune responses of pigs inoculated with a recombinant fowlpox virus coexpressing GP5/GP3 of porcine reproductive and respiratory syndrome virus and swine IL-18. Vaccine **25:**4193-4202.
196. **Shi, M., E. C. Holmes, M. S. Brar, and F. C. Leung.** 2013. Recombination is associated with an outbreak of novel highly pathogenic porcine reproductive and respiratory syndrome viruses in China. Journal of virology **87:**10904-10907.
197. **Shi, M., T. T. Lam, C. C. Hon, R. K. Hui, K. S. Faaberg, T. Wennblom, M. P. Murtaugh, T. Stadejek, and F. C. Leung.** 2010. Molecular epidemiology of PRRSV: a phylogenetic perspective. Virus research **154:**7-17.
198. **Shi, M., T. T. Lam, C. C. Hon, M. P. Murtaugh, P. R. Davies, R. K. Hui, J. Li, L. T. Wong, C. W. Yip, J. W. Jiang, and F. C. Leung.** 2010. Phylogeny-based evolutionary, demographical, and geographical dissection of North American type 2 porcine reproductive and respiratory syndrome viruses. Journal of virology **84:**8700-8711.
199. **Simon-Loriere, E., and E. C. Holmes.** 2011. Why do RNA viruses recombine? Nature reviews. Microbiology **9:**617-626.

200. **Sinkora, M., J. Sun, and J. E. Butler.** 2000. Antibody repertoire development in fetal and neonatal piglets. V. VDJ gene chimeras resembling gene conversion products are generated at high frequency by PCR in vitro. Molecular immunology **37:**1025-1034.
201. **Sinkora, M., J. Sun, J. Sinkorova, R. K. Christenson, S. P. Ford, and J. E. Butler.** 2003. Antibody repertoire development in fetal and neonatal piglets. VI. B cell lymphogenesis occurs at multiple sites with differences in the frequency of in-frame rearrangements. Journal of immunology **170:**1781-1788.
202. **Snijder, E. J., M. Kikkert, and Y. Fang.** 2013. Arterivirus molecular biology and pathogenesis. The Journal of general virology **94:**2141-2163.
203. **Snijder, E. J., and W. J. Spaan.** 2007. Arteriviruses, 5th ed.
204. **Snijder, E. J., H. van Tol, K. W. Pedersen, M. J. Raamsman, and A. A. de Vries.** 1999. Identification of a novel structural protein of arteriviruses. Journal of virology **73:**6335-6345.
205. **Spear, J. B., C. A. Benson, J. C. Pottage, Jr., D. A. Paul, A. L. Landay, and H. A. Kessler.** 1988. Rapid rebound of serum human immunodeficiency virus antigen after discontinuing zidovudine therapy. The Journal of infectious diseases **158:**1132-1133.
206. **Stadejek, T., M. B. Oleksiewicz, D. Potapchuk, and K. Podgorska.** 2006. Porcine reproductive and respiratory syndrome virus strains of exceptional diversity in eastern Europe support the definition of new genetic subtypes. The Journal of general virology **87:**1835-1841.
207. **Stadejek, T., M. B. Oleksiewicz, A. V. Scherbakov, A. M. Timina, J. S. Krabbe, K. Chabros, and D. Potapchuk.** 2008. Definition of subtypes in the European genotype of porcine reproductive and respiratory syndrome virus: nucleocapsid characteristics and geographical distribution in Europe. Archives of virology **153:**1479-1488.
208. **Stadejek, T., A. Stankevicius, M. P. Murtaugh, and M. B. Oleksiewicz.** 2013. Molecular evolution of PRRSV in Europe: current state of play. Veterinary microbiology **165:**21-28.
209. **Stanley, J., L. M. Bhaduri, O. Narayan, and J. E. Clements.** 1987. Topographical rearrangements of visna virus envelope glycoprotein during antigenic drift. Journal of virology **61:**1019-1028.
210. **Steinhauer, D. A., E. Domingo, and J. J. Holland.** 1992. Lack of evidence for proofreading mechanisms associated with an RNA virus polymerase. Gene **122:**281-288.
211. **Subramanian, S., M. Yates, A. A. Vandenbark, and H. Offner.** 2011. Oestrogen-mediated protection of experimental autoimmune encephalomyelitis in the absence of Foxp3+ regulatory T cells implicates compensatory pathways including regulatory B cells. Immunology **132:**340-347.
212. **Sun, J., C. Hayward, R. Shinde, R. Christenson, S. P. Ford, and J. E. Butler.** 1998. Antibody repertoire development in fetal and neonatal piglets. I. Four VH genes account for 80 percent of VH usage during 84 days of fetal life. Journal of immunology **161:**5070-5078.
213. **Sun, L., Y. Li, R. Liu, X. Wang, F. Gao, T. Lin, T. Huang, H. Yao, G. Tong, H. Fan, Z. Wei, and S. Yuan.** 2013. Porcine reproductive and respiratory syndrome virus ORF5a protein is essential for virus viability. Virus research **171:**178-185.
214. **Sun, X., N. Wertz, K. Lager, M. Sinkora, K. Stepanova, G. Tobin, and J. E. Butler.** 2012. Antibody repertoire development in fetal and neonatal piglets. XXII. lambda

Rearrangement precedes kappa rearrangement during B-cell lymphogenesis in swine. Immunology **137:**149-159.

215. **Sun, X., N. Wertz, K. M. Lager, and J. E. Butler.** 2012. Antibody repertoire development in fetal and neonatal piglets. XV. Porcine circovirus type 2 infection differentially affects serum IgG levels and antibodies to ORF2 in piglets free from other environmental factors. Vaccine **31:**141-148.
216. **Sun, X. Z., N. Wertz, K. L. Lager, G. Tobin, and J. E. Butler.** 2012. Antibody repertoire development in fetal and neonatal piglets. XXIII: fetal piglets infected with a vaccine strain of PRRS Virus display the same immune dysregulation seen in isolator piglets. Vaccine **30:**3646-3652.
217. **Sun, Y., M. Han, C. Kim, J. G. Calvert, and D. Yoo.** 2012. Interplay between interferon-mediated innate immunity and porcine reproductive and respiratory syndrome virus. Viruses **4:**424-446.
218. **Sun, Y., F. Xue, Y. Guo, M. Ma, N. Hao, X. C. Zhang, Z. Lou, X. Li, and Z. Rao.** 2009. Crystal structure of porcine reproductive and respiratory syndrome virus leader protease Nsp1alpha. Journal of virology **83:**10931-10940.
219. **Sun, Z., Z. Chen, S. R. Lawson, and Y. Fang.** 2010. The cysteine protease domain of porcine reproductive and respiratory syndrome virus nonstructural protein 2 possesses deubiquitinating and interferon antagonism functions. Journal of virology **84:**7832-7846.
220. **Sun, Z., Y. Li, R. Ransburgh, E. J. Snijder, and Y. Fang.** 2012. Nonstructural protein 2 of porcine reproductive and respiratory syndrome virus inhibits the antiviral function of interferon-stimulated gene 15. Journal of virology **86:**3839-3850.
221. **Tamura, K., J. Dudley, M. Nei, and S. Kumar.** 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular biology and evolution **24:**1596-1599.
222. **Thacker, E. L.** 2001. Immunology of the porcine respiratory disease complex. The Veterinary clinics of North America. Food animal practice **17:**551-565.
223. **Thanawongnuwech, R., A. Amonsin, A. Tatsanakit, and S. Damrongwatanapokin.** 2004. Genetics and geographical variation of porcine reproductive and respiratory syndrome virus (PRRSV) in Thailand. Veterinary microbiology **101:**9-21.
224. **Thanawongnuwech, R., and S. Suradhat.** 2010. Taming PRRSV: revisiting the control strategies and vaccine design. Virus research **154:**133-140.
225. **Tian, D., Z. Wei, J. C. Zevenhoven-Dobbe, R. Liu, G. Tong, E. J. Snijder, and S. Yuan.** 2012. Arterivirus minor envelope proteins are a major determinant of viral tropism in cell culture. Journal of virology **86:**3701-3712.
226. **Tian, K., X. Yu, T. Zhao, Y. Feng, Z. Cao, C. Wang, Y. Hu, X. Chen, D. Hu, X. Tian, D. Liu, S. Zhang, X. Deng, Y. Ding, L. Yang, Y. Zhang, H. Xiao, M. Qiao, B. Wang, L. Hou, X. Wang, X. Yang, L. Kang, M. Sun, P. Jin, S. Wang, Y. Kitamura, J. Yan, and G. F. Gao.** 2007. Emergence of fatal PRRSV variants: unparalleled outbreaks of atypical PRRS in China and molecular dissection of the unique hallmark. PloS one **2:**e526.
227. **Tiegs, S. L., D. M. Russell, and D. Nemazee.** 1993. Receptor editing in self-reactive bone marrow B cells. The Journal of experimental medicine **177:**1009-1020.
228. **Trible, B. R., M. Kerrigan, N. Crossland, M. Potter, K. Faaberg, R. Hesse, and R. R. Rowland.** 2011. Antibody recognition of porcine circovirus type 2 capsid protein

epitopes after vaccination, infection, and disease. Clinical and vaccine immunology : CVI **18:**749-757.

229. **Uze, G., G. Schreiber, J. Piehler, and S. Pellegrini.** 2007. The receptor of the type I interferon family. Current topics in microbiology and immunology **316:**71-95.
230. **Van Breedam, W., H. Van Gorp, J. Q. Zhang, P. R. Crocker, P. L. Delputte, and H. J. Nauwynck.** 2010. The M/GP(5) glycoprotein complex of porcine reproductive and respiratory syndrome virus binds the sialoadhesin receptor in a sialic acid-dependent manner. PLoS pathogens **6:**e1000730.
231. **van der Meer, Y., H. van Tol, J. K. Locker, and E. J. Snijder.** 1998. ORF1a-encoded replicase subunits are involved in the membrane association of the arterivirus replication complex. Journal of virology **72:**6689-6698.
232. **Van Gorp, H., W. Van Breedam, J. Van Doorsselaere, P. L. Delputte, and H. J. Nauwynck.** 2010. Identification of the CD163 protein domains involved in infection of the porcine reproductive and respiratory syndrome virus. Journal of virology **84:**3101-3105.
233. **Van Reeth, K., G. Labarque, H. Nauwynck, and M. Pensaert.** 1999. Differential production of proinflammatory cytokines in the pig lung during different respiratory virus infections: correlations with pathogenicity. Research in veterinary science **67:**47-52.
234. **van Vugt, J. J., T. Storgaard, M. B. Oleksiewicz, and A. Botner.** 2001. High frequency RNA recombination in porcine reproductive and respiratory syndrome virus occurs preferentially between parental sequences with high similarity. The Journal of general virology **82:**2615-2620.
235. **Vashisht, K., T. L. Goldberg, R. J. Husmann, W. Schnitzlein, and F. A. Zuckermann.** 2008. Identification of immunodominant T-cell epitopes present in glycoprotein 5 of the North American genotype of porcine reproductive and respiratory syndrome virus. Vaccine **26:**4747-4753.
236. **Voelkerding, K. V., S. A. Dames, and J. D. Durtschi.** 2009. Next-generation sequencing: from basic research to diagnostics. Clinical chemistry **55:**641-658.
237. **Vollmers, C., R. V. Sit, J. A. Weinstein, C. L. Dekker, and S. R. Quake.** 2013. Genetic measurement of memory B-cell recall using antibody repertoire sequencing. Proceedings of the National Academy of Sciences of the United States of America **110:**13463-13468.
238. **Vu, H. L., B. Kwon, K. J. Yoon, W. W. Laegreid, A. K. Pattnaik, and F. A. Osorio.** 2011. Immune evasion of porcine reproductive and respiratory syndrome virus through glycan shielding involves both glycoprotein 5 as well as glycoprotein 3. Journal of virology **85:**5555-5564.
239. **Wang, X., M. Eaton, M. Mayer, H. Li, D. He, E. Nelson, and J. Christopher-Hennings.** 2007. Porcine reproductive and respiratory syndrome virus productively infects monocyte-derived dendritic cells and compromises their antigen-presenting ability. Archives of virology **152:**289-303.
240. **Wei, X., J. M. Decker, S. Wang, H. Hui, J. C. Kappes, X. Wu, J. F. Salazar-Gonzalez, M. G. Salazar, J. M. Kilby, M. S. Saag, N. L. Komarova, M. A. Nowak, B. H. Hahn, P. D. Kwong, and G. M. Shaw.** 2003. Antibody neutralization and escape by HIV-1. Nature **422:**307-312.
241. **Welch, S. K., and J. G. Calvert.** 2010. A brief review of CD163 and its role in PRRSV infection. Virus research **154:**98-103.

242. **Wensvoort, G., C. Terpstra, J. M. Pol, E. A. ter Laak, M. Bloemraad, E. P. de Kluyver, C. Kragten, L. van Buiten, A. den Besten, F. Wagenaar, and et al.** 1991. Mystery swine disease in The Netherlands: the isolation of Lelystad virus. The Veterinary quarterly **13:**121-130.
243. **Wertz, N., J. Vazquez, K. Wells, J. Sun, and J. E. Butler.** 2013. Antibody repertoire development in fetal and neonatal piglets. XII. Three IGLV genes comprise 70% of the pre-immune repertoire and there is little junctional diversity. Molecular immunology **55:**319-328.
244. **Wiley, D. C., I. A. Wilson, and J. J. Skehel.** 1981. Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. Nature **289:**373-378.
245. **Wills, R. W., J. J. Zimmerman, K. J. Yoon, S. L. Swenson, M. J. McGinley, H. T. Hill, K. B. Platt, J. Christopher-Hennings, and E. A. Nelson.** 1997. Porcine reproductive and respiratory syndrome virus: a persistent infection. Veterinary microbiology **55:**231-240.
246. **Wilson, V., P. Taylor, and U. Desselberger.** 1988. Crossover regions in foot-and-mouth disease virus (FMDV) recombinants correspond to regions of high local secondary structure. Archives of virology **102:**131-139.
247. **Wissink, E. H., M. V. Kroese, H. A. van Wijk, F. A. Rijsewijk, J. J. Meulenberg, and P. J. Rottier.** 2005. Envelope protein requirements for the assembly of infectious virions of porcine reproductive and respiratory syndrome virus. Journal of virology **79:**12495-12506.
248. **Wu, X., T. Zhou, J. Zhu, B. Zhang, I. Georgiev, C. Wang, X. Chen, N. S. Longo, M. Louder, K. McKee, S. O'Dell, S. Perfetto, S. D. Schmidt, W. Shi, L. Wu, Y. Yang, Z. Y. Yang, Z. Yang, Z. Zhang, M. Bonsignori, J. A. Crump, S. H. Kapiga, N. E. Sam, B. F. Haynes, M. Simek, D. R. Burton, W. C. Koff, N. A. Doria-Rose, M. Connors, N. C. S. Program, J. C. Mullikin, G. J. Nabel, M. Roederer, L. Shapiro, P. D. Kwong, and J. R. Mascola.** 2011. Focused evolution of HIV-1 neutralizing antibodies revealed by structures and deep sequencing. Science **333:**1593-1602.
249. **Yang, L., M. L. Frey, K. J. Yoon, J. J. Zimmerman, and K. B. Platt.** 2000. Categorization of North American porcine reproductive and respiratory syndrome viruses: epitopic profiles of the N, M, GP5 and GP3 proteins and susceptibility to neutralization. Archives of virology **145:**1599-1619.
250. **Yoo, D., C. Song, Y. Sun, Y. Du, O. Kim, and H. C. Liu.** 2010. Modulation of host cell responses and evasion strategies for porcine reproductive and respiratory syndrome virus. Virus research **154:**48-60.
251. **Yoon, K. J., L. L. Wu, J. J. Zimmerman, H. T. Hill, and K. B. Platt.** 1996. Antibody-dependent enhancement (ADE) of porcine reproductive and respiratory syndrome virus (PRRSV) infection in pigs. Viral immunology **9:**51-63.
252. **Yoon, K. J., J. J. Zimmerman, S. L. Swenson, M. J. McGinley, K. A. Eernisse, A. Brevik, L. L. Rhinehart, M. L. Frey, H. T. Hill, and K. B. Platt.** 1995. Characterization of the humoral immune response to porcine reproductive and respiratory syndrome (PRRS) virus infection. Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc **7:**305-312.

253. **Yuan, S., D. Mickelson, M. P. Murtaugh, and K. S. Faaberg.** 2001. Complete genome comparison of porcine reproductive and respiratory syndrome virus parental and attenuated strains. Virus research **79:**189-200.
254. **Yuan, S., C. J. Nelsen, M. P. Murtaugh, B. J. Schmitt, and K. S. Faaberg.** 1999. Recombination between North American strains of porcine reproductive and respiratory syndrome virus. Virus research **61:**87-98.
255. **Yun, S. I., and Y. M. Lee.** 2013. Overview: Replication of porcine reproductive and respiratory syndrome virus. Journal of microbiology **51:**711-723.
256. **Zhang, J., and H. M. Temin.** 1994. Retrovirus recombination depends on the length of sequence identity and is not error prone. Journal of virology **68:**2409-2414.
257. **Zheng, Q., D. Chen, P. Li, Z. Bi, R. Cao, B. Zhou, and P. Chen.** 2007. Co-expressing GP5 and M proteins under different promoters in recombinant modified vaccinia virus ankara (rMVA)-based vaccine vector enhanced the humoral and cellular immune responses of porcine reproductive and respiratory syndrome virus (PRRSV). Virus genes **35:**585-595.
258. **Zhu, J., X. Wu, B. Zhang, K. McKee, S. O'Dell, C. Soto, T. Zhou, J. P. Casazza, N. C. S. Program, J. C. Mullikin, P. D. Kwong, J. R. Mascola, and L. Shapiro.** 2013. De novo identification of VRC01 class HIV-1-neutralizing antibodies by next-generation sequencing of B-cell transcripts. Proceedings of the National Academy of Sciences of the United States of America **110:**E4088-4097.
259. **Zinkernagel, R. M., A. LaMarre, A. Ciurea, L. Hunziker, A. F. Ochsenbein, K. D. McCoy, T. Fehr, M. F. Bachmann, U. Kalinke, and H. Hengartner.** 2001. Neutralizing antiviral antibody responses. Advances in immunology **79:**1-53.
260. **Zuckermann, F. A., E. A. Garcia, I. D. Luque, J. Christopher-Hennings, A. Doster, M. Brito, and F. Osorio.** 2007. Assessment of the efficacy of commercial porcine reproductive and respiratory syndrome virus (PRRSV) vaccines based on measurement of serologic response, frequency of gamma-IFN-producing cells and virological parameters of protection upon challenge. Veterinary microbiology **123:**69-85.