STUDY OF RECOMBINATION IN PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (PRRSV) USING A NOVEL *IN-VITRO* SYSTEM

by

RANJNI JAGDISH CHAND

B.S., University of Mumbai, 2008

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine/Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2013

Abstract

Mechanisms for mutations in RNA viruses include random point mutations, insertions, deletions, recombination and re-assortment. Most viruses have more than one of these mechanisms operating during their life cycle. Impact of sequence divergence is seen in the areas of evolution, epidemiology and ecology of these viruses. Immediate negative consequences of genetic diversity include failure of vaccination, resistance to anti-virals, emergence and re-emergence of novel virus isolates with increased virulence or altered tropism. To identify specific sequence features that influence recombination, a new *in-vitro* system was developed using an infectious cDNA clone of PRRS virus that expressed fluorescent proteins. The *in-vitro* experimental system involved the co-transfection of a pair of closely related PRRSV infectious clones: a fully functional non-fluorescent PRRS virus infectious clone that possessed a single mutation in a green fluorescent protein (GFP) and a second infectious clone that contained a defective fluorescent virus. The readout for successful recombination was appearance of a fully functional fluorescent virus. The model system creates the opportunity to study several aspects of recombination, including the requirement for sequence homology between viruses undergoing recombination.

STUDY OF RECOMBINATION IN PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (PRRSV) USING A NOVEL *IN-VITRO* SYSTEM

by

RANJNI JAGDISH CHAND

B.S., University of Mumbai, 2008

A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine/Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2013

Approved by:

Major Professor
Dr. Raymond R. R. Rowland

Copyright

Abstract

Mechanisms for mutations in RNA viruses include random point mutations, insertions, deletions, recombination and re-assortment. Most viruses have more than one of these mechanisms operating during their life cycle. Impact of sequence divergence is seen in the areas of evolution, epidemiology and ecology of these viruses. Immediate negative consequences of genetic diversity include failure of vaccination, resistance to anti-virals, emergence and re-emergence of novel virus isolates with increased virulence or altered tropism. To identify specific sequence features that influence recombination, a new *in-vitro* system was developed using an infectious cDNA clone of PRRS virus that expressed fluorescent proteins. The *in-vitro* experimental system involved the co-transfection of a pair of closely related PRRSV infectious clones: a fully functional non-fluorescent PRRS virus infectious clone that possessed a single mutation in a green fluorescent protein (GFP) and a second infectious clone that contained a defective fluorescent virus. The readout for successful recombination was appearance of a fully functional fluorescent virus. The model system creates the opportunity to study several aspects of recombination, including the requirement for sequence homology between viruses undergoing recombination.

Table of Contents

List of Figures

List of Tables

Acknowledgements

I cannot thank God enough for this opportunity He gave me to come to the United States, live and study here for 4.5 years. I will always be grateful to Dr. Bob Rowland for letting me be part of his research group. He has been gracious to forgive my big and small mistakes several times over these years. I wish I could have been a better student to him and contributed more to his work. I hope someday that will happen. I am thankful to my supervisory committee that includes Dr. Hesse, Dr. Chang, Dr. Shi and Dr. Davis for the time they have devoted to meetings with me. I wish I would have spent more time in discussions with these very talented personalities. I further thank the entire Rowland Lab crew that I have interacted with in so many different ways. I thank my family and friends in India and the US for having adjusted with my busy schedules, not-so-pleasant attitudes and frequent lack of communication. Finally I thank my fiancé, Phillip Schumm, for embracing me the way I am and loving me all day every day.

Dedication

"...To Him who is able to keep you from stumbling,
and to make you stand in the presence
of His glory blameless with great joy,
to the only God our Savior,
through Jesus Christ our Lord,
be glory, majesty, dominion and authority,
before all time and now and forever.
Amen."
Jude 24,25

Chapter 1 - Literature review of PRRS virus

Overview of the dissertation

RNA viruses are known for their rapid rate of mutation and evolution. Point mutations result in genetic drift while recombinations have been found to result in genetic shift. Recombination in RNA viruses is a more recent area of research as compared to point mutations. RNA recombination was first identified in positive-sense RNA animal viruses in the 1960s. It was initially thought to occur only in positive-sense RNA viruses, but soon was discovered in negative-sense RNA viruses, single & double stranded viruses, multipartite viruses and retroviruses as well (Goodrich and Duesberg, 1990; HIRST, 1962; Worobey and Holmes, 1999). Starting from animal viruses, recombination has been identified in plant viruses as well as in bacteriophages. The consequences of such genetic shifts include species jumps and emergence of viruses with altered pathogenicity.

Porcine reproductive and respiratory syndrome virus (PRRSV) is responsible for major economic impacts to the swine industry worldwide. One of the biggest challenges in the control and elimination of this disease is the rapid evolutionary rate of this RNA virus. Recombination has been indicated to be one of the sources of genetic diversity in PRRSV. In this dissertation we describe the use of PRRSV as a model virus to develop an *in-vitro* system to study parameters that influence RNA recombination.

Chapter 1 gives relevant background for PRRS virus and a review of the pathogenic mechanisms of this virus. Chapter 2 is a literature review of RNA recombination. Part A in this chapter includes background knowledge about the molecular mechanism of recombination: 'copy-choice' mechanism, recombination in other members in Order Nidovirales and systems that are used in the study of RNA recombination. Part B in Chapter 2 discusses the current information about PRRSV recombination, knowledge gaps and the goal of this study. Chapter 3

describes the new *in-vitro* system that was established in this research project. Chapter 4 concludes this dissertation with applications of this research in the study of RNA recombination.

1.A. Background for PRRS virus

According to the USDA Foreign Agricultural Service 2012 statistics, pork is the world's most widely eaten meat, which amounts to 42%, followed by chicken, beef and turkey. United States is one of the world's top ten consumers for pork. Besides meat, hogs also make a significant contribution as a source for nearly 20 pharmaceuticals (heart valves, insulin, enzymes) and many more consumer products (fertilizers, garments, cosmetics). Porcine reproductive and respiratory (PRRS) has been the most challenging and devastating disease for producers since it emerged in early 1980s. According to the latest assessment in 2011, the annual production losses to the US swine industry due to PRRS is about $664 million and other additional costs associated with the disease is $447 million (Holtkamp et al., 2011; Neumann et al., 2005). In the US alone an estimated 60% herds are infected with PRRS virus. This disease has been declared to be the biggest economic burden to the swine industry by the National Pork Board. An outbreak of PRRS causes respiratory distress in young pigs, reproductive losses in adult pigs, increased mortality and overall poor growth performance in a herd (Snijder and Spaan, 2007). Within a production system, PRRSV infection predominantly exists as a subclinical infection, participating as a co-factor in various polymicrobial disease syndromes, such as porcine respiratory disease complex (PRDC) and porcine circovirus associated disease (PCVAD). Soon after PRRS virus was first isolated in the Netherlands and United States in the late 1980s, it spread throughout the world and is endemic in most swine-producing countries (Benfield et al., 1992; Wensvoort et al., 1991). The high rate of morbidity and mortality involves severe animal suffering and direct economic impact. In section 1.A. the virus biology, genetic divergence and reverse genetics as an effective tool to study PRRSV are described. This is followed by section 1.B. that discusses the pathogenic mechanisms that PRRSV uses to cause disease.

1.A.i. PRRS virus: genome organization and replication

PRRS first appeared in Midwestern United States in 1987 followed by similar reports from Quebec and Germany (Dea et al., 1992; Hill, H, 1990; Wensvoort et al., 1991). In 1991, the etiological agent of PRRS was confirmed to be a positive-sense single-stranded RNA virus (Terpstra et al., 1991). Based on nucleotide sequencing and amino acid comparison, PRRSV was found to be closely related to lactate dehydrogenase-elevating virus (LDV) and equine arteritis virus (EAV) from the family Arteriviridae (Meng et al., 1994; Meulenberg et al., 1993). Further investigation showed that genome organization and expression of this virus had several similarities with family Coronaviridae that belonged to the same order, Nidovirales (Cavanagh, 1997; Snijder and Meulenberg, 1998).

PRRS virus has limited tropism in continuous cell culture; only African green monkey kidney cells (MA-104) and derivatives (MARC-145) have been found susceptible to the virus (Duan et al., 1998). During infection in swine, target cells are restricted to the monocytic lineage and porcine alveolar macrophages (PAMs) are the primary cells that are infected by PRRSV (Duan et al., 1997). CD 163 is considered to play a critical role in PRRSV infection as it is sufficient to make a cell permissive to this virus (Calvert et al., 2007)(Patton et al., 2009). CD 163 belongs to the scavenger receptor cysteine-rich (SRCR) superfamily which is a macrophage-specific receptor. Physiologically CD 163 may be involved in anti-inflammatory responses and its expression can be modulated by interlukin-10 (IL-10), M-CSF, GM-CSF and IL-4 (Buechler et al., 2000; Moestrup and Møller, 2004). Viral factors that bind with CD 163 have been shown to be GP2 and GP4 (Das et al., 2010). These interactions enable internalization and release of the virus genome inside the cell. Until recently CD169, a sialoadhesin receptor on porcine macrophages was considered essential for the initial binding and entry of PRRSV (Delputte and Nauwynck, 2004). Internalization was suggested to occur by CD169 on cell surface binding with GP5-M heterodimer on the virion surface. However, it has now been demonstrated in CD169 knockout pigs that this receptor is not essential for PRRSV infection in pigs

The length of full-length PRRSV genome is approximately 15 kilobases and it differs based on strain of the virus (Conzelmann et al., 1993). Lelystad, the prototype Eurpean type 1

strain, is 15.1 Kb (GenBank accession number M96262.2) while 16244B, a North American type 2 strain, is 15.4 Kb (GenBank accession number: AF046869.1) in length. As the genomic RNA is positive-stranded it is capable of being directly translated by the host protein synthesis machinery and hence the virus does not have to carry the RNA polymerase in its virion. The genomic RNA has a 5' cap and a 3' polyadenylated tail (Meulenberg et al., 1993; J. J. M. Meulenberg et al., 1998). 5' and 3' ends carry 190-220 nt and 59-117 nt respectively of untranslated regions that flank the polycistronic RNA (Lu et al., 2011; Snijder and Meulenberg, 1998). 5'UTR plays a crucial role in virus replication and translation mechanisms (van den Born et al., 2005).

The PRRS virus genome contains at least ten open reading frames (ORFs) comprising 14 non-structural protein and 8 structural protein coding regions (Fang and Snijder, 2010; Johnson et al., 2011a). The non-structural protein (Nsp's) genes required for virus replication, transcription and translation occupy 75% of the genome at the 5' end (Allende et al., 1999). The remainder of the genome at the 3' end encodes all the structural proteins of the virus. The entire Nsp region is encoded by 2 ORFs, ORF 1a and 1b, that together code for two large replication associated polyproteins pp1a and pp1ab (Ziebuhr et al., 2000). These two polyproteins are translated directly from the genomic RNA. Translation of pp1ab occurs by a -1 ribosomal frame shift signal carried in the overlapping ORF1a/ORF1b region (den Boon et al., 1991). A recently discovered transframe ORF (TF ORF) is present in ORF 1a and is translated by a -2PRF (Fang et al., 2012). The length of ORF 1a is more variable compared with ORF 1b, the main reason being the hypervariable region in Nsp2 within ORF 1a (Wang et al., 2013). After ORF1 is translated from the genomic RNA strand, pp1a and pp1ab are processed into 14 distinct non-structural proteins by a cascade of four virally encoded proteins (Fang and Snijder, 2010). These Nsp's are named Nsp1 to Nsp12 where Nsp1 and Nsp7 are further cleaved into two subunits each: Nsp1α, Nsp1β, Nsp7α and Nsp7β. The four proteinases that carry out the entire polyprotein processing are three papain-like proteases, PLPs encoded by Nsp1α, Nsp1β (den Boon et al., 1995), Nsp2 (Han et al., 2009) and one chymotrypsin-like serine protease, SP encoded by Nsp4 (Tian et al., 2009). ORFs 3 to 8 encode rest of the replicative enzymes which include the two key enzymes: RNA-dependent RNA polymerase (RdRp) encoded by Nsp9 and RNA helicase (Hel) encoded by Nsp10. These key enzymes then participate in the formation of specialized intracellular

compartments called ‘replication transcription complex’ (RTC) which are localized in the perinuclear region in infected cells (Pedersen et al., 1999). RTCs mediate replication of the virus genome as well as the generation of mRNAs.

PRRSV follows the elegant and complex mRNA synthesis process which is a hallmark for the order Nidovirales. This involves the synthesis of a nested set of subgenomic mRNAs (sg-mRNAs) that are translated into eight known structural proteins (Vries et al., 1990). The 3’ end of the genome is transcribed into a set of sg-mRNAs by the process of discontinuous minus-strand synthesis (Pasternak et al., 2006). This mechanism is similar to copy-choice model for RNA recombination (Lai, 1992). These subgenomes are functionally monocistronic except for ORF2 and ORF5, each of which give rise to ‘a’ and ‘b’ subunits (Firth et al., 2011; Snijder et al., 1999). Each of these subgenome length mRNAs have a common ‘leader’ sequence at the 5’ end which is 156-211 nt in length and the 3’ ends of sg-mRNAs are co-terminal. There is a sequence signal called the ‘transcription regulatory sequence’ (TRS) present at 5’ end of each ORF which is complementary to the TRS at the 3’ of the common leader sequence (Marle et al., 1999). During negative strand synthesis the transcription machinery pauses after the synthesis of ‘body TRS’, dissociates and binds to ‘leader TRS’ and completes mRNA synthesis. This mechanism results in generation of negative-sense sg-mRNAs that serve as templates for positive-sense sg-mRNA synthesis. This process of transcription is very sensitive to mutations in Nsp1 and Nsp10 (Tijms et al., 2001; van Dinten et al., 1997). Each of these positive-sense sg-mRNAs is used for translation to generate the virus structural proteins. At present the known structural proteins belong to two groups: glycosylated proteins (GP2a, GP3, GP4, GP5) and non-glycosylated proteins (2b, 5a, M encoded by ORF6, N encoded by ORF7). N protein makes up the nucleocapsid layer by itself, GP5 and M are the major envelope proteins, and the rest of the SPs are called minor envelope proteins (Dokland, 2010).

N protein

The length of nucleocapsid protein in EU strains is 128aa while it is 123aa long in NA strains (369-384 nucleotides in length) (Mardassi et al., 1995; Meulenberg et al., 1995). This is a highly basic protein that weighs between 12-15 KDa. They are rich in phosphoserine residues and the N-terminal of the protein is positively charged which might be the RNA binding domain

(Wootton et al., 2002). The nucleocapsid forms homodimers and encapsidates the ssRNA genome. Symmetry of the capsid was thought to be isometric but recently it is suggested to be pleomorphic (Spilman et al., 2009). Being the 3' most protein to be synthesized it is the most abundant protein, forming 20-40 % of the total protein of the virus. Due to this and the highly conserved nucleotide sequence of ORF 7 the N protein is the most commonly used target protein in diagnosis (Dea et al., 1996; J. J. Meulenberg et al., 1998). Electron microscopic image of the virion shows a roughly spherical and smooth envelope with a diameter of 50-60 nm. The buoyant density of N protein in sucrose was found to be 1.13 to 1.178 g/cm^3 (Mardassi et al., 1994).There are reports showing the localization of this protein in the nucleolus of infected cells (R.Rowland et al., 1999; Rowland and Yoo, 2003).

GP5 and M proteins

These two proteins form heterodimers that are held together by disulfide bonds made in the endoplasmic reticulum (Snijder et al., 2003; Vries et al., 1995). GP5 is 24-26 KDa and M protein is 18-19 KDa in size (Mardassi et al., 1996). GP5 has a localization signal at the N terminal end which is cleaved off during transport from the ER to cell membrane. ORF5 has the most variable nucleotide sequence in the PRRSV genome and its complexity is further increased by the presence of multiple glycosylation sites (Meng et al., 1995; Murtaugh et al., 1995). Most neutralizing antibodies are targeted to epitopes in GP5 protein (Weiland et al., 2000). The role of glycosylation of GP5 and minor envelop proteins in the viral pathogenesis is discussed in detail in section 1.B.i. This protein has an apoptotic effect on PAMs and MARC-145 cells (Suárez et al., 1996). The M protein is non-glycosylated with three hydrophobic domains that may act as transmembrane regions (Faaberg and Plagemann, 1995). M protein plays an important role in virus assembly and budding from cells. The GP5-M heterodimer has been studied for its critical role in binding to cellular receptors in the process of attachment and entry of the virus (Jiang et al., 2007; Xia et al., 2009).

GP2a, GP3, GP4, 2b, 5a proteins

These are the five minor structural proteins present on PRRSV envelope (Wieringa et al., 2004). None of these proteins are required for virus replication but all of them except 5a are

essential for virus infectivity (Molenkamp et al., 2000b; Wissink et al., 2005). GP2a and GP4 are Class 1 integral membrane proteins (Snijder et al., 1999). GP2a carries disulfide resides on its ectodomain that are crucial for forming heterodimers with GP3 and GP4 (Wieringa et al., 2003). GP3 is the most heavily glycosylated envelope protein (de Lima et al., 2009). It has an N-terminal signal sequence and a C-terminal hydrophobic domain. GP4 is a 20-35 KDa envelope protein that generates the most neutralizing antibodies after GP5 (Meulenberg et al., 1997). GP4 has been suggested to interact with GP2 and GP5. This protein binds to the cell receptor CD163 which is important for the virus attachment and internalization process (Das et al., 2010). Protein 2b is a non-glycosylated myristolated membrane protein that has ion channel-like properties (Wu et al., 2001). This protein is mostly embedded in the membrane. Protein 5a is a recently discovered structural protein in PRRSV (Firth et al., 2011). It is hydrophobic in nature and is produced in low levels in cells. The viral envelope proteins are retained in the golgi-complex. RNA-coated nucelocapsids are wrapped in membranes of the ER or golgi complex where extensive processing occurs (Wieringa et al., 2004; Wood et al., 1970). Enveloped viruses bud from here into secretory vesicles which carry them to the plasma membrane to be released outside the cell.

1.A.ii. PRRS virus: genetic diversity

Positive-sense ssRNA viruses encompass the group of RNA viruses with the largest diversity in size (3 to 32 Kb) and replicative mechanisms (Fauquet and International Committee on Taxonomy of Viruses., 2005). The remarkable diversity of RNA viruses is attributed to two features of these viruses:

(1) The RNA-dependent RNA polymerase (RdRp) lacks proofreading mechanism (Steinhauer et al., 1992)
(2) Large population size of the progeny (Eigen and Schuster, 1977)

The knowledge about PRRSV genetic diversity has been expanding with introduction of new molecular sequence data which is added to the PRRSV database through several research

efforts globally. Lelystad and VR-2332 are prototype strains that were first isolated in Europe and North America respectively (Benfield et al., 1992; Meulenberg et al., 1993). Markedly these two strains that were found to cause similar clinical symptoms in pig herds had only 60% similarity at the nucleotide level (Nelsen et al., 1999). They were designated as European genotype 1 and North American genotype 2 strains and since then have rapidly spread globally and diversified genetically (Zimmerman et al., 2006). Further nucleotide divergence up to 20% has been reported within each genotype (Han et al., 2007; Meng, 2000a). GP5 is the most variable structural region while NSP2 is the most variable non-structural region (Murtaugh et al., 1995; Wang et al., 2013). European isolates were classified on the basis of ORF 5 nucleotide sequence into 3 subtypes: Subtype I, II and III (Shi et al., 2010a). Subtype I comprised Western European strain and was further separated in to 12 clades. Subtype II and III comprised Eastern European strains that showed greater diversity compared to Subtype I. The diversity in these isolates was attributed to new strain introductions in to herds, massive trade within Europe, local strain diversification by point mutations and recombination. Type I strain has been introduced into the non-European countries; USA, China, Thailand, Canada and South Korea through vaccinated breeders or modified live vaccines (MLVs are attenuated strains of the virus that elicits an effective immune response without causing clinical disease) (Chae and Choi, 2010; Dewey et al., 2000; Fang et al., 2007a, p. 200; Thanawongnuwech et al., 2004). In both genotypes, MLV vaccine strains have contributed to the diversity. North American genotype viruses were classified initially using the method, restriction fragment length polymorphism (RFLP) (Wesley et al., 1998). However, this method has an important limitation of reliability and hence nucleotide sequencing is the more widely used method. The most recent comprehensive presentation of Type II viruses classified this genotype in to 9 lineages based on ORF 5 nucleotide sequence (Shi et al., 2010b).

The first outbreak referred to as ‘original outbreak’ occurred in late 1980s where the prototype strain was isolated (Collins et al., 1992). Between 1989 and early 1990s this disease spread to a significant number of states in the US. The second epidemic broke out in Iowa in 1996 which was called ‘acute PRRS’ (Eric J Bush et al., 1999). The clinical characterization of this disease was high rate of sow mortality and abortions. This was a peculiar outbreak as the affected herds were vaccinated. Heterogenic sequences were isolated implying multiple

simultaneous outbreaks. The next outbreak occurred in 2001 in Minnesota and Canada (Han et al., 2006). The causative strain of virus was designated 'MN184' due to its RFLP pattern and had unique discontinuous deletions in Nsp2 region. This epidemic was followed by the first emergence in summer 2006 of highly pathogenic Chinese PRRSV 'HP-PRRS' (Tian et al., 2007). This outbreak caused high fever, increased mortality in adult pigs and red body coloration. The causative strain showed a new 30 amino acid deletion in nsp2 but this was found to be not the cause for its virulence. After the epidemic died out there was re-emergence of highly pathogenic PRRS in 2009 in China and other Southeast Asian countries (Zhou et al., 2011). The crucial role that genetic divergence of PRRSV plays in the pathogenic mechanism of this virus is described in section 1.B.ii.

Origin of PRRSV

There are two hypotheses for the theory of origin of distinct type I and type II PRRSV genotypes. Both theories propose that the two genotypes existed in circulation many years prior to the first outbreak, the time of divergence is disputed to be either early or more recent (Forsberg, 2005; Hanada et al., 2005). At present there is no evidence-based theory of emergence of PRRS but there is one potential hypothesis. According to this hypothesis the ancestral virus for PRRSV was a rodent carried virus, as Lactate dehydrogenase elevating virus (LDV) is the closest related Arterivirus member (Plagemann, 2003). This virus was transferred to wild boars in Europe which served as an intermediate host and carried it to US. Oldest records of transport of wild boars from Europe to North Carolina, US were in 1912. After this first possible introduction the virus entered domestic pig populations and evolved separately on the two continents until the initial outbreak. However, this theory leaves many unanswered questions such as the cause for a species jump and lack of larger divergence of PRRSV in wild boars.

1.A.iii. Reverse genetics and use of infectious clones

Classical or forward genetics are procedures that investigate the genetic basis of an observed phenotype while reverse genetics is an approach where mutations are introduced in to

genetic sequences and the resulting phenotype is observed (Lewin, 2000). Introduction of these mutations may be specific or random. Procedures used for specific/site-directed mutations are gene silencing and homologous recombination, while chemical and transposon-mediated mutagenesis result in random mutations. Genetic manipulation of viruses using reverse genetics has become an invaluable tool for study of virus biology and development of viral vaccines. Genetic engineering is performed via homologous recombination with helper virus for larger viruses while 'infectious clones' are constructed for smaller sized viruses. Infectious clones can be defined as a full-length double stranded DNA copy of the viral genome which is carried by bacterial plasmids and generates a functional virus. Infectious clones for DNA viruses including polyomaviruses, papillomaviruses, and adenoviruses have been generated. However, this approach with RNA viruses has additional complications. RNA viruses have mutation frequencies up to 10^5 times greater than DNA viruses (Leider et al., 1988; Steinhauer et al., 1992). The high rate of mutation in RNA viruses creates challenges in the accurate study of genetic features of these viruses and this adds to the difficulty of directly manipulating RNA strands. All RNA viruses besides retroviruses lack a DNA phase in their life cycle and hence cDNA intermediates of RNA viruses are used to generate infectious clones. This approach was first successfully demonstrated in 1981 for poliovirus (Racaniello and Baltimore, 1981). Since then infectious clones for several virus families have been reported including picornaviruses, caliciviruses, orthomyxoviruses, rhabdoviruses, paramyxoviruses, flaviviruses, togaviruses, coronaviruses, arteriviruses, and bacteriophage Qβ (Conzelmann and Meyers, 1996; Taniguchi et al., 1978). The exact molecular mechanism involved in generation of infectious RNA virus from a cDNA clone is not known. It is suggested that after transfection of cells with infectious clone, the bacterial plasmid enters the nucleus where the host machinery begins transcription. This results in generation of mRNA which in this case is full-length positive stranded viral genome. Bacterial plasmid sequences from both termini are eliminated and an infectious RNA virus is produced which initiates the normal virus life cycle.

Construction of infectious clones requires the knowledge of the complete sequence of virus. For PRRSV, several strains have been completely sequenced (Meulenberg et al., 1993; Nelsen et al., 1999; Susan L. Ropp et al., 2004). The first infectious clone constructed for PRRSV carried the complete genome of European prototype Lelystad virus in the bacterial

plasmid pOK12 under the bacteriophage T7 RNA polymerase promoter (J. J. M. Meulenberg et al., 1998). This was followed by a similar infectious clone of the North American strain VR-2332 (Nielsen et al., 2003). For both these clones, RNA transcripts have to be generated *in-vitro* which were then used to transfect BHK-21 cells which made virus that infected PRRS permissive cells. Further improvement was made when infectious clone for a more virulent strain, P129, was built (Lee et al., 2005). The full-length virus genome was under the control of human cytomegalovirus immediate early promoter in pCMVmc1 and hence was the first DNA-launched PRRSV clone. Following this a number of additional infectious clones for PRRS virus have been generated and applied for investigation of PRRS virus biology and generating novel vaccine candidates.

1.B. Pathogenic mechanisms of PRRS virus

The arteriviruses, PRRSV, LDV, SHFV, and EAV possess several novel properties related to viral pathogenesis, including cytopathic replication in macrophages, the capacity to establish a persistent infection, as well as cause severe disease. As a group, the arteriviruses represent the absolute extremes in mammalian pathogenesis. For example, SHFV is nearly 100% fatal in Asian monkeys (Snijder and Spaan, 2007). In contrast, LDV rapidly reaches levels close to 1010 virions per ml in the blood with no apparent clinical signs in mice.

The different outcomes following PRRSV infection are a consequence of a complex set of interactions between the virus and the pig host. The acute phase of viremia covers approximately 28 days and primarily targets alveolar macrophages. The mechanistic basis for acute disease, such as respiratory distress is likely a consequence of the release of inflammatory cytokines in the lung. Following the initial clearance from the blood, viremia periodically re-appears (Boddicker et al., 2011), with lymphoid tissues as the primary site of virus replication. Virus can be isolated from lymph nodes for more than 100 days after infection and virus is easily shed to sentinel pigs during the asymptomatic period. Replication levels gradually decay until the virus eventually becomes extinct (Horter et al., 2002; Rowland et al., 2003). The mechanism for extinction is not clear, but probably relates to the gradual disappearance of permissive cells

combined with only a partially effective immune response. By definition, PRRSV is not a 'persistent' virus. However, since the average lifetime of a production pig is approximately 180 days, PRRSV infection is 'life-long' for the vast majority of pigs. The mechanistic basis for persistence is dependent on a combination of factors including; (1) a complex virion structure that possesses a heavily glycosylated surface, (2) re-direction of the humoral response towards non-surface proteins, (3) antigenic and genetic drift, and (4) subversion of interferon gene induction. This review primarily focuses on recent advances, reported during the past ten years, related to understanding the processes that contribute to persistence.

1.B.i. Humoral immune response and the role of glycan shielding

An example of the humoral response to PRRSV structural and non-structural proteins during experimental infection is shown in Figure 1-4. Following infection, the earliest and strongest antibody response is against the N protein. In contrast, the antibody response against the major surface component, the GP5-M heterodimer, is weak and delayed. In fact, some animals fail to make a detectable antibody response against GP5. The neutralizing antibody response, which is also weak and delayed, follows a similar pattern (Yoon et al., 1994). Interestingly, a strong antibody response is made against non-structural proteins (Nsp's), such as Nsp2. Nsp2 is not a component of the virion and is found only during the infection of cells. Therefore, the antibody response during infection is primarily directed against viral proteins not associated with virus neutralization

The mechanism for the weak response against GP5 is linked to the presence of several N-linked glycosylation sites, identified by the peptide sequence N(X)S/T. After removal of the peptide signal sequence, the ectodomain of GP5 is only about 30 amino acids long. The ectodomain possesses two conserved N-glycosylation sites, located at position N44 and N51 in type 2 viruses and N46 and N 53 in type 1 viruses (Plagemann et al., 2002; Wissink et al., 2004). In addition, the distal asparagine/ serine-rich domain, located between amino acids 30 and 38, possesses a small region containing a variable number of potential N-glycosylation sites. Depending on the virus isolate, the number of N-sites on the distal end of the ectodomain ranges

from 0 to 3 (Dea et al., 2000; Dokland, 2010; Plagemann et al., 2002).The role of the number of N-sites in Arterivirus persistence was first demonstrated by changes in the number of N-glycosylation sites in VP-3 following the infection of mice with LDV. VP-3 of LDV possesses a pattern of glycosylation similar to GP5 of PRRSV with conserved sites located at N45 and N52 (Faaberg and Plagemann, 1995). The preservation of both sites correlates with resistance to neutralizing antibody and persistent infection. In contrast, certain naturally occurring strains of LDV which are neurotropic, lack the N-terminal and N45 glycosylation sites. These isolates are susceptible to neutralizing antibody and exhibit a low level of viremia. Sequence analysis of VP-3 in the residual circulating viruses showed the reacquisition of both glycosylation sites (Plagemann, 2004). Rowland et al. (R. R. Rowland et al., 1999) followed GP5 ectodomain peptide sequences in pigs exposed to VR- 2332 in utero. Within a week after birth, a mutant virus appeared that possessed a D to N mutation in GP5 at amino acid position 34, which created an additional N-glycosylation site on the distal end of the ectodomain (R. R. Rowland et al., 1999). However, the mutant virus did not show increased resistance to neutralizing antibody. Costers et al. (Costers et al., 2010b)studied the appearance of mutations in GP5 during the infection of pigs with a type 1 PRRSV. The results showed the appearance of a D to N mutation at position 37 of GP5, which created an additional N-glycosylation site.

A specific role for glycan shielding in GP5 was demonstrated using reverse genetics of an infectious cDNA clone (Ansari et al., 2006). A panel of recombinant viruses was constructed with different combinations of mutations at N34, N44, and, N51. The results showed viruses without N44 were non-viable in culture, indicating a requirement of the asparagine or sugar residues for replication. The elimination of the N34 and N51 glycosylation sites resulted in the increased sensitivity of recombinant viruses to neutralization by antibody from virus-infected pigs. In addition, the infection of pigs with viruses lacking N34 and/or N51 resulted in the production of increased antibody with enhanced neutralization activity against both mutant and parent viruses. The mechanistic basis for the role of glycans is related to the protection of a conserved B cell epitope located between residues 37-45 (Ostrowski et al., 2002). A similar epitope was identified in the same location relative to N45 and N52 sites in LDV VP3 (Faaberg and Plagemann, 1995).

Using an expression plasmid containing a modified recombinant GP5, which possessed an additional Pan DR T-helper cell epitope (PADRE) combined with the elimination of all glycosylation sites at N30, N34, N35 and N51, an increased anti-GP5 humoral response was detected in mice immunized with the DNA plasmid (Li et al., 2009b). PRRSV-specific neutralizing activity in serum was also increased, including the capacity of serum to neutralize a broad range of PRRSV isolates. A similar approach incorporated the expression of recombinant GP5 by an adenovirus vector (Jiang et al., 2007). Increased PRRSV neutralizing activity was obtained following the infection of mice with constructs possessing deletions in N44, N44/51, N30/44/51, N30/33/44/51 or N30/33.

However, it should be noted that the role of GP5 as the major target for neutralizing antibody is not accepted by all members of the scientific community. Glycan shielding may also play a role in the response to minor glycoproteins. Using a reverse genetics approach, virus neutralization of a recombinant virus lacking N51 in GP5 was further enhanced by the removal of the N131 glycosylation site in GP3 (Vu et al., 2011). Viruses recovered from infected pigs showed the restoration of both glycosylation sites. Martínez-Lobo et al. (Martínez-Lobo et al., 2011) placed 39 European type1 PRRS virus isolates into 4 phenotypes based on sensitivity to neutralization; highly sensitive, sensitive, moderately sensitive and neutralization resistant. There was no correlation between the patterns of glycosylation in the structural proteins with the neutralization phenotype.

1.B.ii. The role of genetic and antigenic drift in persistence

A recent analysis of approximately 8,500 ORF5 (GP5) nucleotide sequences indicates that type 2 viruses can be divided into at least nine distinct groups or lineages (Shi et al., 2010a). The capacity of PRRSV to rapidly change is illustrated in our previous work investigating the emergence of European type 1 isolates in the US (Fang et al., 2007b). Type 1 isolates of European origin first appeared in North America around 1999 (Dewey et al., 2000; Fang et al., 2004; Susan L Ropp et al., 2004) and designated as North American (NA) type 1 PRRSV isolates. Phylogenetic analyses was performed using ORF5 and nsp2 nucleotide sequences from

20 type 1 isolates collected between 1999-2004 from 10 states. The NA type 1 viruses were most closely related to the Lelystad virus. Fifteen of the 20 ORF5 sequences fell into one of two major subgroups, designated group A and group B. The two groups had sufficiently diverged to the extent that antibodies derived from pigs infected with a group A isolate failed to neutralize viruses in group B, and vice versa.

An indication of the origin for the NA type 1 viruses was found in the analysis of nsp2. The nsp2 nucleotide sequences showed a phylogenetic topology similar to ORF5, including the placement of the same isolates into the same group A and B clades. Furthermore, 18 of the 20 isolates possessed a single 51 nt deletion in nsp2 (Fang et al., 2004; Susan L Ropp et al., 2004). The deletion does not play a significant role in pathogenesis, but functions as a marker for tracking the origin of the type 1 isolates. The results indicate that the high degree of genetic and biological diversity was derived from a single virus introduced into the U.S.

Genetic diversity incorporates genetic drift through point mutations and genetic shift through recombination. Estimates of the nucleotide substitution rate for PRRSV range from 4.7-9.8X10^{-2}/site/year, which is the highest rate calculated for any RNA virus to date (Hanada et al., 2005; Jenkins et al., 2002). Peptide sequence variability within the structural proteins is represented in Figure 2-5A. The greatest peptide sequence variation is found in the ectodomain region of GP5, outside of the two conserved N-glycosylation sites. The conserved neutralizing epitope is identified by the dotted arrow. Hypervariability is linked to the presence of decoy epitope located between amino acids in the region between 27 and 30. However, this epitope may actually lie outside of the ectodomain within the GP5 peptide signal sequence. In type 1 viruses, Wissink et al., (Wissink et al., 2003) identified a potential neutralizing epitope between amino acids 29 and 35 of GP5, which is located within a hypervariable region. Another region showing peptide sequence variation in the vicinity of a neutralizing epitope is found in GP4, where the epitope maps to amino acids 51-65 (Costers et al., 2010a, 2010b; Vanhee et al., 2010). Interestingly, the epitope region overlaps the open reading frame that encodes GP3. The arteriviruses incorporate overlapping reading frames, as means to maximize the coding capacity of a relatively small genome. However, the presence of overlapping reading frames does not

appear to create any significant constraint on the capacity of the structural proteins to generate peptide sequence variability.

Peptide sequence variability and hypervariability extends to the non-structural proteins. Examples in Figure 1-5B and C show the peptide sequences for nsp1 and nsp2. Within nsp1, the nsp1β polypeptide shows increased peptide sequence variation compared to nsp1α. The protease site marks the separation between the relatively conserved nsp1α and the variable nsp1β. Another example is nsp2, which possesses peptide sequence insertions and deletions combined with peptide sequence hypervariability. Therefore, peptide sequence variation within the Nsp's appears to be important, but the mechanism of how peptide sequence variation contributes to fitness is not known. One possibility is that mutations in nsp1 and nsp2 may contribute to persistence by altering the overall level of virus replication within the cell. Another possibility is the requirement for physical interactions between structural and non-structural proteins during replication. Therefore, a change in the peptide sequence of a structural protein in response to immune selection may require a corresponding change in the partner Nsp in order to maintain a stable heterodimer.

1.B.iii. Subversion of the type 1 interferon response by non-structural proteins

A number of studies have shown that pretreatment of cells with type 1interferon (IFN) inhibits PRRSV replication (Buddaert et al., 1998; Fang and Snijder, 2010; Overend et al., 2007). Therefore, the study of the escape of PRRSV from the innate immune response has primarily focused on the subversion of IFN gene activation. The principal observation is the down-regulation of IFN type 1 synthesis during virus infection in cultured primary porcine macrophages and cell lines. After uncoating and entry of the virus genome in the cytoplasm of the cells, nsp1α and nsp1β are immediately translated and autocleaved from the pp1a and pp1ab polyprotein. This creates the opportunity to block the induction of interferon during the early stages of replication. A summary of observations related to the inhibition of IFNβ synthesis by nsp1 and other ORF1 polyprotein fragments is presented in Table 1-1. The most common mechanism for the inhibition of IFNβ synthesis is the inhibition of IRF3 phosphorylation, which

can be mediated by nsp1, nsp2, nsp11. (Phosphorylated IRF3 is translocated to the nucleus where it binds to the IFNβ promoter). However, some laboratories report no inhibition of IRF3 phosphorylation or propose other alternative mechanisms for the inhibition of IFNβ gene activation. For example, Kim et al. (Kim et al., 2010) showed that nsp1 induces degradation of CREB-binding protein (CBP) which is a co-activator of phosphorylated IRF3 in the nucleus. An alternative strategy was shown by nsp2, which inhibited the polyubiquitination of IkBα preventing the activation of NF-kB, a transcription factor for IFN genes (Sun et al., 2010). Luo et al. (Luo et al., 2008) observed that PRRSV interferes with the early steps of RIG-1 and TLR3 pathways by blocking the activities of IPS-1 and TRIF. The differences in results may reflect differences in virus isolate used for infection, the cell line or perhaps, the design of the experiment. One intriguing possibility is that multiple viral proteins possess multiple activities in the inhibition of IFN synthesis.

1.B.iv. Conclusion

The persistent nature of PRRSV presents significant challenges for the control and elimination of disease. As summarized in Fig. 1-6, subversion of the innate and humoral responses contributes to PRRSV persistence within a population. The strategies that PRRSV utilizes to neutralize host defenses have placed similar limitations on the effectiveness of the current modified live virus (MLV) vaccines, such as delayed and weak neutralizing antibody, persistent infection, and the inability to provide protection against a broad range of field isolates. With this new knowledge, alternative approaches are being incorporated in the design and development of the next generation of vaccines (Lunney et al., 2011).

1.C. References

1. Aaziz, R., Tepfer, M., 1999. Recombination between genomic RNAs of two cucumoviruses under conditions of minimal selection pressure. Virology 263, 282–289.
2. Alejska, M., Figlerowicz, Magdalena, Malinowska, N., Urbanowicz, A., Figlerowicz, Marek, 2005. A universal BMV-based RNA recombination system—how to search for general rules in RNA recombination. Nucleic Acids Res. 33, e105–e105.
3. Allende, R., Lewis, T.L., Lu, Z., Rock, D.L., Kutish, G.F., Ali, A., Doster, A.R., Osorio, F.A., 1999. North American and European porcine reproductive and respiratory syndrome viruses differ in non-structural protein coding regions. J. Gen. Virol. 80 (Pt 2), 307–315.
4. Ansari, I.H., Kwon, B., Osorio, F.A., Pattnaik, A.K., 2006. Influence of N-Linked Glycosylation of Porcine Reproductive and Respiratory Syndrome Virus GP5 on Virus Infectivity, Antigenicity, and Ability To Induce Neutralizing Antibodies. J. Virol. 80, 3994 –4004.
5. Banner, L.R., Keck, J.G., Lai, M.M., 1990. A clustering of RNA recombination sites adjacent to a hypervariable region of the peplomer gene of murine coronavirus. Virology 175, 548–555.
6. Banner, L.R., Lai, M.M., 1991. Random nature of coronavirus RNA recombination in the absence of selection pressure. Virology 185, 441–445.
7. Benfield, D.A., Nelson, E., Collins, J.E., Harris, L., Goyal, S.M., Robison, D., Christianson, W.T., Morrison, R.B., Gorcyca, D., Chladek, D., 1992. Characterization of swine infertility and respiratory syndrome (SIRS) virus (isolate ATCC VR-2332). J. Vet. Diagn. Investig. Off. Publ. Am. Assoc. Vet. Lab. Diagn. Inc 4, 127–133.
8. Boddicker, N., Waide, E.H., Rowland, R.R.R., Lunney, J.K., Garrick, D.J., Reecy, J.M., Dekkers, J.C.M., 2011. Evidence for a major QTL associated with host response to Porcine Reproductive and Respiratory Syndrome virus challenge. J. Anim. Sci.
9. Bregnard, C., Pacini, G., Danos, O., Basmaciogullari, S., 2012. Suboptimal Provirus Expression Explains Apparent Nonrandom Cell Coinfection with HIV-1. J. Virol. 86, 8810–8820.
10. Brunier, D., Michel, B., Ehrlich, S.D., 1988. Copy choice illegitimate DNA recombination. Cell 52, 883–892.
11. Buddaert, W., Van Reeth, K., Pensaert, M., 1998. In vivo and in vitro interferon (IFN) studies with the porcine reproductive and respiratory syndrome virus (PRRSV). Adv. Exp. Med. Biol. 440, 461–467.
12. Buechler, C., Ritter, M., Orsó, E., Langmann, T., Klucken, J., Schmitz, G., 2000. Regulation of scavenger receptor CD163 expression in human monocytes and macrophages by pro- and antiinflammatory stimuli. J. Leukoc. Biol. 67, 97–103.
13. Calvert, J.G., Slade, D.E., Shields, S.L., Jolie, R., Mannan, R.M., Ankenbauer, R.G., Welch, S.-K.W., 2007. CD163 expression confers susceptibility to porcine reproductive and respiratory syndrome viruses. J. Virol. 81, 7371–7379.
14. Canon, N., Audigé, L., Denac, H., Hofmann, M., Griot, C., 1998. Evidence of freedom from porcine reproductive and respiratory syndrome virus infection in Switzerland. Vet. Rec. 142, 142–143.

15. Cascone, P.J., Carpenter, C.D., Li, X.H., Simon, A.E., 1990. Recombination between satellite RNAs of turnip crinkle virus. EMBO J. 9, 1709–1715.
16. Cavanagh, D., 1997. Nidovirales: a new order comprising Coronaviridae and Arteriviridae. Arch. Virol. 142, 629–633.
17. Chae, J.-S., Choi, K.-S., 2010. Genetic diversity of porcine circovirus type 2 from pigs in Republic of Korea. Res. Vet. Sci. 88, 333–338.
18. Chand, R.J., Trible, B.R., Rowland, R.R., 2012. Pathogenesis of porcine reproductive and respiratory syndrome virus. Curr. Opin. Virol. 2, 256–263.
19. Chang, C.-C., Yoon, K.-J., Zimmerman, J.J., Harmon, K.M., Dixon, P.M., Dvorak, C.M.T., Murtaugh, M.P., 2002. Evolution of porcine reproductive and respiratory syndrome virus during sequential passages in pigs. J. Virol. 76, 4750–4763.
20. Chao, L., 1988. Evolution of sex in RNA viruses. J. Theor. Biol. 133, 99–112.
21. Chen, N., Yu, X., Wang, L., Wu, J., Zhou, Z., Ni, J., Li, X., Zhai, X., Tian, K., 2013. Two natural recombinant highly pathogenic porcine reproductive and respiratory syndrome viruses with different pathogenicities. Virus Genes 46, 473–478.
22. Chetverin, A.B., Chetverina, H.V., Demidenko, A.A., Ugarov, V.I., 1997. Nonhomologous RNA recombination in a cell-free system: evidence for a transesterification mechanism guided by secondary structure. Cell 88, 503–513.
23. Chetverina, H.V., Demidenko, A.A., Ugarov, V.I., Chetverin, A.B., 1999. Spontaneous rearrangements in RNA sequences. FEBS Lett. 450, 89–94.
24. Cho, J.G., Dee, S.A., 2006. Porcine reproductive and respiratory syndrome virus. Theriogenology 66, 655–662.
25. Collins, J.E., Benfield, D.A., Christianson, W.T., Harris, L., Hennings, J.C., Shaw, D.P., Goyal, S.M., McCullough, S., Morrison, R.B., Joo, H.S., 1992. Isolation of swine infertility and respiratory syndrome virus (isolate ATCC VR-2332) in North America and experimental reproduction of the disease in gnotobiotic pigs. J. Vet. Diagn. Investig. Off. Publ. Am. Assoc. Vet. Lab. Diagn. Inc 4, 117–126.
26. Conzelmann, K.-K., Meyers, G., 1996. Genetic engineering of animal RNA viruses. Trends Microbiol. 4, 386–393.
27. Conzelmann, K.K., Visser, N., Van Woensel, P., Thiel, H.J., 1993. Molecular characterization of porcine reproductive and respiratory syndrome virus, a member of the arterivirus group. Virology 193, 329–339.
28. Cooper, P.D., Steiner-Pryor, A., Scotti, P.D., Delong, D., 1974. On the Nature of Poliovirus Genetic Recombinants. J. Gen. Virol. 23, 41–49.
29. Costers, S., Lefebvre, D.J., Van Doorsselaere, J., Vanhee, M., Delputte, P.L., Nauwynck, H.J., 2010a. GP4 of porcine reproductive and respiratory syndrome virus contains a neutralizing epitope that is susceptible to immunoselection in vitro. Arch. Virol. 155, 371–378.
30. Costers, S., Vanhee, M., Van Breedam, W., Van Doorsselaere, J., Geldhof, M., Nauwynck, H.J., 2010b. GP4-specific neutralizing antibodies might be a driving force in PRRSV evolution. Virus Res. 154, 104–113.
31. Dang, Q., Chen, J., Unutmaz, D., Coffin, J.M., Pathak, V.K., Powell, D., KewalRamani, V.N., Maldarelli, F., Hu, W.-S., 2004. Nonrandom HIV-1 infection and double infection via direct and cell-mediated pathways. Proc. Natl. Acad. Sci. U. S. A. 101, 632–637.

32. Das, P.B., Dinh, P.X., Ansari, I.H., Lima, M. de, Osorio, F.A., Pattnaik, A.K., 2010. The Minor Envelope Glycoproteins GP2a and GP4 of Porcine Reproductive and Respiratory Syndrome Virus Interact with the Receptor CD163. J. Virol. 84, 1731–1740.
33. De Lima, M., Ansari, I.H., Das, P.B., Ku, B.J., Martinez-Lobo, F.J., Pattnaik, A.K., Osorio, F.A., 2009. GP3 is a structural component of the PRRSV type II (US) virion. Virology 390, 31–36.
34. Dea, S., Bilodeau, R., Athanassious, R., Sauvageau, R., Martineau, G.P., 1992. Swine reproductive and respiratory syndrome in Qu?bec: Isolation of an enveloped virus serologically-related to Lelystad virus. Can. Vet. J. 33, 801–808.
35. Dea, S., Gagnon, C.A., Mardassi, H., Milane, G., 1996. Antigenic variability among North American and European strains of porcine reproductive and respiratory syndrome virus as defined by monoclonal antibodies to the matrix protein. J. Clin. Microbiol. 34, 1488–1493.
36. Dea, S., Gagnon, C.A., Mardassi, H., Pirzadeh, B., Rogan, D., 2000. Current knowledge on the structural proteins of porcine reproductive and respiratory syndrome (PRRS) virus: comparison of the North American and European isolates. Arch. Virol. 145, 659–688.
37. Dedepsidis, E., Kyriakopoulou, Z., Pliaka, V., Markoulatos, P., 2010. Correlation between recombination junctions and RNA secondary structure elements in poliovirus Sabin strains. Virus Genes 41, 181–191.
38. Dee, S.A., Torremorell, M., Rossow, K., Mahlum, C., Otake, S., Faaberg, K., 2001. Identification of genetically diverse sequences (ORF 5) of porcine reproductive and respiratory syndrome virus in a swine herd. Can. J. Vet. Res. Rev. Can. Rech. Vét. 65, 254–260.
39. Delputte, P.L., Nauwynck, H.J., 2004. Porcine Arterivirus Infection of Alveolar Macrophages Is Mediated by Sialic Acid on the Virus. J. Virol. 78, 8094–8101.
40. Den Boon, J.A., Faaberg, K.S., Meulenberg, J.J., Wassenaar, A.L., Plagemann, P.G., Gorbalenya, A.E., Snijder, E.J., 1995. Processing and evolution of the N-terminal region of the arterivirus replicase ORF1a protein: identification of two papainlike cysteine proteases. J. Virol. 69, 4500–4505.
41. Den Boon, J.A., Snijder, E.J., Chirnside, E.D., de Vries, A.A., Horzinek, M.C., Spaan, W.J., 1991. Equine arteritis virus is not a togavirus but belongs to the coronaviruslike superfamily. J. Virol. 65, 2910–2920.
42. Dewey, C., Charbonneau, G., Carman, S., Hamel, A., Nayar, G., Friendship, R., Eernisse, K., Swenson, S., 2000. Lelystad-like strain of porcine reproductive and respiratory syndrome virus (PRRSV) identified in Canadian swine. Can. Vet. J. Rev. Vét. Can. 41, 493–494.
43. Dokland, T., 2010. The structural biology of PRRSV. Virus Res. 154, 86–97.
44. Duan, X., Nauwynck, H.J., Favoreel, H.W., Pensaert, M.B., 1998. Identification of a Putative Receptor for Porcine Reproductive and Respiratory Syndrome Virus on Porcine Alveolar Macrophages. J. Virol. 72, 4520–4523.
45. Duan, X., Nauwynck, H.J., Pensaert, M.B., 1997. Effects of origin and state of differentiation and activation of monocytes/macrophages on their susceptibility to porcine reproductive and respiratory syndrome virus (PRRSV). Arch. Virol. 142, 2483–2497.
46. Dzianott, A., Bujarski, J.J., 2004. Infection and RNA recombination of Brome mosaic virus in Arabidopsis thaliana. Virology 318, 482–492.

47. Eigen, M., Schuster, P., 1977. The hypercycle. A principle of natural self-organization. Part A: Emergence of the hypercycle. Naturwissenschaften 64, 541–565.
48. Eric J Bush, Barbara Corso, Jeff Zimmerman, Sabrina Swenson, Dave Pyburn, Tom Burkgren, 1999. Update on the acute PRRS investigative study. JSHAP.
49. Faaberg, K.S., Plagemann, P.G., 1995. The envelope proteins of lactate dehydrogenase-elevating virus and their membrane topography. Virology 212, 512–525.
50. Fang, Y., Kim, D.-Y., Ropp, S., Steen, P., Christopher-Hennings, J., Nelson, E.A., Rowland, R.R.R., 2004. Heterogeneity in Nsp2 of European-like porcine reproductive and respiratory syndrome viruses isolated in the United States. Virus Res. 100, 229–235.
51. Fang, Y., Schneider, P., Zhang, W.P., Faaberg, K.S., Nelson, E.A., Rowland, R.R.R., 2007a. Diversity and evolution of a newly emerged North American Type 1 porcine arterivirus: analysis of isolates collected between 1999 and 2004. Arch. Virol. 152, 1009–1017.
52. Fang, Y., Schneider, P., Zhang, W.P., Faaberg, K.S., Nelson, E.A., Rowland, R.R.R., 2007b. Diversity and evolution of a newly emerged North American Type 1 porcine arterivirus: analysis of isolates collected between 1999 and 2004. Arch. Virol. 152, 1009–1017.
53. Fang, Y., Snijder, E.J., 2010. The PRRSV replicase: exploring the multifunctionality of an intriguing set of nonstructural proteins. Virus Res. 154, 61–76.
54. Fang, Y., Treffers, E.E., Li, Y., Tas, A., Sun, Z., Meer, Y. van der, Ru, A.H. de, Veelen, P.A. van, Atkins, J.F., Snijder, E.J., Firth, A.E., 2012. Efficient −2 frameshifting by mammalian ribosomes to synthesize an additional arterivirus protein. Proc. Natl. Acad. Sci. 109, E2920–E2928.
55. Fauquet, C., International Committee on Taxonomy of Viruses., 2005. Virus taxonomy : classification and nomenclature of viruses 8th report of the International Committee on Taxonomy of Viruses. Elsevier/Academic Press, Oxford.
56. Figlerowicz, M., Bujarski, J.J., 1998. RNA recombination in brome mosaic virus, a model plus strand RNA virus. Acta Biochim. Pol. 45, 847–868.
57. Firth, A.E., Zevenhoven-Dobbe, J.C., Wills, N.M., Go, Y.Y., Balasuriya, U.B.R., Atkins, J.F., Snijder, E.J., Posthuma, C.C., 2011. Discovery of a small arterivirus gene that overlaps the GP5 coding sequence and is important for virus production. J. Gen. Virol. 92, 1097–1106.
58. Forsberg, R., 2005. Divergence time of porcine reproductive and respiratory syndrome virus subtypes. Mol. Biol. Evol. 22, 2131–2134.
59. Forsberg, R., Storgaard, T., Nielsen, H.S., Oleksiewicz, M.B., Cordioli, P., Sala, G., Hein, J., Bøtner, A., 2002a. The Genetic Diversity of European Type PRRSV Is Similar to That of the North American Type but Is Geographically Skewed within Europe. Virology 299, 38–47.
60. Forsberg, R., Storgaard, T., Nielsen, H.S., Oleksiewicz, M.B., Cordioli, P., Sala, G., Hein, J., Bøtner, A., 2002b. The genetic diversity of European type PRRSV is similar to that of the North American type but is geographically skewed within Europe. Virology 299, 38–47.
61. Frossard, J.-P., Hughes, G.J., Westcott, D.G., Naidu, B., Williamson, S., Woodger, N.G.A., Steinbach, F., Drew, T.W., 2013. Porcine reproductive and respiratory syndrome virus: Genetic diversity of recent British isolates. Vet. Microbiol. 162, 507–518.

62. Fu, K., Baric, R.S., 1992. Evidence for variable rates of recombination in the MHV genome. Virology 189, 88–102.
63. Georgescu, M.M., Delpeyroux, F., Tardy-Panit, M., Balanant, J., Combiescu, M., Combiescu, A.A., Guillot, S., Crainic, R., 1994. High diversity of poliovirus strains isolated from the central nervous system from patients with vaccine-associated paralytic poliomyelitis. J. Virol. 68, 8089–8101.
64. Goldbach, R.W., 1986. Molecular Evolution of Plant RNA Viruses. Annu. Rev. Phytopathol. 24, 289–310.
65. Goodrich, D.W., Duesberg, P.H., 1990. Retroviral recombination during reverse transcription. Proc. Natl. Acad. Sci. 87, 2052–2056.
66. Gorbalenya, A.E., Koonin, E.V., Lai, M.M.-C., 1991. Putative papain-related thiol proteases of positive-strand RNA viruses Identification of rubi- and aphthovirus proteases and delineation of a novel conserved domain associated with proteases of rubi-, α- and coronaviruses. FEBS Lett. 288, 201–205.
67. Haas, J., Park, E.C., Seed, B., 1996. Codon usage limitation in the expression of HIV-1 envelope glycoprotein. Curr. Biol. CB 6, 315–324.
68. Hahn, C.S., Lustig, S., Strauss, E.G., Strauss, J.H., 1988. Western equine encephalitis virus is a recombinant virus. Proc. Natl. Acad. Sci. 85, 5997–6001.
69. Han, J., Liu, G., Wang, Y., Faaberg, K.S., 2007. Identification of Nonessential Regions of the nsp2 Replicase Protein of Porcine Reproductive and Respiratory Syndrome Virus Strain VR-2332 for Replication in Cell Culture. J. Virol. 81, 9878–9890.
70. Han, J., Rutherford, M.S., Faaberg, K.S., 2009. The porcine reproductive and respiratory syndrome virus nsp2 cysteine protease domain possesses both trans- and cis-cleavage activities. J. Virol. 83, 9449–9463.
71. Han, J., Wang, Y., Faaberg, K.S., 2006. Complete genome analysis of RFLP 184 isolates of porcine reproductive and respiratory syndrome virus. Virus Res. 122, 175–182.
72. Hanada, K., Suzuki, Y., Nakane, T., Hirose, O., Gojobori, T., 2005. The origin and evolution of porcine reproductive and respiratory syndrome viruses. Mol. Biol. Evol. 22, 1024–1031.
73. Hill, H, 1990. Overview and history of mystery swine disease (swine infertility and respiratory syndrome)., in: In Proceedings of the Mystery Swine Disease Committee Meeting. Presented at the Madison, WI: Livestock Conservation Institute, Denver, CO, USA, pp. 29–30.
74. HIRST, G.K., 1962. Genetic recombination with Newcastle disease virus, polioviruses, and influenza. Cold Spring Harb. Symp. Quant. Biol. 27, 303–309.
75. Holtkamp, D., Kliebenstein, J., Neumann, E., Zimmerman, J., Rotto, H., Yoder, T., Wang, C., Yeske, P., Mowrer, C., Haley, C., 2011. Assessment of the economic impact of porcine reproductive and respiratory syndrome virus on U.S. pork producers, in: 2011 International PRRS Symposium. Presented at the 2011 International PRRS Symposium, Chicago Marriot, p. 86.
76. Hopper, S.A., White, M.E., Twiddy, N., 1992. An outbreak of blue-eared pig disease (porcine reproductive and respiratory syndrome) in four pig herds in Great Britain. Vet. Rec. 131, 140–144.
77. Horter, D.C., Pogranichniy, R.M., Chang, C.-C., Evans, R.B., Yoon, K.-J., Zimmerman, J.J., 2002. Characterization of the carrier state in porcine reproductive and respiratory syndrome virus infection. Vet. Microbiol. 86, 213–228.

78. Huang, Y.W., Meng, X.J., 2010. Novel strategies and approaches to develop the next generation of vaccines against porcine reproductive and respiratory syndrome virus (PRRSV). Virus Res. 154, 141–149.
79. Huisman, M.J., Cornelissen, B.J., Groenendijk, C.F., Bol, J.F., van Vloten-Doting, L., 1989. Alfalfa mosaic virus temperature-sensitive mutants. V. The nucleotide sequence of TBTS 7 RNA 3 shows limited nucleotide changes and evidence for heterologous recombination. Virology 171, 409–416.
80. Jarvis, T.C., Kirkegaard, K., 1992. Poliovirus RNA recombination: mechanistic studies in the absence of selection. EMBO J. 11, 3135–3145.
81. Jenkins, G.M., Rambaut, A., Pybus, O.G., Holmes, E.C., 2002. Rates of molecular evolution in RNA viruses: a quantitative phylogenetic analysis. J. Mol. Evol. 54, 156–165.
82. Jia, W., Karaca, K., Parrish, C.R., Naqi, S.A., 1995. A novel variant of avian infectious bronchitis virus resulting from recombination among three different strains. Arch. Virol. 140, 259–271.
83. Jiang, Y., Fang, L., Xiao, S., Zhang, H., Pan, Y., Luo, R., Li, B., Chen, H., 2007. Immunogenicity and protective efficacy of recombinant pseudorabies virus expressing the two major membrane-associated proteins of porcine reproductive and respiratory syndrome virus. Vaccine 25, 547–560.
84. Johnson, C.R., Griggs, T.F., Gnanandarajah, J., Murtaugh, M.P., 2011a. Novel structural protein in porcine reproductive and respiratory syndrome virus encoded by an alternative ORF5 present in all arteriviruses. J. Gen. Virol. 92, 1107–1116.
85. Johnson, C.R., Griggs, T.F., Gnanandarajah, J., Murtaugh, M.P., 2011b. Novel structural protein in porcine reproductive and respiratory syndrome virus encoded by an alternative ORF5 present in all arteriviruses. J. Gen. Virol. 92, 1107–1116.
86. Kapur, V., Elam, M.R., Pawlovich, T.M., Murtaugh, M.P., 1996a. Genetic variation in porcine reproductive and respiratory syndrome virus isolates in the midwestern United States. J. Gen. Virol. 77 (Pt 6), 1271–1276.
87. Kapur, V., Elam, M.R., Pawlovich, T.M., Murtaugh, M.P., 1996b. Genetic variation in porcine reproductive and respiratory syndrome virus isolates in the midwestern United States. J. Gen. Virol. 77 (Pt 6), 1271–1276.
88. Karniychuk, U.U., Geldhof, M., Vanhee, M., Van Doorsselaere, J., Saveleva, T.A., Nauwynck, H.J., 2010. Pathogenesis and antigenic characterization of a new East European subtype 3 porcine reproductive and respiratory syndrome virus isolate. BMC Vet. Res. 6, 30.
89. Khatchikian, D., Orlich, M., Rott, R., 1989. Increased viral pathogenicity after insertion of a 28S ribosomal RNA sequence into the haemagglutinin gene of an influenza virus. Nature 340, 156–157.
90. Kim, D.-Y., Calvert, J.G., Chang, K.-O., Horlen, K., Kerrigan, M., Rowland, R.R.R., 2007. Expression and stability of foreign tags inserted into nsp2 of porcine reproductive and respiratory syndrome virus (PRRSV). Virus Res. 128, 106–114.
91. Kim, M.J., Kao, C., 2001. Factors regulating template switch in vitro by viral RNA-dependent RNA polymerases: implications for RNA-RNA recombination. Proc. Natl. Acad. Sci. U. S. A. 98, 4972–4977.
92. Kim, O., Sun, Y., Lai, F.W., Song, C., Yoo, D., 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.
93. Kirkegaard, K., Baltimore, D., 1986. The mechanism of RNA recombination in poliovirus. Cell 47, 433–443.
94. Koonin, E.V., Dolja, V.V., Morris, T.J., 1993. Evolution and Taxonomy of Positive-Strand RNA Viruses: Implications of Comparative Analysis of Amino Acid Sequences. Crit. Rev. Biochem. Mol. Biol. 28, 375–430.
95. Labarque, G., Reeth, K.V., Nauwynck, H., Drexler, C., Van Gucht, S., Pensaert, M., 2004. Impact of genetic diversity of European-type porcine reproductive and respiratory syndrome virus strains on vaccine efficacy. Vaccine 22, 4183–4190.
96. Lai, M.M., 1992. RNA recombination in animal and plant viruses. Microbiol. Rev. 56, 61–79.
97. Lai, M.M., Baric, R.S., Makino, S., Keck, J.G., Egbert, J., Leibowitz, J.L., Stohlman, S.A., 1985. Recombination between nonsegmented RNA genomes of murine coronaviruses. J. Virol. 56, 449–456.
98. Lai, M.M.C., 1996. Recombination in large RNA viruses: Coronaviruses. Semin. Virol. 7, 381–388.
99. Lauber, C., Ziebuhr, J., Junglen, S., Drosten, C., Zirkel, F., Nga, P.T., Morita, K., Snijder, E.J., Gorbalenya, A.E., 2012. Mesoniviridae: a proposed new family in the order Nidovirales formed by a single species of mosquito-borne viruses. Arch. Virol. 157, 1623–1628.
100. LEDINKO, N., 1963. Genetic recombination with poliovirus type 1. Studies of crosses between a normal horse serum-resistant mutant and several guanidine-resistant mutants of the same strain. Virology 20, 107–119.
101. Lee, C., Calvert, J.G., Welch, S.-K.W., Yoo, D., 2005. A DNA-launched reverse genetics system for porcine reproductive and respiratory syndrome virus reveals that homodimerization of the nucleocapsid protein is essential for virus infectivity. Virology 331, 47–62.
102. Leider, J.M., Palese, P., Smith, F.I., 1988. Determination of the mutation rate of a retrovirus. J. Virol. 62, 3084–3091.
103. Levy, D.N., Aldrovandi, G.M., Kutsch, O., Shaw, G.M., 2004. Dynamics of HIV-1 recombination in its natural target cells. Proc. Natl. Acad. Sci. U. S. A. 101, 4204–4209.
104. Lewin, B., 2000. Genes: VII. Oxford University Press, Incorporated.
105. Li, B., Fang, L., Xu, Z., Liu, S., Gao, J., Jiang, Y., Chen, H., Xiao, S., 2009a. Recombination in Vaccine and Circulating Strains of Porcine Reproductive and Respiratory Syndrome Viruses. Emerg. Infect. Dis. 15, 2032–2035.
106. Li, B., Xiao, S., Wang, Y., Xu, S., Jiang, Y., Chen, H., Fang, L., 2009b. Immunogenicity of the highly pathogenic porcine reproductive and respiratory syndrome virus GP5 protein encoded by a synthetic ORF5 gene. Vaccine 27, 1957–1963.
107. Li, J., Hu, H., Yu, Q., Diel, D.G., Li, D., Miller, P.J., 2012. Generation and characterization of a recombinant Newcastle disease virus expressing the red fluorescent protein for use in co-infection studies. Virol. J. 9, 227.
108. Li, K., Chen, Z., Plagemann, P., 1999. High-Frequency Homologous Genetic Recombination of an Arterivirus, Lactate Dehydrogenase-Elevating Virus, in Mice and Evolution of Neuropathogenic Variants. Virology 258, 73–83.

109. Liu, D., Zhou, R., Zhang, J., Zhou, L., Jiang, Q., Guo, X., Ge, X., Yang, H., 2011. Recombination analyses between two strains of porcine reproductive and respiratory syndrome virus in vivo. Virus Res. 155, 473–486.
110. Lu, J., Gao, F., Wei, Z., Liu, P., Liu, C., Zheng, H., Li, Y., Lin, T., Yuan, S., 2011. A 5'-proximal Stem-loop Structure of 5' Untranslated Region of Porcine Reproductive and Respiratory Syndrome Virus Genome Is Key for Virus Replication. Virol. J. 8, 172.
111. Lunney, J.K., Steibel, J.P., Reecy, J.M., Fritz, E., Rothschild, M.F., Kerrigan, M., Trible, B., Rowland, R.R., 2011. Probing genetic control of swine responses to PRRSV infection: current progress of the PRRS host genetics consortium. BMC Proc. 5 Suppl 4, S30.
112. Luo, R., Xiao, S., Jiang, Y., Jin, H., Wang, D., Liu, M., Chen, H., Fang, L., 2008. Porcine reproductive and respiratory syndrome virus (PRRSV) suppresses interferon-beta production by interfering with the RIG-I signaling pathway. Mol. Immunol. 45, 2839–2846.
113. Mardassi, H., Athanassious, R., Mounir, S., Dea, S., 1994. Porcine reproductive and respiratory syndrome virus: morphological, biochemical and serological characteristics of Quebec isolates associated with acute and chronic outbreaks of porcine reproductive and respiratory syndrome. Can. J. Vet. Res. Rev. Can. Rech. Vét. 58, 55–64.
114. Mardassi, H., Massie, B., Dea, S., 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.
115. Mardassi, H., Mounir, S., Dea, S., 1995. Molecular analysis of the ORFs 3 to 7 of porcine reproductive and respiratory syndrome virus, Québec reference strain. Arch. Virol. 140, 1405–1418.
116. Marle, G. van, Dobbe, J.C., Gultyaev, A.P., Luytjes, W., Spaan, W.J.M., Snijder, E.J., 1999. Arterivirus discontinuous mRNA transcription is guided by base pairing between sense and antisense transcription-regulating sequences. Proc. Natl. Acad. Sci. 96, 12056–12061.
117. Martínez-Lobo, F.J., Díez-Fuertes, F., Simarro, I., Castro, J.M., Prieto, C., 2011. Porcine Reproductive and Respiratory Syndrome Virus isolates differ in their susceptibility to neutralization. Vaccine 29, 6928–6940.
118. Masters, P.S., Koetzner, C.A., Kerr, C.A., Heo, Y., 1994. Optimization of targeted RNA recombination and mapping of a novel nucleocapsid gene mutation in the coronavirus mouse hepatitis virus. J. Virol. 68, 328–337.
119. Masuta, C., Ueda, S., Suzuki, M., Uyeda, I., 1998. Evolution of a quadripartite hybrid virus by interspecific exchange and recombination between replicase components of two related tripartite RNA viruses. Proc. Natl. Acad. Sci. U. S. A. 95, 10487–10492.
120. Mayo, M.A., Jolly, C.A., 1991. The 5'-terminal sequence of potato leafroll virus RNA: evidence of recombination between virus and host RNA. J. Gen. Virol. 72 (Pt 10), 2591–2595.
121. Meng, X.J., 2000a. Heterogeneity of porcine reproductive and respiratory syndrome virus: implications for current vaccine efficacy and future vaccine development. Vet. Microbiol. 74, 309–329.

122. Meng, X.J., 2000b. Heterogeneity of porcine reproductive and respiratory syndrome virus: implications for current vaccine efficacy and future vaccine development. Vet. Microbiol. 74, 309–329.
123. Meng, X.J., Paul, P.S., Halbur, P.G., 1994. Molecular cloning and nucleotide sequencing of the 3'-terminal genomic RNA of the porcine reproductive and respiratory syndrome virus. J. Gen. Virol. 75 (Pt 7), 1795–1801.
124. Meng, X.-J., Paul, P.S., Halbur, P.G., Morozov, I., 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. J. Gen. Virol. 76, 3181–3188.
125. Mengeling, W.L., Lager, K.M., Vorwald, A.C., 1998. Clinical consequences of exposing pregnant gilts to strains of porcine reproductive and respiratory syndrome (PRRS) virus isolated from field cases of "atypical" PRRS. Am. J. Vet. Res. 59, 1540–1544.
126. Mengeling WL., 2002. The potential role of genetic recombination in the evolution of new strains of porcine reproductive and respiratory syndrome virus (PRRSV). J Swine Health Prod 10, 273–275.
127. MESELSON, M., WEIGLE, J.J., 1961. Chromosome brekage accompanying genetic recombination in bacteriophage. Proc. Natl. Acad. Sci. U. S. A. 47, 857–868.
128. Meulenberg, J.J., 2000. PRRSV, the virus. Vet. Res. 31, 11–21.
129. Meulenberg, J.J., Hulst, M.M., de Meijer, E.J., Moonen, P.L., den Besten, A., de Kluyver, E.P., Wensvoort, G., Moormann, R.J., 1993. Lelystad virus, the causative agent of porcine epidemic abortion and respiratory syndrome (PEARS), is related to LDV and EAV. Virology 192, 62–72.
130. Meulenberg, J.J., Petersen-den Besten, A., De Kluyver, E.P., Moormann, R.J., Schaaper, W.M., Wensvoort, G., 1995. Characterization of proteins encoded by ORFs 2 to 7 of Lelystad virus. Virology 206, 155–163.
131. Meulenberg, J.J., van Nieuwstadt, A.P., van Essen-Zandbergen, A., Bos-de Ruijter, J.N., Langeveld, J.P., Meloen, R.H., 1998. Localization and fine mapping of antigenic sites on the nucleocapsid protein N of porcine reproductive and respiratory syndrome virus with monoclonal antibodies. Virology 252, 106–114.
132. Meulenberg, J.J., van Nieuwstadt, A.P., van Essen-Zandbergen, A., Langeveld, J.P., 1997. Posttranslational processing and identification of a neutralization domain of the GP4 protein encoded by ORF4 of Lelystad virus. J. Virol. 71, 6061–6067.
133. Meulenberg, J.J.M., Ruijter, J.N.A.B., Graaf, R. van de, Wensvoort, G., Moormann, R.J.M., 1998. Infectious Transcripts from Cloned Genome-Length cDNA of Porcine Reproductive and Respiratory Syndrome Virus. J. Virol. 72, 380–387.
134. Meyers, G., Rümenapf, T., Thiel, H.J., 1989. Ubiquitin in a togavirus. Nature 341, 491.
135. Meyers, G., Tautz, N., Dubovi, E.J., Thiel, H.J., 1991. Viral cytopathogenicity correlated with integration of ubiquitin-coding sequences. Virology 180, 602–616.
136. Mindich, L., Qiao, X., Onodera, S., Gottlieb, P., Strassman, J., 1992. Heterologous recombination in the double-stranded RNA bacteriophage phi 6. J. Virol. 66, 2605–2610.
137. Moestrup, S.K., Møller, H.J., 2004. CD163: a regulated hemoglobin scavenger receptor with a role in the anti-inflammatory response. Ann. Med. 36, 347–354.

138. Molenkamp, R., Greve, S., Spaan, W.J., Snijder, E.J., 2000a. Efficient homologous RNA recombination and requirement for an open reading frame during replication of equine arteritis virus defective interfering RNAs. J. Virol. 74, 9062–9070.
139. Molenkamp, R., van Tol, H., Rozier, B.C., van der Meer, Y., Spaan, W.J., Snijder, E.J., 2000b. The arterivirus replicase is the only viral protein required for genome replication and subgenomic mRNA transcription. J. Gen. Virol. 81, 2491–2496.
140. Monroe, S.S., Schlesinger, S., 1983. RNAs from two independently isolated defective interfering particles of Sindbis virus contain a cellular tRNA sequence at their 5' ends. Proc. Natl. Acad. Sci. U. S. A. 80, 3279–3283.
141. Mu, C., Lu, X., Duan, E., Chen, J., Li, W., Zhang, F., Martin, D.P., Yang, M., Xia, P., Cui, B., 2013. Molecular evolution of porcine reproductive and respiratory syndrome virus isolates from central China. Res. Vet. Sci.
142. Munishkin, A.V., Voronin, L.A., Chetverin, A.B., 1988. An in vivo recombinant RNA capable of autocatalytic synthesis by Q beta replicase. Nature 333, 473–475.
143. Murtaugh, M.P., Elam, M.R., Kakach, L.T., 1995. Comparison of the structural protein coding sequences of the VR-2332 and Lelystad virus strains of the PRRS virus. Arch. Virol. 140, 1451–1460.
144. Murtaugh, M.P., Xiao, Z., Zuckermann, F., 2002. Immunological responses of swine to porcine reproductive and respiratory syndrome virus infection. Viral Immunol. 15, 533–547.
145. Nagy, P.D., Dzianott, A., Ahlquist, P., Bujarski, J.J., 1995. Mutations in the helicase-like domain of protein 1a alter the sites of RNA-RNA recombination in brome mosaic virus. J. Virol. 69, 2547–2556.
146. Nagy, P.D., Zhang, C., Simon, A.E., 1998. Dissecting RNA recombination in vitro: role of RNA sequences and the viral replicase. EMBO J. 17, 2392–2403.
147. Nelsen, C.J., Murtaugh, M.P., Faaberg, K.S., 1999. Porcine reproductive and respiratory syndrome virus comparison: divergent evolution on two continents. J. Virol. 73, 270–280.
148. Nelson, E.A., Christopher-Hennings, J., Drew, T., Wensvoort, G., Collins, J.E., Benfield, D.A., 1993. Differentiation of U.S. and European isolates of porcine reproductive and respiratory syndrome virus by monoclonal antibodies. J. Clin. Microbiol. 31, 3184–3189.
149. Neumann, E.J., Kliebenstein, J.B., Johnson, C.D., Mabry, J.W., Bush, E.J., Seitzinger, A.H., Green, A.L., Zimmerman, J.J., 2005. Assessment of the economic impact of porcine reproductive and respiratory syndrome on swine production in the United States. J. Am. Vet. Med. Assoc. 227, 385–392.
150. Nielsen, H.S., Liu, G., Nielsen, J., Oleksiewicz, M.B., Bøtner, A., Storgaard, T., Faaberg, K.S., 2003. Generation of an infectious clone of VR-2332, a highly virulent North American-type isolate of porcine reproductive and respiratory syndrome virus. J. Virol. 77, 3702–3711.
151. Ostrowski, M., Galeota, J.A., Jar, A.M., Platt, K.B., Osorio, F.A., Lopez, O.J., 2002. Identification of Neutralizing and Nonneutralizing Epitopes in the Porcine Reproductive and Respiratory Syndrome Virus GP5 Ectodomain. J. Virol. 76, 4241 – 4250.
152. Overend, C., Mitchell, R., He, D., Rompato, G., Grubman, M.J., Garmendia, A.E., 2007. Recombinant swine beta interferon protects swine alveolar macrophages and

MARC-145 cells from infection with Porcine reproductive and respiratory syndrome virus. J. Gen. Virol. 88, 925–931.
153. Pasternak, A.O., Spaan, W.J.M., Snijder, E.J., 2006. Nidovirus transcription: how to make sense...? J. Gen. Virol. 87, 1403–1421.
154. Patton, J.B., Rowland, R.R., Yoo, D., Chang, K.-O., 2009. Modulation of CD163 receptor expression and replication of porcine reproductive and respiratory syndrome virus in porcine macrophages. Virus Res. 140, 161–171.
155. Pedersen, K.W., van der Meer, Y., Roos, N., Snijder, E.J., 1999. Open Reading Frame 1a-Encoded Subunits of the Arterivirus Replicase Induce Endoplasmic Reticulum-Derived Double-Membrane Vesicles Which Carry the Viral Replication Complex. J. Virol. 73, 2016 –2026.
156. Pei, Y., Hodgins, D.C., Wu, J., Welch, S.-K.W., Calvert, J.G., Li, G., Du, Y., Song, C., Yoo, D., 2009. Porcine reproductive and respiratory syndrome virus as a vector: immunogenicity of green fluorescent protein and porcine circovirus type 2 capsid expressed from dedicated subgenomic RNAs. Virology 389, 91–99.
157. Pejsak, Z., Stadejek, T., Markowska-Daniel, I., 1997. Clinical signs and economic losses caused by porcine reproductive and respiratory syndrome virus in a large breeding farm. Vet. Microbiol. 55, 317–322.
158. Pincus, S.E., Diamond, D.C., Emini, E.A., Wimmer, E., 1986. Guanidine-selected mutants of poliovirus: mapping of point mutations to polypeptide 2C. J. Virol. 57, 638–646.
159. Plagemann, P.G.W., 2003. Porcine reproductive and respiratory syndrome virus: origin hypothesis. Emerg. Infect. Dis. 9, 903–908.
160. Plagemann, P.G.W., 2004. GP5 ectodomain epitope of porcine reproductive and respiratory syndrome virus, strain Lelystad virus. Virus Res. 102, 225–230.
161. Plagemann, P.G.W., Rowland, R.R.R., Faaberg, K.S., 2002. The primary neutralization epitope of porcine respiratory and reproductive syndrome virus strain VR-2332 is located in the middle of the GP5 ectodomain. Arch. Virol. 147, 2327–2347.
162. Pond, S.L.K., Murrell, B., Poon, A.F.Y., 2012. Evolution of viral genomes: interplay between selection, recombination, and other forces. Methods Mol. Biol. Clifton NJ 856, 239–272.
163. Racaniello, V.R., Baltimore, D., 1981. Cloned poliovirus complementary DNA is infectious in mammalian cells. Science 214, 916–919.
164. Ropp, Susan L., Mahlum Wees, C.E., Fang, Y., Nelson, E.A., Rossow, K.D., Bien, M., Arndt, B., Preszler, S., Steen, P., Christopher-Hennings, J., Collins, J.E., Benfield, D.A., Faaberg, K.S., 2004. Characterization of Emerging European-Like Porcine Reproductive and Respiratory Syndrome Virus Isolates in the United States. J. Virol. 78, 3684–3703.
165. Ropp, Susan L, Wees, C.E.M., Fang, Y., Nelson, E.A., Rossow, K.D., Bien, M., Arndt, B., Preszler, S., Steen, P., Christopher-Hennings, J., Collins, J.E., Benfield, D.A., Faaberg, K.S., 2004. Characterization of emerging European-like porcine reproductive and respiratory syndrome virus isolates in the United States. J. Virol. 78, 3684–3703.
166. Rowland, R.., Kervin, R., Kuckleburg, C., Sperlich, A., Benfield, D.A., 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 Res. 64, 1–12.

167. Rowland, R.R., Steffen, M., Ackerman, T., Benfield, D.A., 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.
168. Rowland, R.R.R., Lawson, S., Rossow, K., Benfield, D.A., 2003. Lymphoid tissue tropism of porcine reproductive and respiratory syndrome virus replication during persistent infection of pigs originally exposed to virus in utero. Vet. Microbiol. 96, 219–235.
169. Rowland, R.R.R., Yoo, D., 2003. Nucleolar-cytoplasmic shuttling of PRRSV nucleocapsid protein: a simple case of molecular mimicry or the complex regulation by nuclear import, nucleolar localization and nuclear export signal sequences. Virus Res. 95, 23–33.
170. Runckel, C., Westesson, O., Andino, R., DeRisi, J.L., 2013. Identification and Manipulation of the Molecular Determinants Influencing Poliovirus Recombination. PLoS Pathog 9, e1003164.
171. Scheel, T.K.H., Galli, A., Li, Y.-P., Mikkelsen, L.S., Gottwein, J.M., Bukh, J., 2013. Productive Homologous and Non-homologous Recombination of Hepatitis C Virus in Cell Culture. PLoS Pathog 9, e1003228.
172. Serviene, E., Shapka, N., Cheng, C.-P., Panavas, T., Phuangrat, B., Baker, J., Nagy, P.D., 2005. Genome-wide screen identifies host genes affecting viral RNA recombination. Proc. Natl. Acad. Sci. U. S. A. 102, 10545–10550.
173. Shen, S., Kwang, J., Liu, W., Liu, D.X., 2000. Determination of the complete nucleotide sequence of a vaccine strain of porcine reproductive and respiratory syndrome virus and identification of the Nsp2 gene with a unique insertion. Arch. Virol. 145, 871–883.
174. Shi, M., Holmes, E.C., Brar, M.S., Leung, F.C.-C., 2013. Recombination Is Associated with an Outbreak of Novel Highly Pathogenic Porcine Reproductive and Respiratory Syndrome Viruses in China. J. Virol. 87, 10904–10907.
175. Shi, M., Lam, T.T.-Y., Hon, C.-C., Hui, R.K.-H., Faaberg, K.S., Wennblom, T., Murtaugh, M.P., Stadejek, T., Leung, F.C.-C., 2010a. Molecular epidemiology of PRRSV: a phylogenetic perspective. Virus Res. 154, 7–17.
176. Shi, M., Lam, T.T.-Y., Hon, C.-C., Murtaugh, M.P., Davies, P.R., Hui, R.K.-H., Li, J., Wong, L.T.-W., Yip, C.-W., Jiang, J.-W., Leung, F.C.-C., 2010b. Phylogeny-Based Evolutionary, Demographical, and Geographical Dissection of North American Type 2 Porcine Reproductive and Respiratory Syndrome Viruses. J. Virol. 84, 8700–8711.
177. Sibold, C., Meisel, H., Krüger, D.H., Labuda, M., Lysy, J., Kozuch, O., Pejcoch, M., Vaheri, A., Plyusnin, A., 1999. Recombination in Tula hantavirus evolution: analysis of genetic lineages from Slovakia. J. Virol. 73, 667–675.
178. Simon-Loriere, E., Galetto, R., Hamoudi, M., Archer, J., Lefeuvre, P., Martin, D.P., Robertson, D.L., Negroni, M., 2009. Molecular mechanisms of recombination restriction in the envelope gene of the human immunodeficiency virus. PLoS Pathog. 5, e1000418.
179. Simon-Loriere, E., Martin, D.P., Weeks, K.M., Negroni, M., 2010. RNA Structures Facilitate Recombination-Mediated Gene Swapping in HIV-1. J. Virol. 84, 12675–12682.

180. Smits, S.L., Lavazza, A., Matiz, K., Horzinek, M.C., Koopmans, M.P., de Groot, R.J., 2003. Phylogenetic and evolutionary relationships among torovirus field variants: evidence for multiple intertypic recombination events. J. Virol. 77, 9567–9577.
181. Snijder, E.J., den Boon, J.A., Horzinek, M.C., Spaan, W.J., 1991. Comparison of the genome organization of toro- and coronaviruses: evidence for two nonhomologous RNA recombination events during Berne virus evolution. Virology 180, 448–452.
182. Snijder, E.J., Dobbe, J.C., Spaan, W.J.M., 2003. Heterodimerization of the Two Major Envelope Proteins Is Essential for Arterivirus Infectivity. J. Virol. 77, 97–104.
183. Snijder, E.J., Meulenberg, J.J., 1998. The molecular biology of arteriviruses. J. Gen. Virol. 79 (Pt 5), 961–979.
184. Snijder, E.J., Spaan, W.J.M., 2007. Arteriviruses, in: Fields Virology. Lippincott Williams and Wilkins, pp. 1337–1355.
185. Snijder, E.J., Tol, H. van, Pedersen, K.W., Raamsman, M.J.B., Vries, A.A.F. de, 1999. Identification of a Novel Structural Protein of Arteriviruses. J. Virol. 73, 6335–6345.
186. Spilman, M.S., Welbon, C., Nelson, E., Dokland, T., 2009. Cryo-electron tomography of porcine reproductive and respiratory syndrome virus: organization of the nucleocapsid. J. Gen. Virol. 90, 527–535.
187. Steinhauer, D.A., Domingo, E., Holland, J.J., 1992. Lack of evidence for proofreading mechanisms associated with an RNA virus polymerase. Gene 122, 281–288.
188. Strauss, J.H., Strauss, E.G., 1988. Evolution of RNA Viruses. Annu. Rev. Microbiol. 42, 657–683.
189. Suárez, P., Díaz-Guerra, M., Prieto, C., Esteban, M., Castro, J.M., Nieto, A., Ortín, J., 1996. Open reading frame 5 of porcine reproductive and respiratory syndrome virus as a cause of virus-induced apoptosis. J. Virol. 70, 2876–2882.
190. Sun, Z., Chen, Z., Lawson, S.R., Fang, Y., 2010. The Cysteine Protease Domain of Porcine Reproductive and Respiratory Syndrome Virus Nonstructural Protein 2 Possesses Deubiquitinating and Interferon Antagonism Functions. J. Virol. 84, 7832–7846.
191. Taniguchi, T., Palmieri, M., Weissmann, C., 1978. A Qbeta DNA-containing hybrid plasmid giving rise to Qbeta phage formation in the bacterial host [proceedings]. Ann. Microbiol. (Paris) 129 B, 535–536.
192. Terpstra, C., Wensvoort, G., Pol, J.M., 1991. Experimental reproduction of porcine epidemic abortion and respiratory syndrome (mystery swine disease) by infection with Lelystad virus: Koch's postulates fulfilled. Vet. Q. 13, 131–136.
193. Thanawongnuwech, R., Thacker, B., Halbur, P., Thacker, E.L., 2004. Increased Production of Proinflammatory Cytokines following Infection with Porcine Reproductive and Respiratory Syndrome Virus and Mycoplasma hyopneumoniae. Clin. Diagn. Lab. Immunol. 11, 901 –908.
194. Tian, K., Yu, X., Zhao, T., Feng, Y., Cao, Z., Wang, C., Hu, Y., Chen, X., Hu, D., Tian, X., Liu, D., Zhang, S., Deng, X., Ding, Y., Yang, L., Zhang, Y., Xiao, H., Qiao, M., Wang, B., Hou, L., Wang, X., Yang, X., Kang, L., Sun, M., Jin, P., Wang, S., Kitamura, Y., Yan, J., Gao, G.F., 2007. Emergence of fatal PRRSV variants: unparalleled outbreaks of atypical PRRS in China and molecular dissection of the unique hallmark. PloS One 2, e526.

195. Tian, X., Lu, G., Gao, F., Peng, H., Feng, Y., Ma, G., Bartlam, M., Tian, K., Yan, J., Hilgenfeld, R., Gao, G.F., 2009. Structure and cleavage specificity of the chymotrypsin-like serine protease (3CLSP/nsp4) of Porcine Reproductive and Respiratory Syndrome Virus (PRRSV). J. Mol. Biol. 392, 977–993.
196. Tijms, M.A., Dinten, L.C. van, Gorbalenya, A.E., Snijder, E.J., 2001. A zinc finger-containing papain-like protease couples subgenomic mRNA synthesis to genome translation in a positive-stranded RNA virus. Proc. Natl. Acad. Sci. 98, 1889–1894.
197. Tolskaya, E.A., Romanova, L.I., Blinov, V.M., Viktorova, E.G., Sinyakov, A.N., Kolesnikova, M.S., Agol, V.I., 1987. Studies on the recombination between RNA genomes of poliovirus: the primary structure and nonrandom distribution of crossover regions in the genomes of intertypic poliovirus recombinants. Virology 161, 54–61.
198. Urbanowicz, A., Alejska, M., Formanowicz, P., Blazewicz, J., Figlerowicz, M., Bujarski, J.J., 2005. Homologous crossovers among molecules of brome mosaic bromovirus RNA1 or RNA2 segments in vivo. J. Virol. 79, 5732–5742.
199. Van den Born, E., Posthuma, C.C., Gultyaev, A.P., Snijder, E.J., 2005. Discontinuous subgenomic RNA synthesis in arteriviruses is guided by an RNA hairpin structure located in the genomic leader region. J. Virol. 79, 6312–6324.
200. Van Dinten, L.C., den Boon, J.A., Wassenaar, A.L., Spaan, W.J., Snijder, E.J., 1997. An infectious arterivirus cDNA clone: identification of a replicase point mutation that abolishes discontinuous mRNA transcription. Proc. Natl. Acad. Sci. U. S. A. 94, 991–996.
201. Van Vugt, J.J., Storgaard, T., Oleksiewicz, M.B., Bøtner, A., 2001. High frequency RNA recombination in porcine reproductive and respiratory syndrome virus occurs preferentially between parental sequences with high similarity. J. Gen. Virol. 82, 2615–2620.
202. Vanhee, M., Costers, S., Van Breedam, W., Geldhof, M.F., Van Doorsselaere, J., Nauwynck, H.J., 2010. A variable region in GP4 of European-type porcine reproductive and respiratory syndrome virus induces neutralizing antibodies against homologous but not heterologous virus strains. Viral Immunol. 23, 403–413.
203. Vries, A.A. de, Post, S.M., Raamsman, M.J., Horzinek, M.C., Rottier, P.J., 1995. The two major envelope proteins of equine arteritis virus associate into disulfide-linked heterodimers. J. Virol. 69, 4668–4674.
204. Vries, A.A.F. de, Chrinside, E.D., Bredenbeek, P.J., Gravestein, L.A., Horzinek, M.C., Spaan, W.J.M., 1990. All sibdgenomic mRNAs of equine arteritis virus contain am common leader sequence. Nucleic Acids Res. 18, 3241–3241.
205. Vu, H.L.X., Kwon, B., Yoon, K.-J., Laegreid, W.W., Pattnaik, A.K., Osorio, F.A., 2011. Immune Evasion of Porcine Reproductive and Respiratory Syndrome Virus through Glycan Shielding Involves both Glycoprotein 5 as Well as Glycoprotein 3. J. Virol. 85, 5555 –5564.
206. Wang, F.-X., Song, N., Chen, L.-Z., Cheng, S.-P., Wu, H., Wen, Y.-J., 2013. Non-structural protein 2 of the porcine reproductive and respiratory syndrome (PRRS) virus: a crucial protein in viral pathogenesis, immunity and diagnosis. Res. Vet. Sci. 95, 1–7.
207. Weiland, E., Bolz, S., Weiland, F., Herbst, W., Raamsman, M.J., Rottier, P.J., De Vries, A.A., 2000. Monoclonal antibodies directed against conserved epitopes on the

nucleocapsid protein and the major envelope glycoprotein of equine arteritis virus. J. Clin. Microbiol. 38, 2065–2075.
208. Welch, S.-K.W., Jolie, R., Pearce, D.S., Koertje, W.D., Fuog, E., Shields, S.L., Yoo, D., Calvert, J.G., 2004. Construction and evaluation of genetically engineered replication-defective porcine reproductive and respiratory syndrome virus vaccine candidates. Vet. Immunol. Immunopathol. 102, 277–290.
209. Wenhui, L., Zhongyan, W., Guanqun, Z., Zhili, L., JingYun, M., Qingmei, X., Baoli, S., Yingzuo, B., 2012. Complete Genome Sequence of a Novel Variant Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) Strain: Evidence for Recombination between Vaccine and Wild-Type PRRSV Strains. J. Virol. 86, 9543–9543.
210. Wensvoort, G., Terpstra, C., Pol, J.M., ter Laak, E.A., Bloemraad, M., de Kluyver, E.P., Kragten, C., van Buiten, L., den Besten, A., Wagenaar, F., 1991. Mystery swine disease in The Netherlands: the isolation of Lelystad virus. Vet. Q. 13, 121–130.
211. Wesley, R.D., Mengeling, W.L., Lager, K.M., Clouser, D.F., Landgraf, J.G., Frey, M.L., 1998. Differentiation of a Porcine Reproductive and Respiratory Syndrome Virus Vaccine Strain from North American Field Strains by Restriction Fragment Length Polymorphism Analysis of ORF 5. J. Vet. Diagn. Invest. 10, 140–144.
212. White, K.A., Morris, T.J., 1995. RNA determinants of junction site selection in RNA virus recombinants and defective interfering RNAs. RNA N. Y. N 1, 1029–1040.
213. Wieringa, R., de Vries, A.A.F., Rottier, P.J.M., 2003. Formation of disulfide-linked complexes between the three minor envelope glycoproteins (GP2b, GP3, and GP4) of equine arteritis virus. J. Virol. 77, 6216–6226.
214. Wieringa, R., de Vries, A.A.F., van der Meulen, J., Godeke, G.-J., Onderwater, J.J.M., van Tol, H., Koerten, H.K., Mommaas, A.M., Snijder, E.J., Rottier, P.J.M., 2004. Structural protein requirements in equine arteritis virus assembly. J. Virol. 78, 13019–13027.
215. Wijegoonawardane, P.K.M., Sittidilokratna, N., Petchampai, N., Cowley, J.A., Gudkovs, N., Walker, P.J., 2009. Homologous genetic recombination in the yellow head complex of nidoviruses infecting Penaeus monodon shrimp. Virology 390, 79–88.
216. Wilson, V., Taylor, P., Desselberger, U., 1988. Crossover regions in foot-and-mouth disease virus (FMDV) recombinants correspond to regions of high local secondary structure. Arch. Virol. 102, 131–139.
217. Wissink, E.H.J., Kroese, M.V., Maneschijn-Bonsing, J.G., Meulenberg, J.J.M., van Rijn, P.A., Rijsewijk, F. a. M., Rottier, P.J.M., 2004. Significance of the oligosaccharides of the porcine reproductive and respiratory syndrome virus glycoproteins GP2a and GP5 for infectious virus production. J. Gen. Virol. 85, 3715 – 3723.
218. Wissink, E.H.J., Kroese, M.V., van Wijk, H.A.R., Rijsewijk, F.A.M., Meulenberg, J.J.M., Rottier, P.J.M., 2005. Envelope Protein Requirements for the Assembly of Infectious Virions of Porcine Reproductive and Respiratory Syndrome Virus. J. Virol. 79, 12495–12506.
219. Wissink, E.H.J., van Wijk, H.A.R., Kroese, M.V., Weiland, E., Meulenberg, J.J.M., Rottier, P.J.M., van Rijn, P.A., 2003. The major envelope protein, GP5, of a European porcine reproductive and respiratory syndrome virus contains a neutralization epitope in its N-terminal ectodomain. J. Gen. Virol. 84, 1535–1543.

220. Wiuf, C., Christensen, T., Hein, J., 2001. A simulation study of the reliability of recombination detection methods. Mol. Biol. Evol. 18, 1929–1939.
221. Wood, O., Tauraso, N., Liebhaber, H., 1970. Electron Microscopic Study of Tissue Cultures Infected with Simian Haemorrhagic Fever Virus. J. Gen. Virol. 7, 129–136.
222. Wootton, S.K., Rowland, R.R.R., Yoo, D., 2002. Phosphorylation of the Porcine Reproductive and Respiratory Syndrome Virus Nucleocapsid Protein. J. Virol. 76, 10569–10576.
223. Worobey, M., Holmes, E.C., 1999. Evolutionary aspects of recombination in RNA viruses. J. Gen. Virol. 80 (Pt 10), 2535–2543.
224. Wu, W.H., Fang, Y., Farwell, R., Steffen-Bien, M., Rowland, R.R., Christopher-Hennings, J., Nelson, E.A., 2001. A 10-kDa structural protein of porcine reproductive and respiratory syndrome virus encoded by ORF2b. Virology 287, 183–191.
225. Xia, P. an, Dang, Z. guo, Qiu, H., Fan, X., Zhou, B., Cui, B. an, Chen, P. yan, 2009. Function of PRRSV GP5 envelope protein by using pseudotyped virus. Vet. Microbiol. 138, 297–303.
226. Yoon, I.J., Joo, H.S., Goyal, S.M., Molitor, T.W., 1994. A modified serum neutralization test for the detection of antibody to porcine reproductive and respiratory syndrome virus in swine sera. J. Vet. Diagn. Investig. Off. Publ. Am. Assoc. Vet. Lab. Diagn. Inc 6, 289–292.
227. Yuan, S., Nelsen, C.J., Murtaugh, M.P., Schmitt, B.J., Faaberg, K.S., 1999a. Recombination between North American strains of porcine reproductive and respiratory syndrome virus. Virus Res. 61, 87–98.
228. Yuan, S., Nelsen, C.J., Murtaugh, M.P., Schmitt, B.J., Faaberg, K.S., 1999b. Recombination between North American strains of porcine reproductive and respiratory syndrome virus. Virus Res. 61, 87–98.
229. Zhang, C.X., Cascone, P.J., Simon, A.E., 1991. Recombination between satellite and genomic RNAs of turnip crinkle virus. Virology 184, 791–794.
230. Zhou, Z., Ni, J., Cao, Z., Han, X., Xia, Y., Zi, Z., Ning, K., Liu, Q., Cai, L., Qiu, P., Deng, X., Hu, D., Zhang, Q., Fan, Y., Wu, J., Wang, L., Zhang, M., Yu, X., Zhai, X., Tian, K., 2011. The epidemic status and genetic diversity of 14 highly pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV) isolates from China in 2009. Vet. Microbiol. 150, 257–269.
231. Ziebuhr, J., Snijder, E.J., Gorbalenya, A.E., 2000. Virus-encoded proteinases and proteolytic processing in the Nidovirales. J. Gen. Virol. 81, 853–879.
232. Zimmerman, J., Benfield, D.A., Murtaugh, M.P., Osorio, F., Stevenson, G.W., Torremorell, M., 2006. Porcine reproductive and respiratory syndrome virus (porcine arterivirus). Dis. Swine 387–417.

Figure 1-1 PRRS-free regions of the world

PRRS is globally endemic in most swine-producing countries.The red arrows show the countries in the world that are currently considered free of PRRSV.

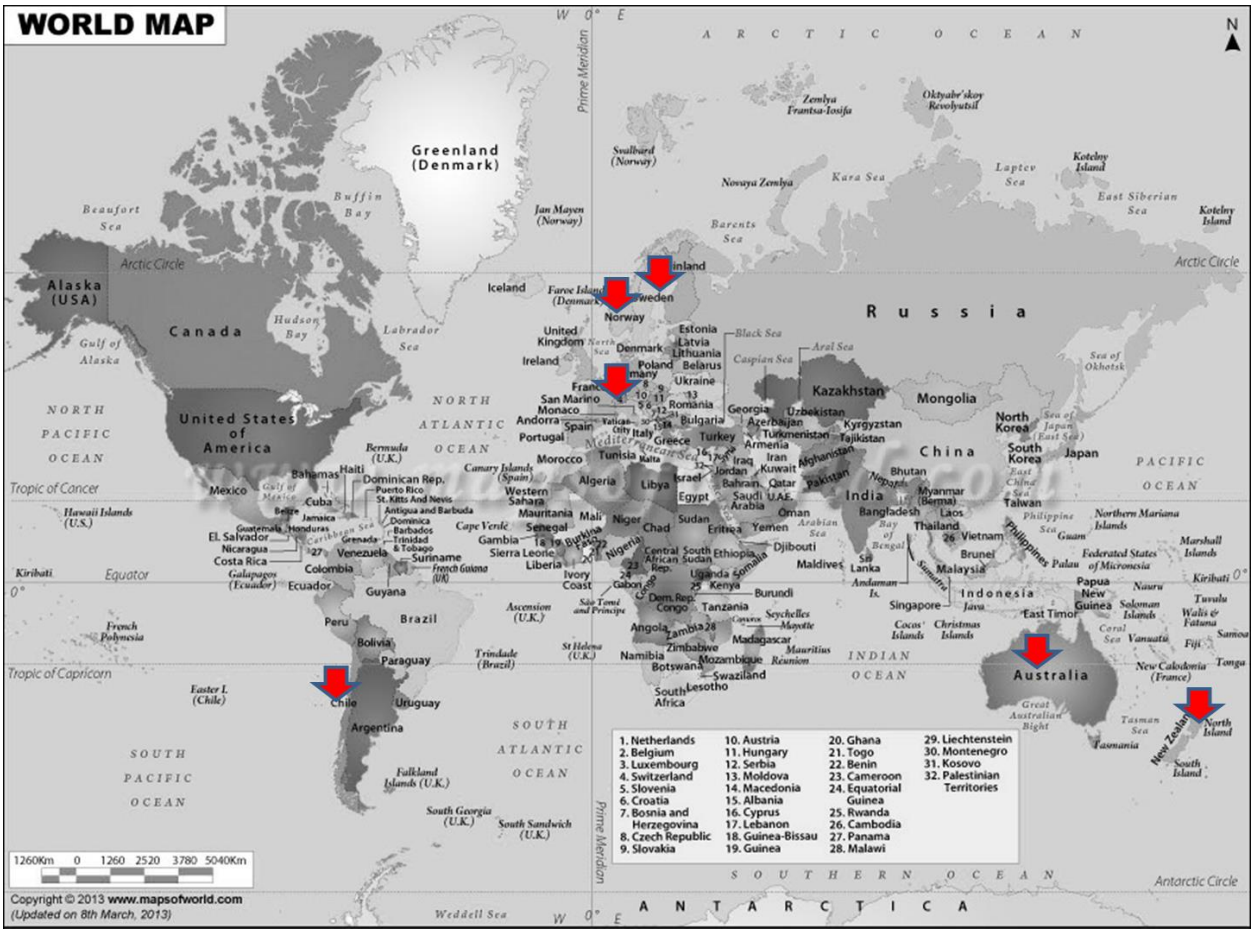
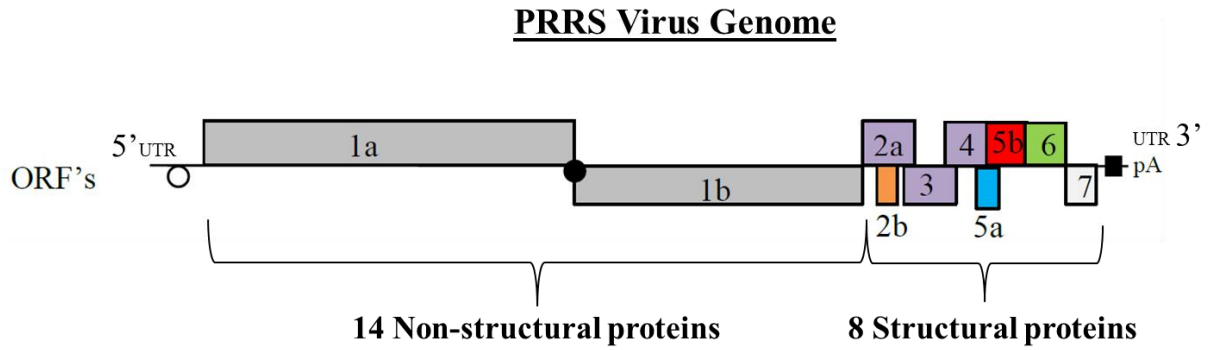
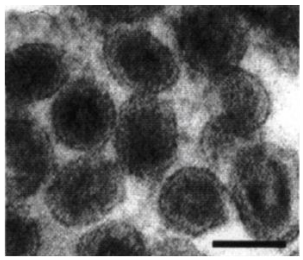


Figure 1-2 PRRS virus genome and virion structure

PRRS virus has a single stranded positive-sense RNA genome which is approximately 15 kilobases in length. In the schematic representation below the proteins encoded by the genome are color coded in the virion.

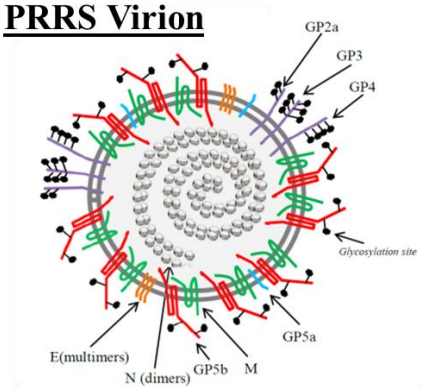


PRRSV electron micrograph



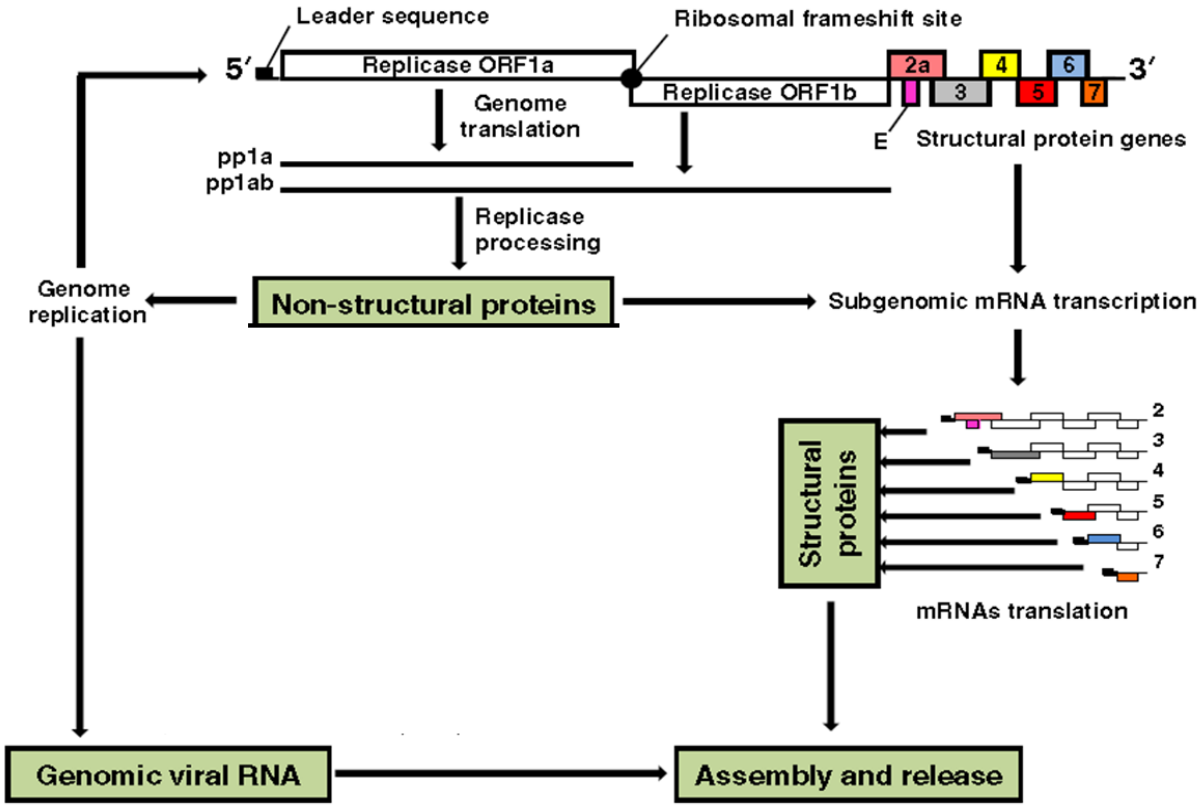
ICTV database

PRRS Virion



(courtesy B. Trible, Rowland Lab)

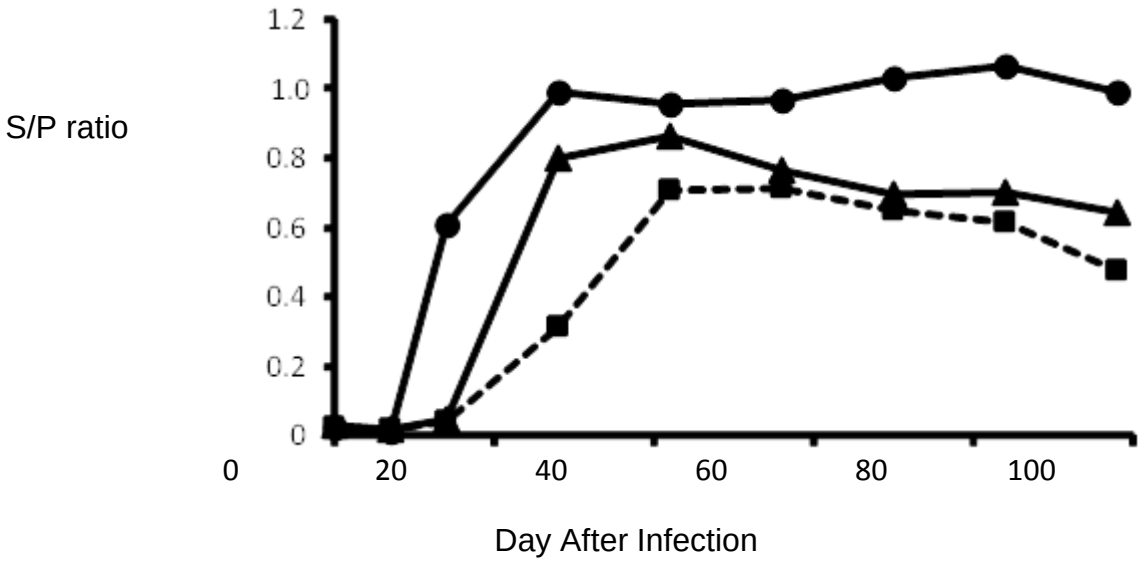
Figure 1-3 PRRS virus replication, transcription and translation machinery



(Music and Gagnon, 2010)

Figure 1-4 Antibody response to major structural and nonstructural proteins

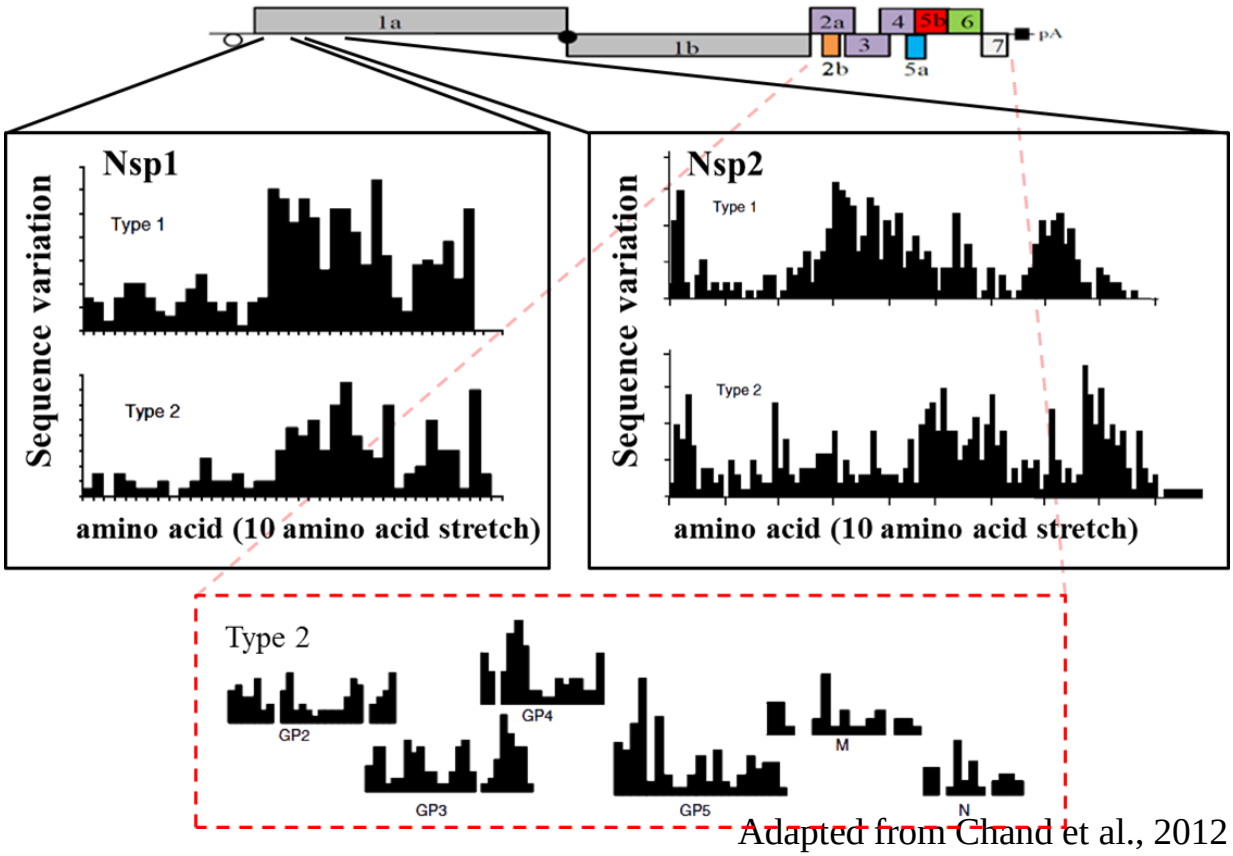
Ten pigs were experimentally infected with PRRSV and antibody measured against the N protein (circles), nsp2 (triangles) and the major virion surface components, GP5-M (squares with dotted line). Antibody was measured by ELISA and results shown as the mean of the sample to positive (S/P) ratio.



Chand et al., 2012

Figure 1-5 Peptide sequence variability and hypervariability in structural and nonstructural proteins

Sequence variation is presented as the number of amino acid changes within a 10 amino acid stretch for type 2 structural proteins (A), Type 1 and type 2 nsp1 (B), and type 1 and type 2 nsp2 (C). Peptide sequence information was collected from GenBank.



Adapted from Chand et al., 2012

Figure 1-6 Mechanism of PRRSV persistence

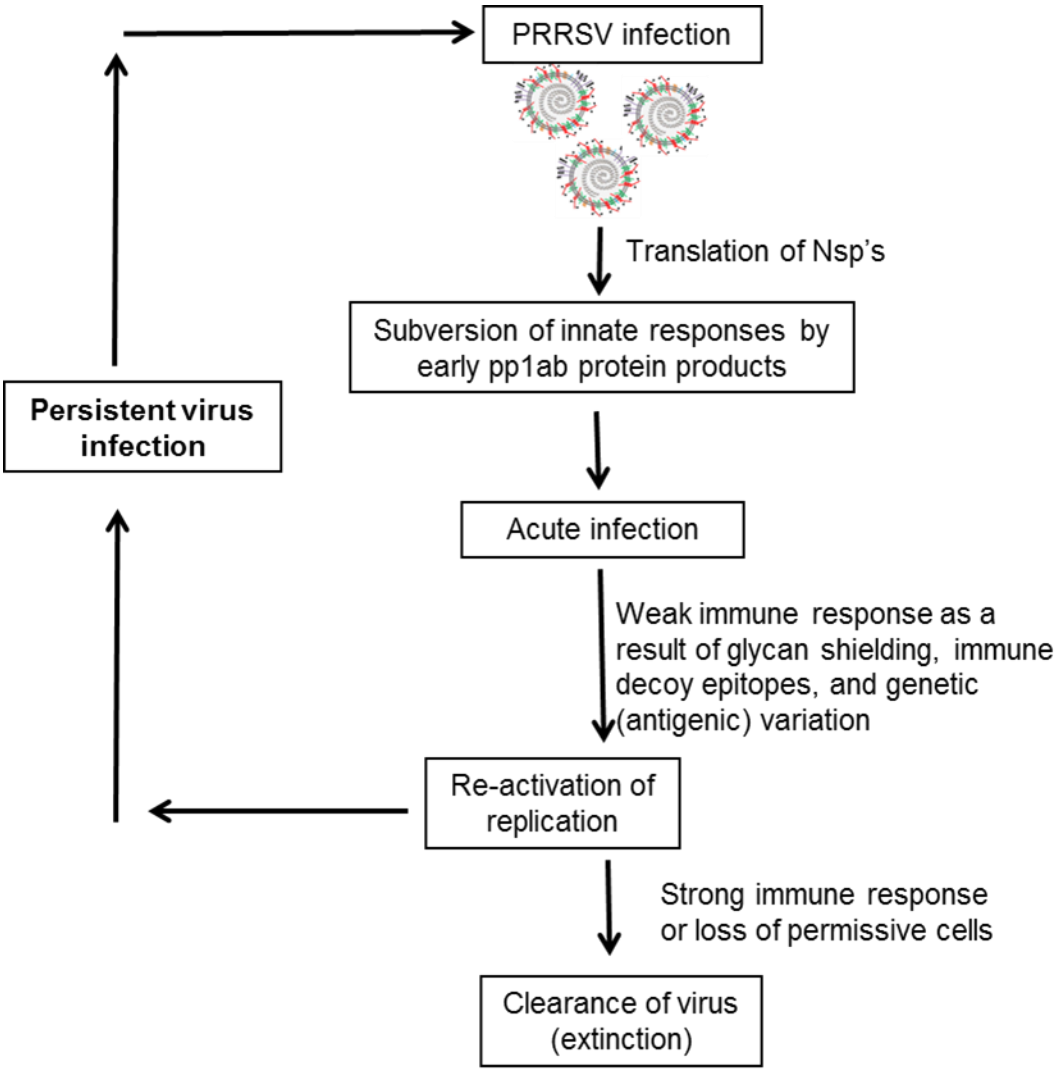


Table 1-1 Effects of PRRSV pp1ab proteins on the activation of IFNβ

Nsp	Cell System	Observation	References
nsp1	MARC-145, HEK-293T	Reduced phosphorylation of IRF3	(Beura et al., 2010; Shi et al., 2010)
nsp1	MARC-145 HeLa	Degradation of CREB-binding protein	(Kim et al., 2010)
nsp1α	HeLa	Reduced IκB phosphorylation and nuclear translocation	(Song et al., 2010)
nsp1α, nsp1β	HEK-293T	Decreased IFNβ promoter activation No effect on IRF3 phosphorylation	(Chen et al., 2010)
nsp2	HEK-293T	Reduced polyubiquitination of IκBα	(Sun et al., 2010)
nsp2	HEK-293T	Reduced IRF3 phosphorylation	(Li et al., 2010)
nsp1α, nsp1β, nsp11	HEK-293, HT1080	Reduced IRF3-mediated gene activation	(Beura et al., 2010)
nsp1, nsp2, nsp4, nsp11	HeLa	Decreased IFNβ promoter activation	(Beura et al., 2010)

Chapter 2 - Literature review of Recombination

2.A. General RNA recombination

2.A.i. Molecular mechanism: Copy-choice method

RNA recombination was first identified in 1960s in the animal viruses Newcastle disease virus, poliovirus and influenza virus (HIRST, 1962; LEDINKO, 1963). Soon after, recombination was reported in bacteriophages, plant viruses and other animal viruses such as FMDV, MHV, BMV (Lai, 1992). Phylogenetic relationships showed evidence of recombination as a source of genetic diversity in RNA viruses (Koonin et al., 1993). Early studies of genome sequences, organization and amino acid similarities also suggested that recombination played a critical role in virus evolution (Goldbach, 1986; Strauss and Strauss, 1988). Further confirmation of recombination events among RNA viruses came from sequence rearrangements in natural strains of viruses such as: alfalfa mosaic virus, turnip crinkle virus, luteoviruses (Cascone et al., 1990; Huisman et al., 1989; Mayo and Jolly, 1991) and identification of host sequences in genomes of bovine diarrhea virus (ubiquitin-coding sequence) and Sindbis virus (tRNA sequence) (Meyers et al., 1991; Monroe and Schlesinger, 1983). Based on the types of RNA molecules involved and cross-over sites, RNA recombination can be divided into 2 types: homologous and non-homologous recombination (Lai, 1992). Homologous recombination involves cross-over between two closely related RNA molecules or genetic regions as commonly seen in picornaviruses and coronaviruses (Jarvis and Kirkegaard, 1992; Lai et al., 1985). Non-homologous recombination involves cross-over between distantly related RNA molecules or genetic regions as observed in QB bacteriophage (Munishkin et al., 1988). Homologous recombination usually results in conservation of the genome structure while non-homologous cross-over results in gene rearrangement or duplication, insertions and deletions. This property has been suggested to result in greater survival frequency of viruses that undergo homologous recombination events as compared with non-homologous recombinants. Another layer of

complexity is added to the process of recombination as we frequently find both types of recombination occurring in viruses such as BMV (Figlerowicz and Bujarski, 1998).

The established mechanism for double-stranded DNA recombination is the 'breaking and joining' method while 'copy-choice' has been found to be a rare occurrence in these viruses (Brunier et al., 1988; MESELSON and WEIGLE, 1961) . The most widely accepted mechanism for RNA recombination is the 'copy-choice' or 'template-switch' mechanism (Kirkegaard and Baltimore, 1986). The fundamental difference between these two mechanisms is that copy-choice mechanism occurs only during RNA synthesis while breaking-joining is a non-replicative model. There are a few reports for evidence of non-replicative mechanisms for RNA recombination, mainly in Qβ replicase systems. Trans-esterification and replicase independent self-recombination mechanisms have been proposed(Chetverin et al., 1997; Chetverina et al., 1999) to occur in cell-free systems. However, copy-choice mechanism is the most widely accepted model and has been suggested to operate for PRRS virus recombination and hence this model will be discussed in further details in this dissertation.

Choice-choice model

Figure 1-7 shows a schematic representation of this model. As the copy-choice model is a replicative mechanism it operates only during RNA synthesis and parent RNA molecules are not part of the newly recombined molecules. According to this model, RNA synthesis pauses due to a secondary RNA structure or some specific undetermined nucleotide sequence followed by a dissociation step. As RNA synthesis cannot be initiated independently by the nascent RNA strand, it was proposed that the entire transcription complex consisting of nascent RNA, transcription proteins and RNA polymerase dissociates from the template strand. This dissociated complex finds a new RNA template in the proximity and binds at a comparable site. Based on structural features of the nascent RNA transcript and/or the acceptor template, RNA binding would result in homologous or non-homologous recombination. This binding is followed by continuation of RNA synthesis of the RNA transcript to generate the new recombined molecule. In some cases nascent transcripts might be processed by endo- or exo-nucleases at the

3’ end before binding to new template (Banner and Lai, 1991). Such processing and imprecise binding lead to insertions and deletions in the progeny recombined molecule.

Determination of hot-spots for recombination is the most intriguing area in the study of recombination. So far, a number of studies in poliovirus and FMDV have shown that sites of secondary RNA structures are hot-spots for recombination (Tolskaya et al., 1987; Wilson et al., 1988) This is because they promote RNA polymerase pausing as well as act as an anchor for selection as a binding site. Functionality of the resulting recombined protein is a critical determinant for the fitness and hence survival of recombined viruses. Hypervariable regions may be more tolerant to structural mutation and hence this was suggested to be the reason for the hypervariable region in MHV spike gene to be a hot-spot (Banner et al., 1990). Further studies to detect hot-spots and interpretation of results must be performed with caution considering the influence of selection pressure on the nature of recovered recombined viruses in *in-vitro* experimental systems. This criterion was brought to attention by the different pattern of recombinants obtained when selection pressure was removed in a MHV-2/A59 system for recombination studies (Banner and Lai, 1991). The first experimental evidence for copy-choice mechanism was from an *in-vitro* system with polioviruses (Kirkegaard and Baltimore, 1986). This was done by co-infection of a wild-type virus (5’ guanidine sensitive, 3’ temperature resistant) with a double mutant (5’ guanidine resistant, 3’ temperature sensitive). Guanidine blocked positive- and negative-strand synthesis in wild-type virus, while non-permissive temperature blocked negative-strand synthesis of the double mutant. It was seen that recombined viruses (5’ guanidine resistant, 3’ temperature resistant) were generated under non-permissive temperature but were absent in the presence of guanidine. This study showed that RNA synthesis of at least the donor template is required for recombination to occur and also suggested that cross-over occurred primarily during negative-strand synthesis.

Biological significance and mechanistic constraints of recombination

Recombination is one of the mechanisms of genetic exchange that contributes to the diversity of RNA viruses. Biological significance of recombination has been mainly suggested in two areas. Firstly, recombination is considered to be an efficient process to clear RNA genomes

of deleterious mutations that accumulate through the high rate of mutation (Chao, 1988) and promote the spread of beneficial mutations (Worobey and Holmes, 1999). Secondly, heterologous recombination that can introduce large insertions and deletions has been suggested to influence genome size and organization of RNA viruses. This has been observed in members of the Order Nidovirales and discussed in the following section. Evidences of recombination resulting in beneficial purging are seen in examples such as virulent recombined viruses generated from attenuated vaccine strains in poliovirus (Georgescu et al., 1994) and in infectious bronchitis virus (Jia et al., 1995). Reports of recombination resulting in new beneficial variants include that of a non-cytopathogenic BVDV recombining with a host gene to generate a cytopathogenic recombined virus (Meyers et al., 1989). Implication of recombination on phylogenetic data discourages the use of portions of viral genome as accurate representations of the virus. This review also brings to notice the co-occurrence of re-assortment and recombination mechanisms in many viruses such as hantavirus (Sibold et al., 1999), influenza A virus (Khatchikian et al., 1989), cucomovirus (Masuta et al., 1998) and bacteriophage φ6 (Mindich et al., 1992). Although recombination provides significant advantages to viruses, this mechanism has not been found universally in all RNA viruses and also the frequency of RNA recombination is highly variable between viruses. The reasons for this could be a number of mechanistic constraints for successful recombination. A successful recombination involves occurrence of cross-over that results in a fitness advantage that will enable establishment and spread of the recombined virus. The constraints can be classified to be at 5 levels of the recombination process: simultaneous infection of a host, co-infection of a cell within that host, compatibility for co-replication, physical detachment for switching the RNA template and finally overcoming the selective pressures of the host (Worobey and Holmes, 1999). A successful recombined virus has to clear each of these 5 constraints and further be efficiently transmitted for establishment in a population.

2.A.ii. Recombination in Order Nidovirales

PRRS virus belongs to the order Nidovirales, which includes three families: Arteriviridae (genus Arterivirus), Coronaviridae (genus Coronavirus and Torovirus) and Roniviridae (genus

Okavirus). Recently a fourth family, Mesoniviridae (genus Alphamesonivirus), has been proposed to be included in this order (Lauber et al., 2012). Nidoviruses have shown clear evidence of recombination being an important evolutionary mechanism. Many initial studies investigating RNA recombination were performed on coronaviruses, which showed up to a 30% recombination frequency over the entire genome (Lai, 1996). Toroviruses that cause enteric disease in cattle, horses and possibly humans were also shown to undergo frequent recombination (Smits et al., 2003). Little is known about Okaviruses that infect shrimp, but recently homologous recombination was reported in an okavirus, Yellow-head virus (YHD) (Wijegoonawardane et al., 2009). This is an important discovery because it is the first evidence of recombination in a virus infecting an invertebrate host. Arteriviruses are more extensively studied and there are several reports of homologous and non-homologous recombination in EAV, PRRSV and LDV (Li et al., 1999; Molenkamp et al., 2000a; Yuan et al., 1999a). Recombination has been suggested to have resulted in duplication and relocation of genes between different viruses in the order Nidovirales. Evidence for this was indicated in the presence of multiple protease (PL^{pro}) proteins in corona- and arteriviruses (den Boon et al., 1995; Gorbalenya et al., 1991). Heterologous recombination was speculated to have resulted in relocation of CPD and HE genes in toro- and coronaviruses (Snijder et al., 1991).

2.A.iii. Systems to study recombination

Systems for investigation of recombination can be classified in to two broad categories:

(i) *In vitro* systems

(ii) *In silico* systems

In vitro systems are fewer in number as demonstration of recombination is a challenging task. However, experimental systems provide insights in to this mechanism that cannot be perceived through phylogenetic analysis. The popular *in-vitro* methods include subjecting viral RNA to single stranded chain polymorphism, hybridization, restriction fragment length polymorphism or denaturing gradient gel electrophoresis. These techniques enable detection of changes in binding patterns of probes and restriction sites which indicate presence of a recombined or new strain of virus. Isolation of viruses or amplification of virus fragments by RT-PCR followed by sequencing yield useful data which is analyzed using a number of different

software that search for recombination footprints. A multitude of programs that are being used for *in silico* recombination analysis include GARD, RDP, SimPlot, 3 SEQ, MaxChi, BOOTSCAN, Lard and GENECONV.

Some of the experimental systems used to study recombination in viruses that infect plant, animal and bacteria are discussed in this section. In Brome-mosaic virus (BMV), a virus that infects bromegrass and barley, insertions in the UTRs that impeded virus replication was the system used to study recombination mechanism. Recombination in this system was detected by restoration of functional UTRs in progeny viruses (Alejska et al., 2005). Modification of this concept was used by another system where mutations were incorporated in genes encoding the viral RNA polymerase (Nagy et al., 1995). The effects of these mutations were observed to be different for non-homologous and homologous recombination. Another BMV-based system involved the use of two parental viruses with different silent markers such as restriction sites, in the same region (Urbanowicz et al., 2005). Analyses of restriction patterns in the progeny viruses demonstrated different cross-over regions. Some systems in Turnip crinkle virus (TCV) and Cucumber necrosis virus (CNV) used a combination of genomic and satellite or defective RNAs respectively to determine impact of specific mutations on recombination in these viruses (White and Morris, 1995; Zhang et al., 1991). This was done by co-inoculating the host with a pair of viruses where one carried a deleterious mutation and only recombination restored this mutation. A similar system was designed recently for Hepatitis C virus (Scheel et al., 2013). Co-transfection of hepatoma cells with a replicating HCV strain that lacked envelope genes and a non-replicating HCV strain that carried envelope genes gave rise to viruses that had undergone homologous or non-homologous recombination. Introduction of cell-free systems enabled minimization of selection pressure and the capacity to easily modify the host environment (Kim and Kao, 2001; Nagy et al., 1998). These experiments demonstrated the requirement of secondary RNA structure in TCV and AU-rich stretches in BMV for cross-over. Transgenic plants that expressed viral or host factors were used to study contribution of these factors to recombination in infecting BMV and cucomovirus recombination RNA (Aaziz and Tepfer, 1999; Dzianott and Bujarski, 2004). A gene-knockout yeast system was developed to study host factors essential for RNA recombination of infecting viruses (Serviene et al., 2005). Classical systems for detection of recombination between parent viruses carrying different genetic markers such as

temperature sensitivity, guanidine resistance, antibody neutralization resistance and resistance to horse serum treatments have been used for picornavirus and coronavirus studies (HIRST, 1962; Pincus et al., 1986). The latest model developed to study recombination in poliovirus uses co-infection of a wild-type and a genetically synthesized virus followed by deep sequencing of the resulting recombined viruses (Runckel et al., 2013). The synthetic genome carried 368 synonymous mutations and enabled demonstration of predictive nature of recombination hotspots. A single cycle tissue culture assay was developed for HIV-1 recombination where the *Env* protein gene was inserted in plasmids with either a *LacZ'* gene or a truncated *MalT* gene preceding the *Env* gene (Simon-Loriere et al., 2009). Recombinants were identified by a presence of a specific restriction site and functional *LacZ'* gene when cloned in to bacterial cells. Experimental systems for PRRS virus recombination studies have not been explored greatly so far. The only available system is a simplistic model that involves co-infection of PRRSV permissive cells with different strains of the virus followed by PCR amplification using differential primers. The nature and shortcomings of these studies will be expanded further in the following section.

2.B. PRRS virus recombination

2.B.i. Previous studies

Genetic diversity in PRRSV is largely the result of point mutations and recombination (Chang et al., 2002; Dee et al., 2001). Phylogenetic analyses provided the first evidence for recombination in PRRSV. In 1996, Kapur et al. reported on the phylogenetic analysis of ORFs 2 through 7 in 10 NA isolates (Kapur et al., 1996a). There was evidence of intragenic recombination in all the structural ORFs except ORF6 which may be due to negative selection of recombinant forms in this region. In another study, 50 US field isolates were sequenced and one natural recombinant isolate, 93-15416, was found (Yuan et al., 1999a,). Cross-over between the two parent-isolates occurred within a 31 bp identical stretch in the ORF 5 region. Phylogenetic incongruity in a group of European isolates followed by SimPlot analysis identified an Italian isolate that had undergone cross-over in the end of ORF6 region (Forsberg et al., 2002a).

Similarly, cross-over was found to occur in nsp2/3 junction region in a NA type 1 isolate (Fang et al., 2007a). More recently, recombination was suggested to contribute to the genetic diversity of PRRSV in the British isolates where double cross-over was found to occur at the 5' and 3' ends of ORF 5 (Frossard et al., 2013). Recombination can be demonstrated in the laboratory after the co-infection of two different isolates. Yuan et. al. reported the co-infection of MA-104 cells and porcine alveolar macrophages (PAMs) with two attenuated PRRSV vaccine strains (Yuan et al., 1999a). RespPRRS and Prime Pac strains showed 92% sequence homology in the 1182 bp region analyzed that comprised part of ORF 3 through ORF 5. Recombinants were detected by PCR using isolate-specific primers followed by sequencing. Recombination frequency was estimated at 2-10 % by analysis of band density. Real time RT-PCR approach was used to study inter- and intragenotype recombination in Danish and North American PRRSV isolates (van Vugt et al., 2001). Intra-type recombination of viruses with 94% homology in ORF5 was detected at a frequency of 0.1 to 2.5% while recombination was not detected between type I and type II isolates (60% homology). Cross-over regions between type II viruses possessed identical stretches in the range of 5-104 bases in length. A Chinese isolate EM2007 was found to be a recombined virus between field and vaccine strains that had crossed over in the Nsp2 and Nsp9 regions (Li et al., 2009a). An in-vivo study model that isolated recombined viruses by plaque assay found a variety of complex cross-over patterns, especially within Nsp2 region (Liu et al., 2011).

2.B.ii. Implications about PRRSV recombination

Results from previous work have given a few insights into PRRSV recombination. Recombination was demonstrated to occur among European, North American and Asian isolates. This showed that the mechanism was not exclusive to a specific genotype or geographic region but rather occurred frequently. Intra-genotype recombination frequency was found to be in the range of 0.1 to 10% while inter-genotype recombination was not detected. *In-vitro* studies using isolate-specific PCR demonstrated that flanking region homology promotes cross-over. Imprecise cross-over resulting in deletions was detected, which suggested the presence of non-homologous recombination. Two characteristics regarding sequence homology that influenced

recombination were: length of identical stretches within a region and overall homology of a region. Length of identical stretches over which cross-over occurred was in the range of 5 to 104 bp. Minimum overall homology required for recombination in PRRSV was found to be 89% which was over a 603 bp region in ORF 5. Long regions of high degree of identity that flank short homologous regions were suggested to promote recombination.

2.B.iii. Limitations of these studies

PCR based detection of recombined viruses is the only *in-vitro* system to study PRRSV recombination available at present. In this system two parent viruses are used to co-infect PRRSV permissive cells. After 72 hours of infection the supernatant or cell lysate was used for viral RNA isolation. Isolate specific primers were used for RT-PCR amplification of a specific region of the virus where recombination is expected to occur. Alteration of number of PCR cycles, plaque assay and real-time RT-PCR were techniques used to determine the frequency of recombination in these studies. Although PCR based experiments provide some insight in to the mechanism there are a number of limitations to this technique. The most important limitation is that the source of PCR template in the serum or cell culture sample cannot be restricted to functional virus genomes. Hence, sg-mRNA, genomic or defective RNA could serve as template for PCR for the region under analysis. Therefore, viability of the recombined virus carrying the detected recombination event is not known. Secondly, sequencing of PCR products has shown the occurrence of complex pattern of cross-overs. As PCR primers are targeted to a specific region of the genome all cross-overs occurring outside the targeted region are neglected. Therefore this system would underestimate the recombination frequency of the virus. Thirdly, as only RNA sequence is detected and not the entire virion, the impact of the recombination event on the phenotype of the virus is unknown. Furthermore, random mutations in primer attachment regions might result in false-negative results for PCR and hence be unable to detect recombined viruses in the sample.

2.B.iv. Significance of the study of PRRSV recombination

Features of PRRSV that make it a good candidate for recombination are as follows:

(i) Discontinuous replication mechanism involves an inherent pausing and detachment step of the RNA polymerase machinery which is a crucial factor for recombination to occur (Snijder and Meulenberg, 1998).

(ii) PRRSV has been shown to propagate as a 'quasispecies' starting from a single strain infecting an animal (R. R. Rowland et al., 1999). This creates opportunity for intra-strain recombination and thus circumventing the need for dual infection.

(iii) One of the main challenges with elimination of PRRS is the persistent nature of the virus (Chand et al., 2012). This results in prolonged periods of association of virus in the animal and hence increases the possibility of co-infection of cells.

Implications of recombination are expected in two broad areas within PRRSV research:

Diagnostics and vaccination: Recombination directly impacts genetic diversity and hence there would be an important implication of recombination on vaccination and diagnostic strategies (Forsberg et al., 2002a; Meng, 2000a). Detection of emergence of a recombined isolate between a vaccine and a field strain in China proposed the question: Are live vaccines capable to shape evolution of PRRSV through recombination? (Li et al., 2009a). Isolation of viable recombined viruses resulting from cross-over events between vaccine and field strain have been detected in a number of studies from Britain and China (Frossard et al., 2013; Li et al., 2009a; Wenhui et al., 2012). MLV vaccine is used extensively in for the control of this disease and hence the potential for recombination with field strains is a significant concern.

Epidemiology and evolution: Tracing recombination events during the evolution of a virus is a challenging task (Wiuf et al., 2001). Knowing the contribution of recombination could help explain some of the genetic divergence observed over time and geographical locations. A study that detected a recombination event among NA type 1 viruses pointed out that recombination events are important in epidemiological studies of this PRRSV genotype (Fang et

al., 2007a). Recombination causes construction of phylogenetic trees to stagger (Pond et al., 2012). Cross-over has most often been detected in ORF 5 and this is the region most commonly used for classification and genotyping. Therefore, regular screening of sequence databases for recombination is required. A study that identified two recombined HP-PRRS viruses made an interesting observation regarding their pathogenicities (Chen et al., 2013). Two highly pathogenic parent strains generated a highly pathogenic recombined virus, while when high and low pathogenic strains recombined, a low pathogenic virus was generated. This suggested the possibility of recombination events generating highly pathogenic viruses. In recombination studies for PRRSV recombination has been detected throughout the genomic region and no defined hotspots have been confirmed.

Recombination could also be the source of emergence and re-emergence of unique and highly pathogenic strains that cause outbreaks. These include the following outbreaks: acute PRRS in the Midwest in 1996, HP-PRRS in China in 2006 and 2009, and isolation of East European sub-type3 strain named 'Lena' (Karniychuk et al., 2010; Mengeling et al., 1998; Tian et al., 2007). In a commentary regarding PRRSV recombination it was suggested that consequence of recombination in PRRSV would depend on the nature of the parental strains: recombination between attenuated strains, between virulent strains and between attenuated and virulent strains (Mengeling WL., 2002). In each of these cases the possibility of the occurrence of recombination and survival of the recombined virus may be different. If two virulent strains co-infect a pig, their replication rates would be comparable which would enable recombination to happen in co-infected cells in the pig. However, as every virus has a replication threshold; it might not be theoretically possible that the progeny would have a higher replication competency to outgrow its parents. However, if this occurs it would have serious adverse consequences. Recombination between attenuated strains might occur in case a multi-strain vaccine approach is applied. Emergence of a recombined strain showing higher virulence cannot be easily explained. However, in case the resulting recombined virus has a higher replication rate than the parents it might not supersede the virulence of the original field strain of the attenuated parent. Lastly, co-infection of virulent (field strain) and attenuated (vaccine strain) strains seems to be the most commonly occurring situation. As the replication rate of these two strains would be considerably different, prolonged co-existence and hence chances of co-infection of cells within the pig appear

to be lower. However, reports have shown that this type of recombination events are the most commonly reported cases. Furthermore, in some cases the progeny has gained higher pathogenicity than the parents. This implies the significance of natural recombination in the field as well as exemplifies our limited understanding of the process. Better knowledge of recombination patterns would help make accurate derivations of evolutionary history of PRRSV and also predict strains that might emerge through recombination. Recently, there has been one *in silico* study that has associated recombination with an outbreak (Shi et al., 2013). Currently circulating strains since the 2009 re-emergence of HP-PRRSV in China were shown to have resulted from a recombination event that took place between two highly pathogenic strains from the 2006 outbreak. This recombination event did preserve the pathogenicity of the virus as well as had the fitness required to replace the parental viruses that were in circulation. Although this study does not provide evidence to comment about the influence of the recombination event on the pathogenicity of the virus, it provides support for the significance of study of recombination in PRRSV. Phylogenetic inconsistencies between different ORFs and rejection of molecular clocks are factors commonly considered indicative of recombination in PRRSV (Forsberg et al., 2002a).

Lastly, recombination studies would help answer some of the questions that are still unanswered about the biology of PRRSV. Factors such as increased replication rate, immune selection and cell receptor affinity have been suggested to give the recombined virus selective advantage over the parent viruses. If the recombination event in a structural gene changes the conformation of a neutralizing epitope then this recombined virus has the potential to escape immune pressure and hence survive better than the parent virus. Similarly, if the recombination event changes the viral protein involved in attachment to a cell receptor such that it is now susceptible to new tissue this feature would change the cellular tropism of the recombined virus. However, the role of each of these factors has not been specifically investigated. Cross-over has been detected in Nsp2, 7, 9 and 11 and in all the structural ORFs. However, there is no evidence to confirm if this is a random process or if there are specific hotspots along the PRRSV genome. Furthermore, viral determinants of recombination frequency have not been identified yet. At present there are no studies investigating how recombination is impacted by host factors such as, tissue microenvironment, immune pressure and timing of recombination events during the course

of infection. The frequency at which recombination occurs in the field may be underestimated due to limitations in detection techniques. It is possible that the frequency may be overestimated as most field reports of recombination have been identified as a single recombined virus and not an established strain in a herd (Forsberg et al., 2002b; Li et al., 2009a; Mu et al., 2013). This could be due to the fitness cost that the recombination event has on the virus.

2.B.v. Goal of the research in this dissertation

The primary requirement to investigate recombination mechanism is the establishment of a robust *in-vitro* system. These limitations in the *in-vitro* tool combined with the knowledge gap in recombination in PRRSV motivated this project to develop an *in-vitro* experimental model. Specifically the overall goal of this project was to establish a model system and study recombination. The system was required to have the following features:

(i) Detect viable recombined viruses in absence of selection pressure

(ii) Easy detection method

(iii) Conduct genetic manipulation to investigate mechanism & frequency of recombination

2.C. References

1. Aaziz, R., Tepfer, M., 1999. Recombination between genomic RNAs of two cucumoviruses under conditions of minimal selection pressure. Virology 263, 282–289.
2. Alejska, M., Figlerowicz, Magdalena, Malinowska, N., Urbanowicz, A., Figlerowicz, Marek, 2005. A universal BMV-based RNA recombination system—how to search for general rules in RNA recombination. Nucleic Acids Res. 33, e105–e105.
3. Banner, L.R., Keck, J.G., Lai, M.M., 1990. A clustering of RNA recombination sites adjacent to a hypervariable region of the peplomer gene of murine coronavirus. Virology 175, 548–555.
4. Banner, L.R., Lai, M.M., 1991. Random nature of coronavirus RNA recombination in the absence of selection pressure. Virology 185, 441–445.
5. Brunier, D., Michel, B., Ehrlich, S.D., 1988. Copy choice illegitimate DNA recombination. Cell 52, 883–892.
6. Cascone, P.J., Carpenter, C.D., Li, X.H., Simon, A.E., 1990. Recombination between satellite RNAs of turnip crinkle virus. EMBO J. 9, 1709–1715.
7. Chand, R.J., Trible, B.R., Rowland, R.R., 2012. Pathogenesis of porcine reproductive and respiratory syndrome virus. Curr. Opin. Virol. 2, 256–263.
8. Chang, C.-C., Yoon, K.-J., Zimmerman, J.J., Harmon, K.M., Dixon, P.M., Dvorak, C.M.T., Murtaugh, M.P., 2002. Evolution of porcine reproductive and respiratory syndrome virus during sequential passages in pigs. J. Virol. 76, 4750–4763.
9. Chao, L., 1988. Evolution of sex in RNA viruses. J. Theor. Biol. 133, 99–112.
10. Chen, N., Yu, X., Wang, L., Wu, J., Zhou, Z., Ni, J., Li, X., Zhai, X., Tian, K., 2013. Two natural recombinant highly pathogenic porcine reproductive and respiratory syndrome viruses with different pathogenicities. Virus Genes 46, 473–478.
11. Chetverin, A.B., Chetverina, H.V., Demidenko, A.A., Ugarov, V.I., 1997. Nonhomologous RNA recombination in a cell-free system: evidence for a transesterification mechanism guided by secondary structure. Cell 88, 503–513.
12. Chetverina, H.V., Demidenko, A.A., Ugarov, V.I., Chetverin, A.B., 1999. Spontaneous rearrangements in RNA sequences. FEBS Lett. 450, 89–94.
13. Dee, S.A., Torremorell, M., Rossow, K., Mahlum, C., Otake, S., Faaberg, K., 2001. Identification of genetically diverse sequences (ORF 5) of porcine reproductive and respiratory syndrome virus in a swine herd. Can. J. Vet. Res. Rev. Can. Rech. Vét. 65, 254–260.
14. Den Boon, J.A., Faaberg, K.S., Meulenberg, J.J., Wassenaar, A.L., Plagemann, P.G., Gorbalenya, A.E., Snijder, E.J., 1995. Processing and evolution of the N-terminal region of the arterivirus replicase ORF1a protein: identification of two papainlike cysteine proteases. J. Virol. 69, 4500–4505.
15. Dzianott, A., Bujarski, J.J., 2004. Infection and RNA recombination of Brome mosaic virus in Arabidopsis thaliana. Virology 318, 482–492.
16. Fang, Y., Schneider, P., Zhang, W.P., Faaberg, K.S., Nelson, E.A., Rowland, R.R.R., 2007. Diversity and evolution of a newly emerged North American Type 1 porcine arterivirus: analysis of isolates collected between 1999 and 2004. Arch. Virol. 152, 1009–1017.
17. Figlerowicz, M., Bujarski, J.J., 1998. RNA recombination in brome mosaic virus, a model plus strand RNA virus. Acta Biochim. Pol. 45, 847–868.

18. Forsberg, R., Storgaard, T., Nielsen, H.S., Oleksiewicz, M.B., Cordioli, P., Sala, G., Hein, J., Bøtner, A., 2002a. The Genetic Diversity of European Type PRRSV Is Similar to That of the North American Type but Is Geographically Skewed within Europe. Virology 299, 38–47.
19. Forsberg, R., Storgaard, T., Nielsen, H.S., Oleksiewicz, M.B., Cordioli, P., Sala, G., Hein, J., Bøtner, A., 2002b. The genetic diversity of European type PRRSV is similar to that of the North American type but is geographically skewed within Europe. Virology 299, 38–47.
20. Frossard, J.-P., Hughes, G.J., Westcott, D.G., Naidu, B., Williamson, S., Woodger, N.G.A., Steinbach, F., Drew, T.W., 2013. Porcine reproductive and respiratory syndrome virus: Genetic diversity of recent British isolates. Vet. Microbiol. 162, 507–518.
21. Georgescu, M.M., Delpeyroux, F., Tardy-Panit, M., Balanant, J., Combiescu, M., Combiescu, A.A., Guillot, S., Crainic, R., 1994. High diversity of poliovirus strains isolated from the central nervous system from patients with vaccine-associated paralytic poliomyelitis. J. Virol. 68, 8089–8101.
22. Goldbach, R.W., 1986. Molecular Evolution of Plant RNA Viruses. Annu. Rev. Phytopathol. 24, 289–310.
23. Gorbalenya, A.E., Koonin, E.V., Lai, M.M.-C., 1991. Putative papain-related thiol proteases of positive-strand RNA viruses Identification of rubi- and aphthovirus proteases and delineation of a novel conserved domain associated with proteases of rubi-, α- and coronaviruses. FEBS Lett. 288, 201–205.
24. HIRST, G.K., 1962. Genetic recombination with Newcastle disease virus, polioviruses, and influenza. Cold Spring Harb. Symp. Quant. Biol. 27, 303–309.
25. Huisman, M.J., Cornelissen, B.J., Groenendijk, C.F., Bol, J.F., van Vloten-Doting, L., 1989. Alfalfa mosaic virus temperature-sensitive mutants. V. The nucleotide sequence of TBTS 7 RNA 3 shows limited nucleotide changes and evidence for heterologous recombination. Virology 171, 409–416.
26. Jarvis, T.C., Kirkegaard, K., 1992. Poliovirus RNA recombination: mechanistic studies in the absence of selection. EMBO J. 11, 3135–3145.
27. Jia, W., Karaca, K., Parrish, C.R., Naqi, S.A., 1995. A novel variant of avian infectious bronchitis virus resulting from recombination among three different strains. Arch. Virol. 140, 259–271.
28. Kapur, V., Elam, M.R., Pawlovich, T.M., Murtaugh, M.P., 1996. Genetic variation in porcine reproductive and respiratory syndrome virus isolates in the midwestern United States. J. Gen. Virol. 77 (Pt 6), 1271–1276.
29. Karniychuk, U.U., Geldhof, M., Vanhee, M., Van Doorsselaere, J., Saveleva, T.A., Nauwynck, H.J., 2010. Pathogenesis and antigenic characterization of a new East European subtype 3 porcine reproductive and respiratory syndrome virus isolate. BMC Vet. Res. 6, 30.
30. Khatchikian, D., Orlich, M., Rott, R., 1989. Increased viral pathogenicity after insertion of a 28S ribosomal RNA sequence into the haemagglutinin gene of an influenza virus. Nature 340, 156–157.
31. Kim, M.J., Kao, C., 2001. Factors regulating template switch in vitro by viral RNA-dependent RNA polymerases: implications for RNA-RNA recombination. Proc. Natl. Acad. Sci. U. S. A. 98, 4972–4977.

32. Kirkegaard, K., Baltimore, D., 1986. The mechanism of RNA recombination in poliovirus. Cell 47, 433–443.
33. Koonin, E.V., Dolja, V.V., Morris, T.J., 1993. Evolution and Taxonomy of Positive-Strand RNA Viruses: Implications of Comparative Analysis of Amino Acid Sequences. Crit. Rev. Biochem. Mol. Biol. 28, 375–430.
34. Lai, M.M., 1992. RNA recombination in animal and plant viruses. Microbiol. Rev. 56, 61–79.
35. Lai, M.M., Baric, R.S., Makino, S., Keck, J.G., Egbert, J., Leibowitz, J.L., Stohlman, S.A., 1985. Recombination between nonsegmented RNA genomes of murine coronaviruses. J. Virol. 56, 449–456.
36. Lai, M.M.C., 1996. Recombination in large RNA viruses: Coronaviruses. Semin. Virol. 7, 381–388.
37. Lauber, C., Ziebuhr, J., Junglen, S., Drosten, C., Zirkel, F., Nga, P.T., Morita, K., Snijder, E.J., Gorbalenya, A.E., 2012. Mesoniviridae: a proposed new family in the order Nidovirales formed by a single species of mosquito-borne viruses. Arch. Virol. 157, 1623–1628.
38. LEDINKO, N., 1963. Genetic recombination with poliovirus type 1. Studies of crosses between a normal horse serum-resistant mutant and several guanidine-resistant mutants of the same strain. Virology 20, 107–119.
39. Li, B., Fang, L., Xu, Z., Liu, S., Gao, J., Jiang, Y., Chen, H., Xiao, S., 2009. Recombination in Vaccine and Circulating Strains of Porcine Reproductive and Respiratory Syndrome Viruses. Emerg. Infect. Dis. 15, 2032–2035.
40. Li, K., Chen, Z., Plagemann, P., 1999. High-Frequency Homologous Genetic Recombination of an Arterivirus, Lactate Dehydrogenase-Elevating Virus, in Mice and Evolution of Neuropathogenic Variants. Virology 258, 73–83.
41. Liu, D., Zhou, R., Zhang, J., Zhou, L., Jiang, Q., Guo, X., Ge, X., Yang, H., 2011. Recombination analyses between two strains of porcine reproductive and respiratory syndrome virus in vivo. Virus Res. 155, 473–486.
42. Masuta, C., Ueda, S., Suzuki, M., Uyeda, I., 1998. Evolution of a quadripartite hybrid virus by interspecific exchange and recombination between replicase components of two related tripartite RNA viruses. Proc. Natl. Acad. Sci. U. S. A. 95, 10487–10492.
43. Mayo, M.A., Jolly, C.A., 1991. The 5'-terminal sequence of potato leafroll virus RNA: evidence of recombination between virus and host RNA. J. Gen. Virol. 72 (Pt 10), 2591–2595.
44. Meng, X.J., 2000. Heterogeneity of porcine reproductive and respiratory syndrome virus: implications for current vaccine efficacy and future vaccine development. Vet. Microbiol. 74, 309–329.
45. Mengeling, W.L., Lager, K.M., Vorwald, A.C., 1998. Clinical consequences of exposing pregnant gilts to strains of porcine reproductive and respiratory syndrome (PRRS) virus isolated from field cases of "atypical" PRRS. Am. J. Vet. Res. 59, 1540–1544.
46. Mengeling WL., 2002. The potential role of genetic recombination in the evolution of new strains of porcine reproductive and respiratory syndrome virus (PRRSV). J Swine Health Prod 10, 273–275.
47. MESELSON, M., WEIGLE, J.J., 1961. Chromosome brekage accompanying genetic recombination in bacteriophage. Proc. Natl. Acad. Sci. U. S. A. 47, 857–868.
48. Meyers, G., Rümenapf, T., Thiel, H.J., 1989. Ubiquitin in a togavirus. Nature 341, 491.

49. Meyers, G., Tautz, N., Dubovi, E.J., Thiel, H.J., 1991. Viral cytopathogenicity correlated with integration of ubiquitin-coding sequences. Virology 180, 602–616.
50. Mindich, L., Qiao, X., Onodera, S., Gottlieb, P., Strassman, J., 1992. Heterologous recombination in the double-stranded RNA bacteriophage phi 6. J. Virol. 66, 2605–2610.
51. Molenkamp, R., Greve, S., Spaan, W.J., Snijder, E.J., 2000. Efficient homologous RNA recombination and requirement for an open reading frame during replication of equine arteritis virus defective interfering RNAs. J. Virol. 74, 9062–9070.
52. Monroe, S.S., Schlesinger, S., 1983. RNAs from two independently isolated defective interfering particles of Sindbis virus contain a cellular tRNA sequence at their 5' ends. Proc. Natl. Acad. Sci. U. S. A. 80, 3279–3283.
53. Mu, C., Lu, X., Duan, E., Chen, J., Li, W., Zhang, F., Martin, D.P., Yang, M., Xia, P., Cui, B., 2013. Molecular evolution of porcine reproductive and respiratory syndrome virus isolates from central China. Res. Vet. Sci.
54. Munishkin, A.V., Voronin, L.A., Chetverin, A.B., 1988. An in vivo recombinant RNA capable of autocatalytic synthesis by Q beta replicase. Nature 333, 473–475.
55. Nagy, P.D., Dzianott, A., Ahlquist, P., Bujarski, J.J., 1995. Mutations in the helicase-like domain of protein 1a alter the sites of RNA-RNA recombination in brome mosaic virus. J. Virol. 69, 2547–2556.
56. Nagy, P.D., Zhang, C., Simon, A.E., 1998. Dissecting RNA recombination in vitro: role of RNA sequences and the viral replicase. EMBO J. 17, 2392–2403.
57. Pincus, S.E., Diamond, D.C., Emini, E.A., Wimmer, E., 1986. Guanidine-selected mutants of poliovirus: mapping of point mutations to polypeptide 2C. J. Virol. 57, 638–646.
58. Pond, S.L.K., Murrell, B., Poon, A.F.Y., 2012. Evolution of viral genomes: interplay between selection, recombination, and other forces. Methods Mol. Biol. Clifton NJ 856, 239–272.
59. Rowland, R.R., Steffen, M., Ackerman, T., Benfield, D.A., 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.
60. Runckel, C., Westesson, O., Andino, R., DeRisi, J.L., 2013. Identification and Manipulation of the Molecular Determinants Influencing Poliovirus Recombination. PLoS Pathog 9, e1003164.
61. Scheel, T.K.H., Galli, A., Li, Y.-P., Mikkelsen, L.S., Gottwein, J.M., Bukh, J., 2013. Productive Homologous and Non-homologous Recombination of Hepatitis C Virus in Cell Culture. PLoS Pathog 9, e1003228.
62. Serviene, E., Shapka, N., Cheng, C.-P., Panavas, T., Phuangrat, B., Baker, J., Nagy, P.D., 2005. Genome-wide screen identifies host genes affecting viral RNA recombination. Proc. Natl. Acad. Sci. U. S. A. 102, 10545–10550.
63. Shi, M., Holmes, E.C., Brar, M.S., Leung, F.C.-C., 2013. Recombination Is Associated with an Outbreak of Novel Highly Pathogenic Porcine Reproductive and Respiratory Syndrome Viruses in China. J. Virol. 87, 10904–10907.
64. Sibold, C., Meisel, H., Krüger, D.H., Labuda, M., Lysy, J., Kozuch, O., Pejcoch, M., Vaheri, A., Plyusnin, A., 1999. Recombination in Tula hantavirus evolution: analysis of genetic lineages from Slovakia. J. Virol. 73, 667–675.

65. Simon-Loriere, E., Galetto, R., Hamoudi, M., Archer, J., Lefeuvre, P., Martin, D.P., Robertson, D.L., Negroni, M., 2009. Molecular mechanisms of recombination restriction in the envelope gene of the human immunodeficiency virus. PLoS Pathog. 5, e1000418.
66. Smits, S.L., Lavazza, A., Matiz, K., Horzinek, M.C., Koopmans, M.P., de Groot, R.J., 2003. Phylogenetic and evolutionary relationships among torovirus field variants: evidence for multiple intertypic recombination events. J. Virol. 77, 9567–9577.
67. Snijder, E.J., den Boon, J.A., Horzinek, M.C., Spaan, W.J., 1991. Comparison of the genome organization of toro- and coronaviruses: evidence for two nonhomologous RNA recombination events during Berne virus evolution. Virology 180, 448–452.
68. Snijder, E.J., Meulenberg, J.J., 1998. The molecular biology of arteriviruses. J. Gen. Virol. 79 (Pt 5), 961–979.
69. Strauss, J.H., Strauss, E.G., 1988. Evolution of RNA Viruses. Annu. Rev. Microbiol. 42, 657–683.
70. Tian, K., Yu, X., Zhao, T., Feng, Y., Cao, Z., Wang, C., Hu, Y., Chen, X., Hu, D., Tian, X., Liu, D., Zhang, S., Deng, X., Ding, Y., Yang, L., Zhang, Y., Xiao, H., Qiao, M., Wang, B., Hou, L., Wang, X., Yang, X., Kang, L., Sun, M., Jin, P., Wang, S., Kitamura, Y., Yan, J., Gao, G.F., 2007. Emergence of fatal PRRSV variants: unparalleled outbreaks of atypical PRRS in China and molecular dissection of the unique hallmark. PloS One 2, e526.
71. Tolskaya, E.A., Romanova, L.I., Blinov, V.M., Viktorova, E.G., Sinyakov, A.N., Kolesnikova, M.S., Agol, V.I., 1987. Studies on the recombination between RNA genomes of poliovirus: the primary structure and nonrandom distribution of crossover regions in the genomes of intertypic poliovirus recombinants. Virology 161, 54–61.
72. Urbanowicz, A., Alejska, M., Formanowicz, P., Blazewicz, J., Figlerowicz, M., Bujarski, J.J., 2005. Homologous crossovers among molecules of brome mosaic bromovirus RNA1 or RNA2 segments in vivo. J. Virol. 79, 5732–5742.
73. Van Vugt, J.J., Storgaard, T., Oleksiewicz, M.B., Bøtner, A., 2001. High frequency RNA recombination in porcine reproductive and respiratory syndrome virus occurs preferentially between parental sequences with high similarity. J. Gen. Virol. 82, 2615–2620.
74. Wenhui, L., Zhongyan, W., Guanqun, Z., Zhili, L., JingYun, M., Qingmei, X., Baoli, S., Yingzuo, B., 2012. Complete Genome Sequence of a Novel Variant Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) Strain: Evidence for Recombination between Vaccine and Wild-Type PRRSV Strains. J. Virol. 86, 9543–9543.
75. White, K.A., Morris, T.J., 1995. RNA determinants of junction site selection in RNA virus recombinants and defective interfering RNAs. RNA N. Y. N 1, 1029–1040.
76. Wijegoonawardane, P.K.M., Sittidilokratna, N., Petchampai, N., Cowley, J.A., Gudkovs, N., Walker, P.J., 2009. Homologous genetic recombination in the yellow head complex of nidoviruses infecting Penaeus monodon shrimp. Virology 390, 79–88.
77. Wilson, V., Taylor, P., Desselberger, U., 1988. Crossover regions in foot-and-mouth disease virus (FMDV) recombinants correspond to regions of high local secondary structure. Arch. Virol. 102, 131–139.
78. Wiuf, C., Christensen, T., Hein, J., 2001. A simulation study of the reliability of recombination detection methods. Mol. Biol. Evol. 18, 1929–1939.

79. Worobey, M., Holmes, E.C., 1999. Evolutionary aspects of recombination in RNA viruses. J. Gen. Virol. 80 (Pt 10), 2535–2543.
80. Yuan, S., Nelsen, C.J., Murtaugh, M.P., Schmitt, B.J., Faaberg, K.S., 1999. Recombination between North American strains of porcine reproductive and respiratory syndrome virus. Virus Res. 61, 87–98.
81. Zhang, C.X., Cascone, P.J., Simon, A.E., 1991. Recombination between satellite and genomic RNAs of turnip crinkle virus. Virology 184, 791–794.

Figure 2-1 General mechanism for RNA recombination: Template-switch model/copy-choice model established in 1974

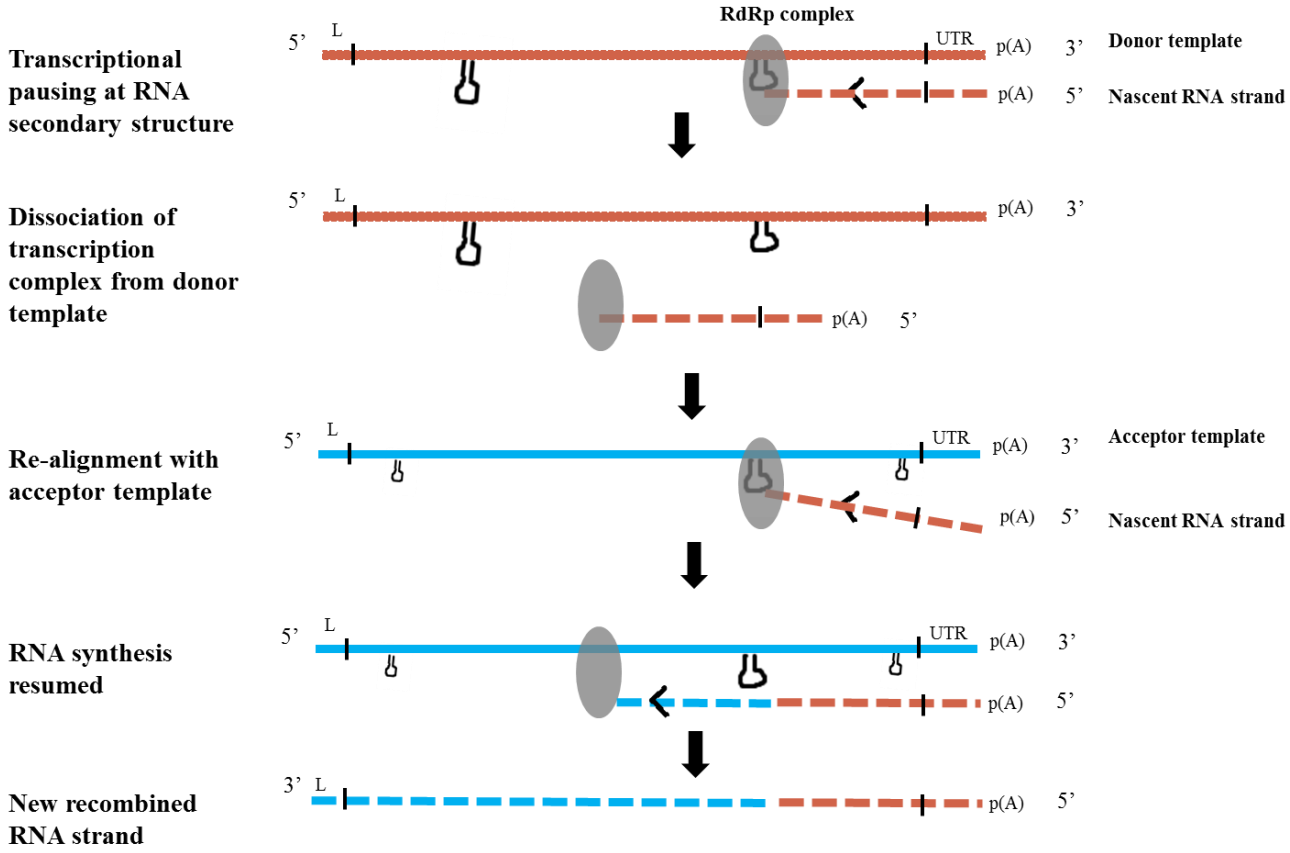
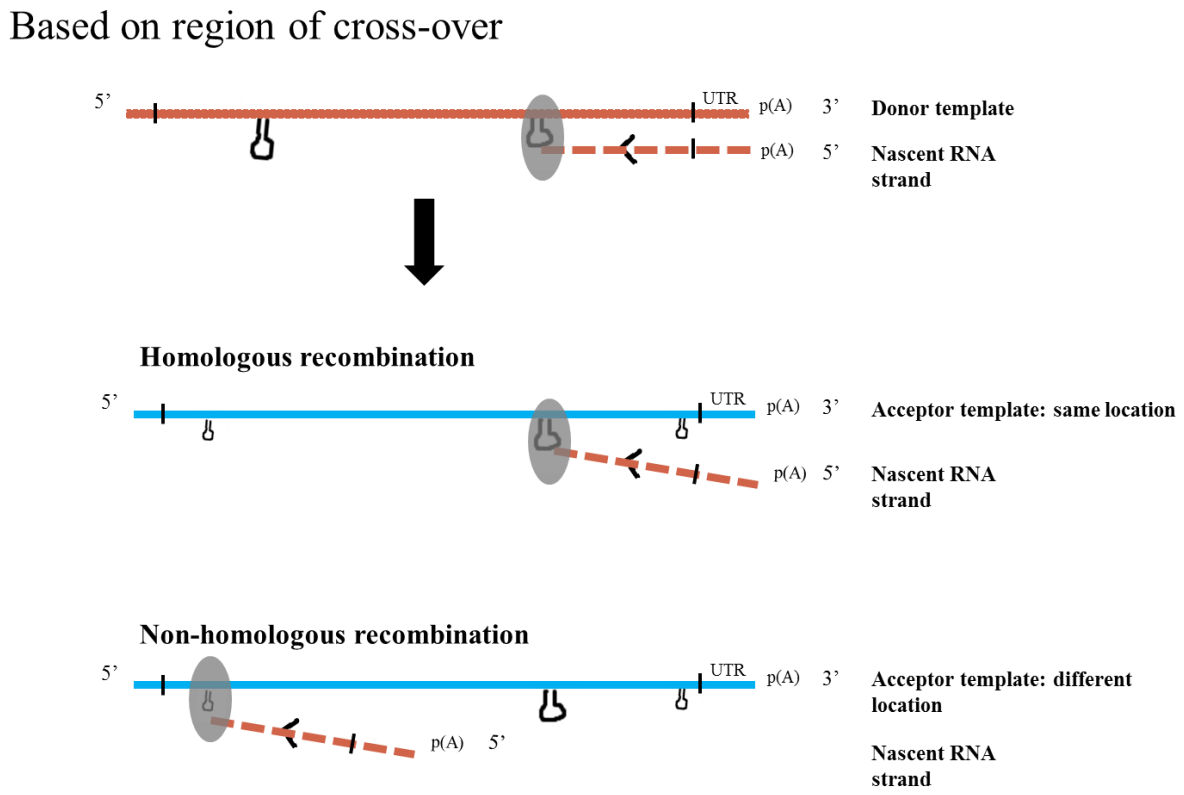


Figure 2-2 Types of recombination: Homologous and Non-homologous



Chapter 3 - Incorporation of green fluorescent protein for the study of recombination in porcine reproductive and respiratory syndrome virus (PRRSV)

Ranjni Chand, Yu Wang, and Raymond R. R. Rowland

Department of Diagnostic Medicine and Pathobiology, Kansas State University, Manhattan, KS 66506, USA

Abstract

A model system incorporating infectious clones that expressed green fluorescent protein (GFP) or destabilized red fluorescent protein (RFP) was developed to investigate recombination in porcine reproductive and respiratory syndrome virus (PRRSV). The co-infection of MARC-145 and porcine alveolar macrophage (PAM) cells with RFP and GFP viruses demonstrated the capacity of permissive cells to be superinfected with PRRSV, a necessary first step in recombination. To study recombination, a full-length PRRSV-enhanced GFP (EGFP) infectious cDNA clone was prepared that possessed an Arg to Cys mutation at position 97 of EGFP (EGFP-97C), which resulted in a non-fluorescent EGFP. The P129-EGFP-97C infectious clone plasmid was co-transfected along with a defective infectious cDNA clone, P129-EGFP-d(2-6), which lacked ORFs 2 through 6, but expressed a native EGFP. The result for successful recombination between full-length and defective genomes was the recovery of virus that expressed a fluorescent EGFP. The role of sequence homology in recombination was investigated by substituting EGFP with GFP, which possess 83% sequence identity. Co-transfection of EGFP and GFP failed to yield a fluorescent virus. However, successful recombination was obtained after replacing a 290 nucleotide segment in GFP with sequence identical to EGFP. Fluorescent viruses were not obtained when EGFP was expressed as a separate cytoplasmic mRNA, suggesting a requirement of the viral replication complex for efficient recombination.

3.A. Introduction

Porcine reproductive and respiratory syndrome (PRRS) incurs significant economic losses to the pork industry worldwide. In the late 1980's, PRRS appeared almost simultaneously in North America and Europe (Benfield et al., 1992; Collins et al., 1992; Wensvoort et al., 1991). Clinical signs of PRRS include respiratory disease in young pigs and reproductive failure in pregnant sows (Hopper et al., 1992; Pejsak et al., 1997). The disease is endemic in all major pork-producing countries, with only the Australian continent free of disease, along with countries, such as New Zealand, Norway, Chile, Sweden and Switzerland (Canon et al., 1998; Cho and Dee, 2006). The etiological agent is an enveloped single-stranded, positive-sense RNA virus, PRRS virus (PRRSV), which belongs to the family Arteriviridae in the order Nidovirales (Cavanagh, 1997; Meulenberg, 2000; Snijder and Meulenberg, 1998). PRRSV isolates are further divided into two distinct genotypes; European (EU) type 1 and North American (NA) type 2 strains (Allende et al., 1999; Nelsen et al., 1999). In 1999, European-like type 1 viruses were identified in North America and designated as NA type 1 (Fang et al., 2007a; Susan L. Ropp et al., 2004). The PRRSV genome is approximately 15 kb in length and contains at least ten open reading frames (ORFs) (Dokland, 2010; Johnson et al., 2011b). Virus replication incorporates the production of a nested set of subgenomic mRNAs, which possess a common leader and poly-A tail. After entry and uncoating, ORFs 1a and 1b are directly translated into pp1a and pp1ab proteins, which are proteolytically cleaved into 14 non-structural proteins. Located on the 3' end of the genome are ORFs 2, 3, 4 and 5, which code for glycosylated envelope proteins, GP2a, GP3, GP4, and GP5. ORFs 2b, 5a and 6 code for the non-glycosylated envelope proteins namely: 2b, ORF5a protein and M, respectively. ORF 7 codes for the nucleocapsid (N) protein.

PRRSV is considered one of the most genetically diverse animal viruses with an estimated mutation rate of 10^{-2}/site/year (Hanada et al., 2005; Jenkins et al., 2002). One outcome is the divergence of type 1 and type II viruses, which possess about 60% identity at the nucleotide level (Nelson et al., 1993). The analysis of ORF5 sequences shows that strains within each genotype can possess up to 20% variability (Han et al., 2007; Meng, 2000b). The consequences of genetic diversity are vaccine failure in the field, as well as the emergence of

novel viruses with increased virulence. In general, vaccines provide the best protection against viruses similar to the vaccine strain with cross-protection dropping off as peptide sequence diverges from the vaccine strain (Labarque et al., 2004; Meng, 2000b; Murtaugh et al., 2002).

The first step in recombination is the superinfection of cells with two or more viruses. Once inside the cell, recombination occurs during the synthesis of genomic RNA or subgenomic mRNA replication. Recombination occurs through a template switch or 'copy-choice' mechanism, first described for polioviruses (Cooper et al., 1974). Three prerequisites for template switching to occur are: 1) RNA replication machinery must dissociate from the parent template; 2) the acceptor template must occur in the vicinity of the replication complex, and 3) the acceptor template must promote landing of the replication complex and resume RNA synthesis. Secondary RNA structures and sequence homology between donor and acceptor templates are factors that influence selection of a recombination site. Recombination in coronaviruses has been extensively studied in murine hepatitis virus (MHV) using temperature sensitive mutants (Lai et al., 1985). Coronavirus possess a recombination frequency of about 25% across the entire genome (Lai, 1996). Recombination has been found to occur between viral genome and defective-interfering RNAs or plasmid derived mRNAs. These properties have been used as a tool to manipulate the coronavirus genome (Masters et al., 1994).

Based on the analysis of sequences from field viruses, Kapur et al reported the first evidence for recombination within the structural genes of PRRSV (Kapur et al., 1996b). In a later study, 50 US field isolates were sequenced and one natural recombinant virus was identified, the result of a cross-over within a 31bp region of ORF 5 (Yuan et al., 1999b). Phylogenetic incongruity in a group of EU isolates identified an Italian isolate that had undergone a recombination event in ORF6 (Forsberg et al., 2002a). Similarly, Fang et al. identified a recombined NA type 1 isolate that possessed a crossover site in the nsp2/3 junction region (Fang et al., 2007a).

Recombination between PRRSV isolates has also been demonstrated in the laboratory. Cells are co-infected with two isolates followed by differential PCR, using combinations of isolate-specific primers (van Vugt et al., 2001; Yuan et al., 1999b). This experimental approach

has demonstrated several properties associated with recombination. First, recombination frequencies can be as high as 10% when cells are infected with two closely related isolates (Yuan et al., 1999b). Secondly, the frequency of recombination is inversely related to the degree of sequence homology between the two viruses; as nucleotide sequence homology diverges, the frequency of recombination decreases to the extent that recombination between type 1 and type II viruses becomes an uncommon event (van Vugt et al., 2001). And finally, imprecise recombination can yield insertions and deletions at crossover sites.

In this study, a model system for investigating recombination has been described. This model incorporates reverse genetics and infectious PRRSV clones that express a fluorescent protein gene as a separate subgenomic mRNA. Successful recombination is easily identified by gain of biological function; i.e. fluorescence. This approach can be used to investigate the properties of recombination in a virally expressed gene that is non-essential for virus replication.

3.B. Materials and Methods

3.B.i. Cells and infectious clones

All procedures were performed after approval by the Kansas State University institutional biosafety and animal use care committees. MARC-145 cells and HEK-293T cells were propagated and maintained in Eagle's Minimum Essential Medium (Sigma-Aldrich, St. Louis, USA) with 7% fetal bovine serum (Sigma-Aldrich, St. Louis, USA) and supplemented with 0.01% pen/strep and 0.008% Fungizone. Porcine alveolar macrophage (PAM) cells were collected from 3 to 4 week old piglets by lung lavage in sterile 1 X phosphate buffered saline (PBS). PAMs were maintained in 1 X RPMI (Gibco-Life Technologies, Grand Island, USA) supplemented with 10% FBS, 0.01% pen/strep, 0.008% Fungizone and 1% L-glutamine (Life Technologies, Grand Island, USA). Cells were maintained at 37^{O}C with 5% CO_2. Full-length and defective viruses were derived from the DNA-launched P129-GFP infectious clone (Welch et al., 2004). The parent infectious clone was derived from a North American PRRSV strain, P129 (GenBank Accession #AF494042). The original P129 clone was further modified to

express GFP from an independent sub-genomic mRNA inserted between ORF1 and ORF2 (Pei et al., 2009). Modifications of the P129-GFP clone used in these experiments are diagrammed in Figure 1A. The EGFP-97C and DsRed genes were inserted between AflII and MluI restriction sites present in the original infectious clone P129-GFP (Pei et al., 2009). With reference to P129-GFP, AflII is at position 12057 and MluI is at 12785. These 2 restriction sites were inserted between ORF1b and ORF2 after nucleotide number 12056. P129-RFP was constructed by replacing GFP with DsRed obtained from pDsRed-N1 (courtesy of Yongming Sang). P129-EGFP-97C was constructed by replacing GFP with a mutated EGFP gene that possessed a single nucleotide, C to T, change at nucleotide position 289, which resulted in an arginine to cysteine change at amino acid 97 in the native EGFP gene (derived from pEGFP-N1, Clontech, Mountain View, USA) (GenBank Accession #U55762). The defective replicating virus, P129-GFP-d(2-6), was constructed by deleting the structural genes, ORFs 2-6, in the parent P129-GFP plasmid. This was accomplished in two steps; first, a *Mlu*I site was placed in front of the subgenomic transcription-regulating sequence (TRS) located in front of ORF7. A second *Mlu*I site was already present at the 3' end of GFP. The intervening ORFs 2-6 were removed by *Mlu*I digestion followed by re-ligation. The remaining defective viruses, P129-EGFP-d(2-6) and P129-GFPm-d(2-6), were constructed by replacing the GFP gene in P129-GFP-d(2-6) with the native EGFP gene and GFPm (Figure 1B). Existing AflII and MluI restriction sites were used for cloning GFP, EGFP and GFPm genes into the P129 genome (Pei et al., 2009). The GFPm gene, which possessed a 290 bp stretch of identity between GFP and EGFP (see Figure 1B), was prepared by commercial gene synthesis (GenScript, Piscataway, USA).

3.B.ii. Transfection of HEK 293T cells and infection of MARC-145 cells

Viruses were derived by first transfecting HEK cells with infectious clone plasmid DNA followed by incubation for 48 hours at 37 ^{O}C and 5% CO_2. Transfection of HEK cells on 24-well plates was performed with 0.54 μg plasmid DNA using Fugene HD transfection reagent (Promega, Madison, USA) according to manufacturer's recommendations. Successful replication was confirmed by presence of fluorescence and/or positive staining for the expression of nucleocapsid (N) protein. For N protein staining, cells were washed two times with PBS and then

fixed with 4% paraformaldehyde for 30 min. The fixed cells were then permeabilized with 0.02% saponin and then stained with mAb SDOW-17 at a 1:1000 dilution in PBS with 10% goat serum (PBS-GS). After 30 minutes, the cells were washed with PBS. SDOW was detected using a 1:500 dilution of secondary antibody goat anti-mouse IgG conjugated with Alexa fluor 594 nm (Invitrogen-Life Technologies, Grand Island, USA). After one hour incubation at room temperature, the cells were washed with PBS and visualized under a fluorescence microscope.

For preparation of virus stocks, infectious clone-transfected HEK cells were freeze-thawed and media centrifuged to remove cell debris, and the virus stored at -80^{O}C until further use. Viruses were further propagated in MARC-145 cells. Three days after infection of confluent MARC-145 cells, the presence of infection was confirmed by green fluorescence and/or the presence of cytopathic effect (CPE). Viruses were further amplified by passage on MARC-145 cells.

3.B.iii. Flow cytometry and fluorescence microscopy

Flow cytometry was performed at 48 hours after infection or transfection, when fluorescence was clearly visible under the fluorescence microscope. Plates were washed with PBS and cells were removed after adding a few drops of 0.25% Trypsin-EDTA (Gibco-Life Technologies, Grand Island, USA). Cells were resuspended in PBS with 1% paraformaldehyde and analyzed using a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, USA). Data were analyzed using BD CellQuest Pro software. For confocal microscopy, MARC-145 cells were cultured in 35 mm glass bottom microwell dishes (MatTek, Ashland, USA) with 500 µl of medium. At 48 hours after infection/transfection, cells were imaged by a confocal microscope (Nikon Eclipse TE 2000-E, Melville, USA) with a Quantem 512 SC camera and NIS Elements 3.0 software was used to merge images.

3.B.iv. Preparation of EGFP-expressing HEK and MARC-145 cell lines

MARC-145 or HEK cells were transfected with pEGFP-N1 (Clontech, Mountain View, USA)). Forty-eight hours later, cells were reseeded to 50% confluency in a new flask and 500 μg/mL G418 added. G418 is an antibiotoic that belongs to the aminoglycoside group of compounds similar to gentamicin. Neomycin resistance gene (*Neo*) carried by plasmids such as pEGFP-N1 confers resistance to G418. This is used as a selection system in mammalian cell lines. Medium was changed every 3 to 4 days to remove dead cells. Cells were maintained with G418 at a concentration of 200 μg/mL.

3.B.v. PCR amplification of EGFP/GFP and DNA sequencing

MARC-145 cells, obtained at three days after infection, were enriched for viruses that expressed native EGFP by two rounds cell sorting that selected for cells that expressed green fluorescence (BioRad S3 Cell Sorter, Hercules, USA). RNA extraction of sorted cells was performed according to manufacturer's protocol using the MagMax Viral RNA Isolation Kit (Ambion -Life Technologies, Grand Island, USA). RT-PCR was performed using a One Step RT-PCR kit (Qiagen, Valencia, USA) according to manufacturer's instructions. The forward and reverse primers amplified a 737 bp region between AflII and MluI restriction sites, which covered the entire EGFP/GFP. The sequences for the GFP-AflII forward and GFP-MluI primers were cttaaggccaccatgagcaagggcgaggagctgttc and acgcgtcacttgtacagctcgtc, respectively (restriction sites underlined). The RT-PCR amplified fragments were gel purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA) and cloned in the pCR 2.1 Topo vector (Invitrogen-Life Technologies, Grand Island, USA) and sequenced (ACGT, Wheeling, USA). Sequences were analyzed using Mega 5.2 software.

3.C. Results

3.C.i. Co-infection of MARC-145 cells and PAMs with GFP and RFP PRRS viruses

Since the first step in recombination is the superinfection of single cells, MARC-145 and PAM cells were evaluated for the presence of dual fluorescence after co-infection with P129-

GFP and P129-RFP viruses. Except for the differences in the nucleotide sequence of the two fluorescent genes, both viruses were identical. These viruses exhibited similar growth kinetics, achieving levels of between 10^7 and 10^8 $TCID_{50}$/ml within 36 hrs after infection of MARC-145 cells (Figure 2). The maximum number of fluorescent cells at 48 hrs after infection was achieved using a MOI of 10. Even at 10 MOI, only about 10% of cells were fluorescent after the first round of replication (data not shown).

To demonstrate that cells were capable of simultaneously expressing GFP and RFP proteins, HEK cells were co-transfected with P129-GFP and P129-RFP plasmids, at a ratio of 1:1. Each plasmid was used at 0.27 μg to make up 0.54 μg total DNA for every transfection reaction in a 24-well plate. After 48 hours, the co-localization of green and red fluorescence was observed in single cells (see Figure 3A). MARC-145 cells were co-infected with P129-GFP and P129-RFP viruses at a ratio of 1:1 and MOI 10 which was the total MOI of the two viruses when combined. . An example of co-localization of red and green fluorescence in a single infected MARC-145 cell is shown in Figure 3B. PAM cells were also co-infected with red and green viruses. A representative example of a PAM cell with green and red fluorescence is shown in Figure 3C. Cells expressing dual fluorescence were further analyzed using flow cytometry. MARC-145 cells were infected with different ratios of P129-GFP and P129-RFP viruses. The results, presented in Figure 4, showed the cells in all four scatter plot quadrants; fluorescence negative cells, GFP single-positive cells, RFP single-positive cells and double-positive cells. Even though a total MOI of 10 was used for infection, at least 42% of cells remained negative for fluorescence. The maximum percentage of double-positive cells was achieved using a ratio of 8:1, P129-GFP:P129-RFP. At this infection ratio, 9.6% of total cells or 17% of fluorescent cells were double-positive. Together, these data demonstrate that MARC-145 cells can be easily superinfected and under appropriate experimental conditions, the rate of co-infection is relatively high.

3.C.ii. Recombination within EGFP

The above results demonstrated that MARC-145 cells can be co-infected with at least two PRRS viruses. Recombination within EGFP was studied by constructing a non-fluorescent infectious virus clone that contained a single mutation in EGFP (see Figure 1). The arginine at amino acid position 97 of EGFP, or position 96 of GFP, is required for fluorescence. Changing a single C to T at nucleotide position 289 of EGFP changed the arginine to cysteine, which results in a corresponding loss of fluorescence (Kim et al., 2007). The model system illustrating recombination within mutant EGFP to yield a fluorescent protein is shown in Figure 5. Consistent with the template switch model, the production of a fluorescent virus can occur by single or multiple cross-over events. The minimum requirement for a detectable recombined virus in this system would be a single recombination event within the 426 nucleotide region located between the 97C mutation and the MluI site on the 3' end of EGFP. After transfection of HEK cells with only the P129-EGFP-97C plasmid, cells were negative for green fluorescence, but positive for the expression of the N protein, indicating that the construct was replication competent (see Figure 6A). The resulting virus was capable of productively infecting MARC-145 cells (data not shown). Infected cells remained negative for EGFP fluorescence and positive for N protein staining after six sequential passages of the P129-EGFP-97C virus on MARC-145cells. This demonstrated that the 97C mutation was stable and infectious was produced during each passage. The second plasmid construct contained a modified P129-EGFP infectious clone, which contained a deletion of ORFs 2 through 6, to create the defective virus, P129-EGFP-d(2-6). Transfection of HEK cells with the P129-EGFP-d(2-6) plasmid resulted in green fluorescence (see Figure 6D). The same cells stained positive for the N protein (Figure 6C); therefore, ORFs 2-6 did not affect the synthesis of EGFP or N protein subgenomic mRNAs. The media recovered from transfected cells, when placed on MARC-145 cells, did not produce green fluorescence or positive staining for N protein (data not shown), demonstrating that the defective virus, which lacked all of the surface proteins, was not capable of infecting MARC cells. At 48 hrs after co-transfection of HEK cells with the full-length and defective infectious clones, the media was transferred to MARC cells. The result was the appearance of cells that expressed the N protein and green fluorescence, indicating the presence of recombination between the full-length and defective viral genomes (see Figure 6, panels E and F). The recovery of recombined fluorescent viruses was confirmed after a second passage on MARC cells.

To estimate the frequency of recombination, media from MARC-145 cells was removed at 48 hrs after infection and virus titration performed. $TCID_{50}$/mL values for the non-fluorescent P129-EGFP-97C virus and recombined green fluorescent virus were 1.5 X 10^5 and 5 X 10^2, respectively. Therefore, the ratio of parent and recombined viruses was approximately 300 fluorescent negative viruses for every fluorescent virus or a recombination frequency of about 0.3%.

The EGFP nucleotide and peptide sequences represent modifications of the native GFP isolated from the jellyfish, *Aequorea victoria*. The EGFP peptide sequence is a single amino acid longer than GFP and possesses two amino acid substitutions, which result in a red shift in fluorescence. Furthermore, the EGFP cDNA is codon optimized for optimal expression in mammalian cells (Haas et al., 1996). The result is a 124 base pair difference or 83% nucleotide identity between GFP and EGFP. For the purpose of comparison, the overall nucleotide identity between Type I and Type II genotype PRRS viruses is approximately 60 to 70%. Within the Type II PRRSV genotype group, isolates possess homologies as low as 85% in ORF5 (personal observation) and 60% in nsp2 .

The nucleotide sequence differences were used to determine if recombination in the 97-C region would occur between GFP and EGFP. HEK cells were co-transfected with P129-GFP-d(2-6) and P129-EGFP-97C plasmids at ratios of 1:10, 1:5, 1:2.5 and 1:1. At 48 hrs after transfection, media were transferred to MARC-145 cells and cells visualized for green fluorescence 48 hrs later. The results, presented in Figure 7 (panels A and B), showed that the P129-GFP-d(2-6) construct successfully expressed the N protein and GFP fluorescence in HEK cells. Viruses derived from the co-transfection of P129-GFP-d(2-6) and P129-EGFP-97C plasmids were able to infect MARC-145 cells productively (Figure 7C), but remained negative for fluorescence for all plasmid ratios (Figure 7D). These results demonstrate that P129-GFP-d(2-6) is replication competent, but GFP and EGFP failed to recombine.

One possibility for a negative recombination result was the lack of sufficient nucleotide sequence homology between GFP and EGFP. To test this possibility, the experiment was repeated using a defective virus that expressed GFPm, a modified GFP that possessed a

large region of nucleotide identity with EGFP (see Figure 1B). The region of identity flanked both sides of the 97-C mutation. When transfected into HEK cells, the P129-GFPm-d(2-6) plasmid produced green fluorescence and expressed the N protein demonstrating that the construct was fluorescence positive and replication competent (Figure 7C and D). Viruses derived from the co-transfection of the HEK cells with P129-EGFP-97C and P129-GFPm-d(2-6) plasmids were able to produce green fluorescence in MARC-145 cells, indicating successful recombination between EGFP-97C and GFPm genes. Fluorescence-activated cell sorting was used to enrich for fluorescent cells. After RNA extraction, the region between the AflII and MluI restrictions sites on the ends of the EGFP and GFPm were amplified by PCR and cloned into pCR2.1 vector. Six plasmids were selected and sequenced. All cloned products showed the same DNA sequence, which included a single crossover site located between nucleotide position 651and 672 (see Figure 8).

3.C.iii. Virus recombination with cytoplasmic mRNA

This study also determined if the P129-EGFP-97C virus could recombine with EGFP when EGFP was expressed as a cytoplasmic mRNA. MARC-145 and HEK cell lines were created that stably expressed an EGFP placed under the control of a CMV promoter. EGFP-MARC cells were infected with P129-EGFP-97C virus and media harvested 5 days later. There was no evidence of fluorescence. The experiment was repeated by transfecting EGFP-HEK cells with plasmid P129-EGFP-97C. Media were harvested 48 hrs later and then placed on MARC-145 cells. Cells were productively infected, but failed to express fluorescence (data not shown). Under these experimental conditions, the results suggest that recombination does not occur when EGFP is expressed as separate cytoplasmic mRNA.

3.D. Discussion

In this study, the use of fluorescent protein genes, infectious cDNA clones, and reverse genetics as a model system to investigate the properties of recombination in PRRSV have

been described. The experimental model incorporates native and mutated fluorescent protein genes expressed during PRRSV replication via a separate viral subgenomic mRNA fragment, located between ORF1b and ORF2 of the PRRSV genome. One advantage in the use of a fluorescent protein expressed as a separate mRNA is that mutations and other modifications can be made to the fluorescent protein gene without disrupting viral genes required for virus replication. A second advantage is that successful recombination is easily detected by the acquisition of fluorescence in a replication competent virus.

Although recombination has been found to occur in a large number of RNA viruses this is a complex process with at least five levels mechanistic constraints. These include: simultaneous infection of a host, co-infection of a cell within that host, compatibility for co-replication, physical detachment for switching the RNA template and finally overcoming the selective pressures of the host (Worobey and Holmes, 1999). A recombined virus has to clear each of these 5 constraints and further be efficiently transmitted for establishment in a population. Therefore, the first step in recombination which is superinfection of cells with two or more viruses was demonstrated to occur in the experimental system to study recombination. As shown in Figure 3, the co-infection with P129-GFP and P129-RFP viruses produced cells that contained both proteins, demonstrating that MARC-145 cells and PAM cells readily support superinfection. Under optimal experimental conditions, up to 17% of MARC cells could be co-infected, which is similar to results for other viruses. For example, co-infection DF-1 cells with Newcastle disease viruses expressing RFP and EGFP resulted in 27% dual fluorescence (Li et al., 2012). For HIV-1, the co-infection frequency was only 2.3% in four different cell lines (Bregnard et al., 2012), but increased to around 40% when T lymphocytes were the target of superinfection (Dang et al., 2004; Levy et al., 2004).

Recombination within EGFP was studied by co-transfecting HEK cells with P129-EGFP-97C and P129-EGFP-d(2-6) infectious clone plasmids. The P129-EGFP-97C infectious clone codes for a full-length virus that expresses a non-fluorescent mutant EGFP. The 97C mutation was discovered by sequencing non-fluorescent EGFP genes cloned into the nsp2 region of PRRSV (Kim et al., 2007). During the formation of the fluorophore, arginine 97 stabilizes the carbonyl group of the imidazolidinone ring formed by Tyr-67/Gly-68. Together, all three amino

acids participate in the post-translational modifications needed for fluorophore formation. To obtain a fluorescent virus, recombination between the full-length and defective genomes would have to occur within the 426 nucleotides between site of the 97C mutation and the MluI restriction site (see Figure 5). Infected cells remained negative for EGFP fluorescence and positive for N protein staining after six sequential passages of the P129-EGFP-97C virus on MARC-145 cells. This demonstrated that the 97C mutation was stable and infectious virus was produced during each passage. The successful production of EGFP-97C protein was demonstrated by a dual staining experiment of P129-EGFP-97C-transfected HEK cells with anti-GFP (green) and anti N-protein (red). Figure 9 shows cells that are simultaneously producing EGFP-97C and N protein. The stability of this mutation over serial passages cannot be tested in HEK cells because these cells although susceptible, are non-permissive to PRRS virus. The recombination frequency within the 426 nt region of EGFP was estimated to be 0.3%, a value consistent with the results of previous studies, which incorporated PCR as the means to identify a recombination event (van Vugt et al., 2001). For instance, the infection of MARC cells with two closely related Type I viruses revealed a recombination frequency of 0.1 to 2.5% within a 621 nt region located between the 3' end of ORF3 through the 3' end of ORF5 (van Vugt et al., 2001).

EGFP and GFP gene sequences possess 124 mismatches or 83% identity at the nucleotide level (see Figure 1B). Within the 426 nt region where recombination was predicted to occur, there are 64 mismatches, with two 20 nucleotide stretches of identity. Recombination was not detected between the GFP and EGFP-97C viruses. A 164 nt stretch of 100% identity was inserted 3' of the 97C mutation to create the construct, P129-GFPm-d(2-6), see Figure 1B. Co-transfection of this plasmid with the P129-EGFP-97C yielded a fluorescent virus. However, the recombination crossover site was located downstream of the 164 nt region of homology, within a second region that possessed a 20 nt stretch of homology (see Figure 8). The same crossover region was identified in 6 of 6 clones in a population of infected MARC-145 cells selected for fluorescence. The mechanistic basis for the selection of this site remains to be determined.

Recombination between P129-EGFP-97C and EGFP was not detected when EGFP was expressed as a separate mRNA. One explanation for this negative result is that the EGFP mRNA, may not have access to double walled membrane vesicles, the location of virus

replication complexes (Pedersen et al., 1999). Therefore, the development of targeted recombination techniques for PRRSV and other arteriviruses may require the specific localization of the regions of interest to the virus replication complex.

3.E. References

1. Allende, R., Lewis, T.L., Lu, Z., Rock, D.L., Kutish, G.F., Ali, A., Doster, A.R., Osorio, F.A., 1999. North American and European porcine reproductive and respiratory syndrome viruses differ in non-structural protein coding regions. J. Gen. Virol. 80 (Pt 2), 307–315.
2. Benfield, D.A., Nelson, E., Collins, J.E., Harris, L., Goyal, S.M., Robison, D., Christianson, W.T., Morrison, R.B., Gorcyca, D., Chladek, D., 1992. Characterization of swine infertility and respiratory syndrome (SIRS) virus (isolate ATCC VR-2332). J. Vet. Diagn. Investig. Off. Publ. Am. Assoc. Vet. Lab. Diagn. Inc 4, 127–133.
3. Bregnard, C., Pacini, G., Danos, O., Basmaciogullari, S., 2012. Suboptimal Provirus Expression Explains Apparent Nonrandom Cell Coinfection with HIV-1. J. Virol. 86, 8810–8820.
4. Canon, N., Audigé, L., Denac, H., Hofmann, M., Griot, C., 1998. Evidence of freedom from porcine reproductive and respiratory syndrome virus infection in Switzerland. Vet. Rec. 142, 142–143.
5. Cavanagh, D., 1997. Nidovirales: a new order comprising Coronaviridae and Arteriviridae. Arch. Virol. 142, 629–633.
6. Cho, J.G., Dee, S.A., 2006. Porcine reproductive and respiratory syndrome virus. Theriogenology 66, 655–662.
7. Collins, J.E., Benfield, D.A., Christianson, W.T., Harris, L., Hennings, J.C., Shaw, D.P., Goyal, S.M., McCullough, S., Morrison, R.B., Joo, H.S., 1992. Isolation of swine infertility and respiratory syndrome virus (isolate ATCC VR-2332) in North America and experimental reproduction of the disease in gnotobiotic pigs. J. Vet. Diagn. Investig. Off. Publ. Am. Assoc. Vet. Lab. Diagn. Inc 4, 117–126.
8. Cooper, P.D., Steiner-Pryor, A., Scotti, P.D., Delong, D., 1974. On the Nature of Poliovirus Genetic Recombinants. J. Gen. Virol. 23, 41–49.
9. Dang, Q., Chen, J., Unutmaz, D., Coffin, J.M., Pathak, V.K., Powell, D., KewalRamani, V.N., Maldarelli, F., Hu, W.-S., 2004. Nonrandom HIV-1 infection and double infection via direct and cell-mediated pathways. Proc. Natl. Acad. Sci. U. S. A. 101, 632–637.
10. Dokland, T., 2010. The structural biology of PRRSV. Virus Res. 154, 86–97.
11. Fang, Y., Schneider, P., Zhang, W.P., Faaberg, K.S., Nelson, E.A., Rowland, R.R.R., 2007. Diversity and evolution of a newly emerged North American Type 1 porcine arterivirus: analysis of isolates collected between 1999 and 2004. Arch. Virol. 152, 1009–1017.
12. Forsberg, R., Storgaard, T., Nielsen, H.S., Oleksiewicz, M.B., Cordioli, P., Sala, G., Hein, J., Bøtner, A., 2002. The Genetic Diversity of European Type PRRSV Is Similar to That of the North American Type but Is Geographically Skewed within Europe. Virology 299, 38–47.
13. Haas, J., Park, E.C., Seed, B., 1996. Codon usage limitation in the expression of HIV-1 envelope glycoprotein. Curr. Biol. CB 6, 315–324.

14. Han, J., Liu, G., Wang, Y., Faaberg, K.S., 2007. Identification of Nonessential Regions of the nsp2 Replicase Protein of Porcine Reproductive and Respiratory Syndrome Virus Strain VR-2332 for Replication in Cell Culture. J. Virol. 81, 9878–9890.
15. Hanada, K., Suzuki, Y., Nakane, T., Hirose, O., Gojobori, T., 2005. The origin and evolution of porcine reproductive and respiratory syndrome viruses. Mol. Biol. Evol. 22, 1024–1031.
16. Hopper, S.A., White, M.E., Twiddy, N., 1992. An outbreak of blue-eared pig disease (porcine reproductive and respiratory syndrome) in four pig herds in Great Britain. Vet. Rec. 131, 140–144.
17. Jenkins, G.M., Rambaut, A., Pybus, O.G., Holmes, E.C., 2002. Rates of molecular evolution in RNA viruses: a quantitative phylogenetic analysis. J. Mol. Evol. 54, 156–165.
18. Johnson, C.R., Griggs, T.F., Gnanandarajah, J., Murtaugh, M.P., 2011. Novel structural protein in porcine reproductive and respiratory syndrome virus encoded by an alternative ORF5 present in all arteriviruses. J. Gen. Virol. 92, 1107–1116.
19. Kapur, V., Elam, M.R., Pawlovich, T.M., Murtaugh, M.P., 1996. Genetic variation in porcine reproductive and respiratory syndrome virus isolates in the midwestern United States. J. Gen. Virol. 77 (Pt 6), 1271–1276.
20. Kim, D.-Y., Calvert, J.G., Chang, K.-O., Horlen, K., Kerrigan, M., Rowland, R.R.R., 2007. Expression and stability of foreign tags inserted into nsp2 of porcine reproductive and respiratory syndrome virus (PRRSV). Virus Res. 128, 106–114.
21. Labarque, G., Reeth, K.V., Nauwynck, H., Drexler, C., Van Gucht, S., Pensaert, M., 2004. Impact of genetic diversity of European-type porcine reproductive and respiratory syndrome virus strains on vaccine efficacy. Vaccine 22, 4183–4190.
22. Lai, M.M., Baric, R.S., Makino, S., Keck, J.G., Egbert, J., Leibowitz, J.L., Stohlman, S.A., 1985. Recombination between nonsegmented RNA genomes of murine coronaviruses. J. Virol. 56, 449–456.
23. Lai, M.M.C., 1996. Recombination in large RNA viruses: Coronaviruses. Semin. Virol. 7, 381–388.
24. Levy, D.N., Aldrovandi, G.M., Kutsch, O., Shaw, G.M., 2004. Dynamics of HIV-1 recombination in its natural target cells. Proc. Natl. Acad. Sci. U. S. A. 101, 4204–4209.
25. Li, J., Hu, H., Yu, Q., Diel, D.G., Li, D., Miller, P.J., 2012. Generation and characterization of a recombinant Newcastle disease virus expressing the red fluorescent protein for use in co-infection studies. Virol. J. 9, 227.
26. Masters, P.S., Koetzner, C.A., Kerr, C.A., Heo, Y., 1994. Optimization of targeted RNA recombination and mapping of a novel nucleocapsid gene mutation in the coronavirus mouse hepatitis virus. J. Virol. 68, 328–337.
27. Meng, X.J., 2000. Heterogeneity of porcine reproductive and respiratory syndrome virus: implications for current vaccine efficacy and future vaccine development. Vet. Microbiol. 74, 309–329.
28. Meulenberg, J.J., 2000. PRRSV, the virus. Vet. Res. 31, 11–21.
29. Murtaugh, M.P., Xiao, Z., Zuckermann, F., 2002. Immunological responses of swine to porcine reproductive and respiratory syndrome virus infection. Viral Immunol. 15, 533–547.

30. Nelsen, C.J., Murtaugh, M.P., Faaberg, K.S., 1999. Porcine reproductive and respiratory syndrome virus comparison: divergent evolution on two continents. J. Virol. 73, 270–280.
31. Nelson, E.A., Christopher-Hennings, J., Drew, T., Wensvoort, G., Collins, J.E., Benfield, D.A., 1993. Differentiation of U.S. and European isolates of porcine reproductive and respiratory syndrome virus by monoclonal antibodies. J. Clin. Microbiol. 31, 3184–3189.
32. Pedersen, K.W., van der Meer, Y., Roos, N., Snijder, E.J., 1999. Open Reading Frame 1a-Encoded Subunits of the Arterivirus Replicase Induce Endoplasmic Reticulum-Derived Double-Membrane Vesicles Which Carry the Viral Replication Complex. J. Virol. 73, 2016 –2026.
33. Pei, Y., Hodgins, D.C., Wu, J., Welch, S.-K.W., Calvert, J.G., Li, G., Du, Y., Song, C., Yoo, D., 2009. Porcine reproductive and respiratory syndrome virus as a vector: immunogenicity of green fluorescent protein and porcine circovirus type 2 capsid expressed from dedicated subgenomic RNAs. Virology 389, 91–99.
34. Pejsak, Z., Stadejek, T., Markowska-Daniel, I., 1997. Clinical signs and economic losses caused by porcine reproductive and respiratory syndrome virus in a large breeding farm. Vet. Microbiol. 55, 317–322.
35. Ropp, S.L., Mahlum Wees, C.E., Fang, Y., Nelson, E.A., Rossow, K.D., Bien, M., Arndt, B., Preszler, S., Steen, P., Christopher-Hennings, J., Collins, J.E., Benfield, D.A., Faaberg, K.S., 2004. Characterization of Emerging European-Like Porcine Reproductive and Respiratory Syndrome Virus Isolates in the United States. J. Virol. 78, 3684–3703.
36. Snijder, E.J., Meulenberg, J.J., 1998. The molecular biology of arteriviruses. J. Gen. Virol. 79 (Pt 5), 961–979.
37. Van Vugt, J.J., Storgaard, T., Oleksiewicz, M.B., Bøtner, A., 2001. High frequency RNA recombination in porcine reproductive and respiratory syndrome virus occurs preferentially between parental sequences with high similarity. J. Gen. Virol. 82, 2615–2620.
38. Welch, S.-K.W., Jolie, R., Pearce, D.S., Koertje, W.D., Fuog, E., Shields, S.L., Yoo, D., Calvert, J.G., 2004. Construction and evaluation of genetically engineered replication-defective porcine reproductive and respiratory syndrome virus vaccine candidates. Vet. Immunol. Immunopathol. 102, 277–290.
39. Wensvoort, G., Terpstra, C., Pol, J.M., ter Laak, E.A., Bloemraad, M., de Kluyver, E.P., Kragten, C., van Buiten, L., den Besten, A., Wagenaar, F., 1991. Mystery swine disease in The Netherlands: the isolation of Lelystad virus. Vet. Q. 13, 121–130.
40. Worobey, M., Holmes, E.C., 1999. Evolutionary aspects of recombination in RNA viruses. J. Gen. Virol. 80 (Pt 10), 2535–2543.
41. Yuan, S., Nelsen, C.J., Murtaugh, M.P., Schmitt, B.J., Faaberg, K.S., 1999. Recombination between North American strains of porcine reproductive and respiratory syndrome virus. Virus Res. 61, 87–98.

Figure 3-1 PRRSV constructs used in this study.

A. PRRSV P129 strain is the parent virus used to create the constructs shown below. This schematic representation shows the position of foreign gene (GFP, DsRed, EGFP-97C, EGFP, GFPm) to be between non-structural (ORF1) and structural (ORFs2-7) regions of the virus genome. The V-shaped line indicates a deletion in that region. B. White lines indicate nucleotide mismatches between GFP or GFPm with EGFP gene.

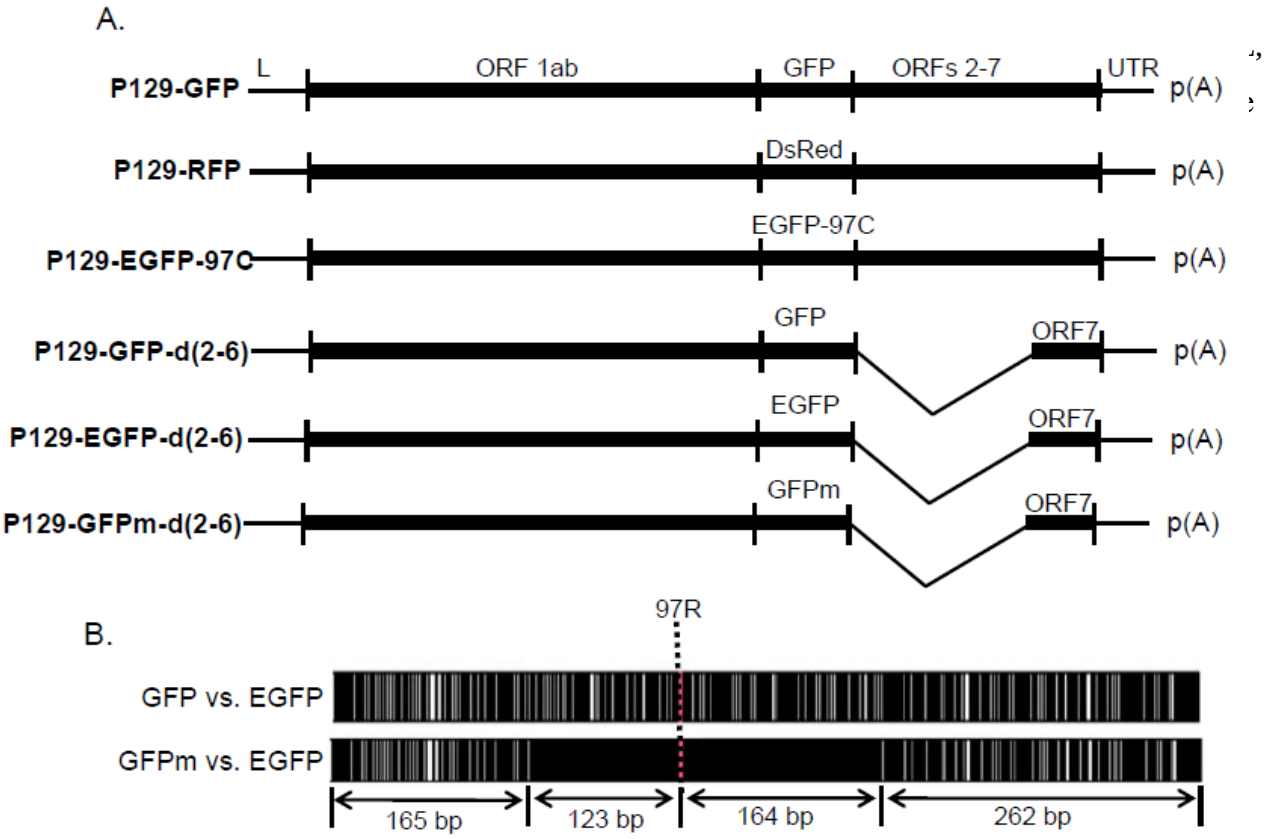


Figure 3-2 Growth curves for P129-GFP and P129-RFP viruses.

Panel A shows the schematic representation of the two infectious clones used for this experiement. Panel B shows the growth curves. Growth of P129-GFP (open circles) and P129-RFP (closed circles) viruses was performed on MARC-145 cells. Viruses were prepared by transfection of HEK cells with plasmid cDNA followed by 6 passages on MARC-145 cells.

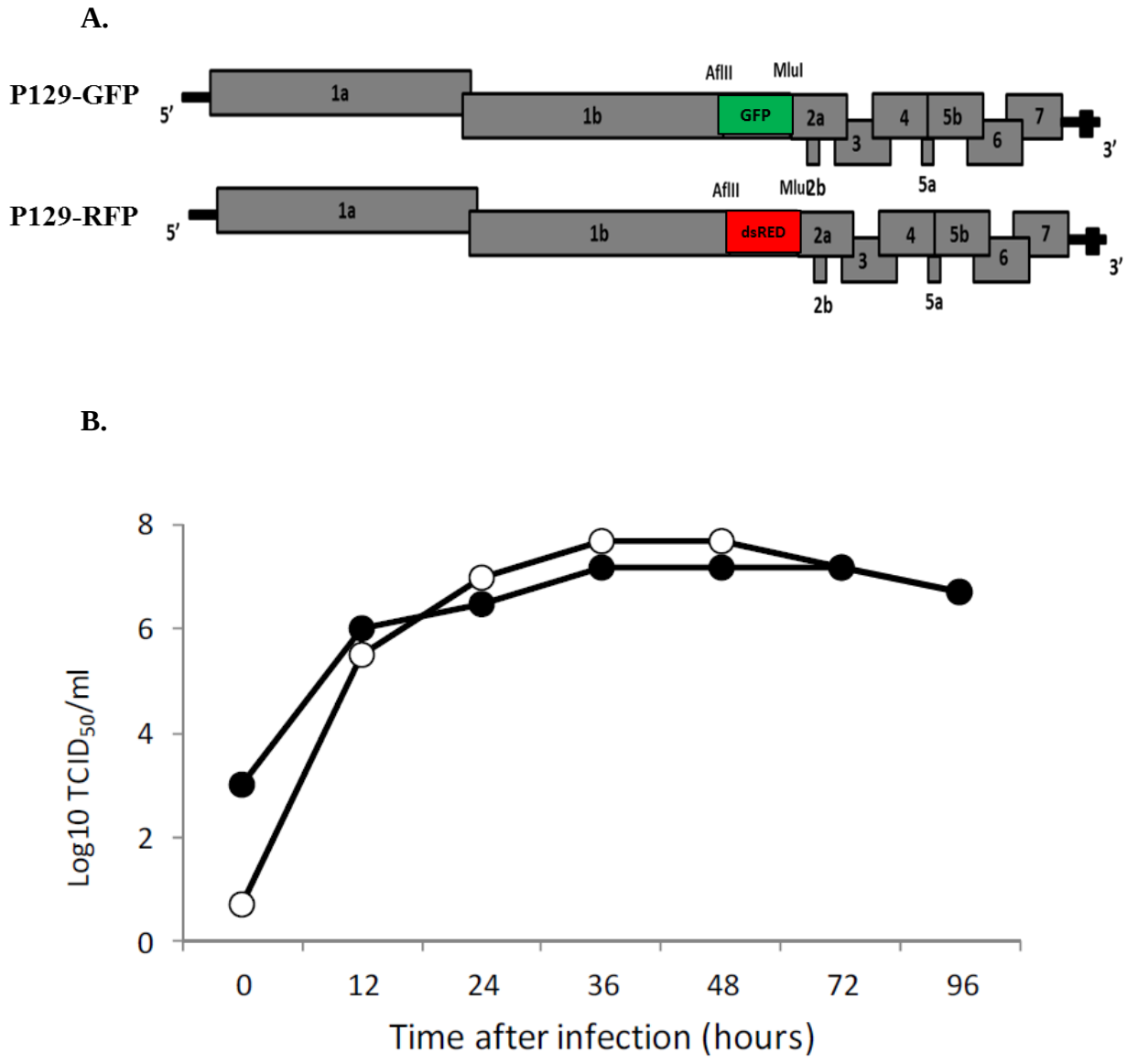


Figure 3-3 Co-infection/transfection of cells with P129-GFP and P129-RFP viruses/plasmids.

Panel A shows HEK cells co-transfected with equal quantities of P129-GFP and P129-RFP plasmids. The amount of each plasmid used for transfection was 0.27 µg . Panel B shows a single MARC cell at 48 hrs after co-infection with equal amounts of P129-RFP and P129-GFP viruses on MARC cells. Panel C shows a representative PAM cell at 24 hrs. after co-infection.

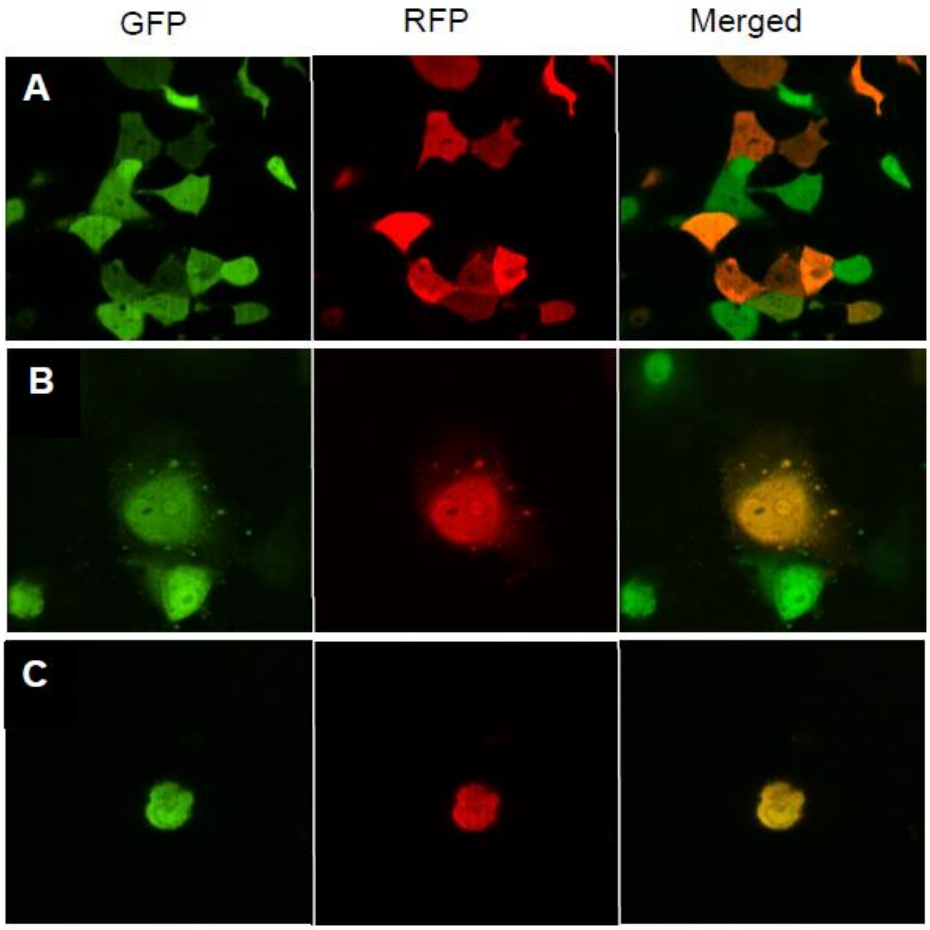


Figure 3-4 P129-GFP and P129-RFP expression in MARC-145 cells.

Top panels show infection of MARC cells by individual viruses. Bottom panels show co-infection of MARC cells using different ratios of viruses. The numbers are the percentage of cells in each quadrant. Data were collected at 48 hrs after infecting cells with an MOI of 10.

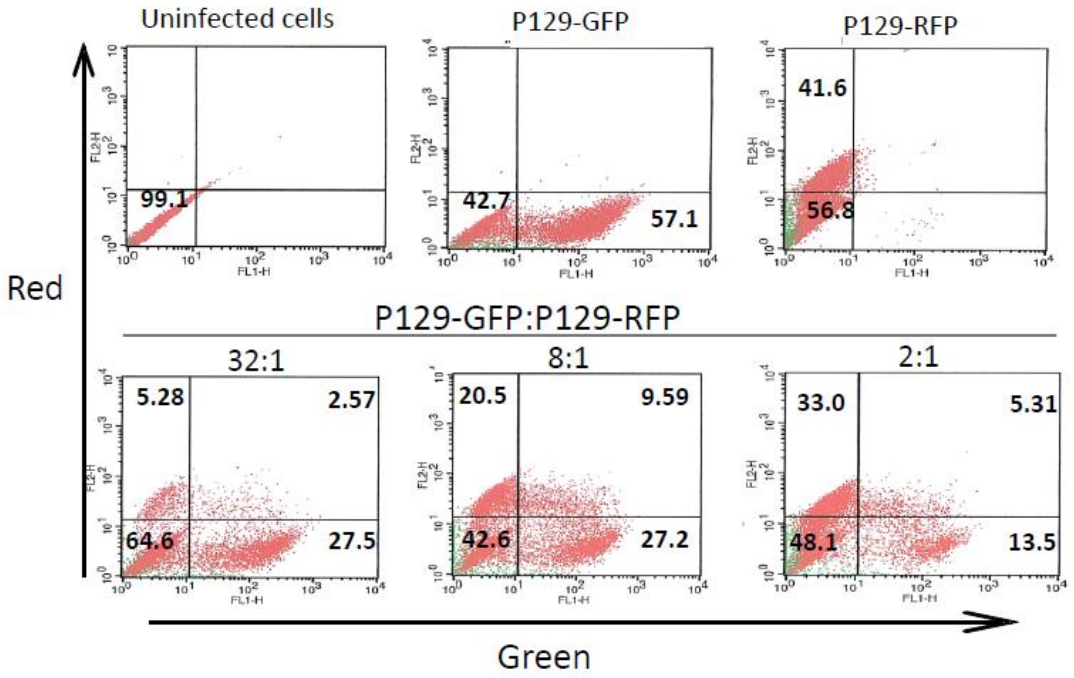


Figure 3-5 Experimental design for traditional PCR method used for PRRSV recombination

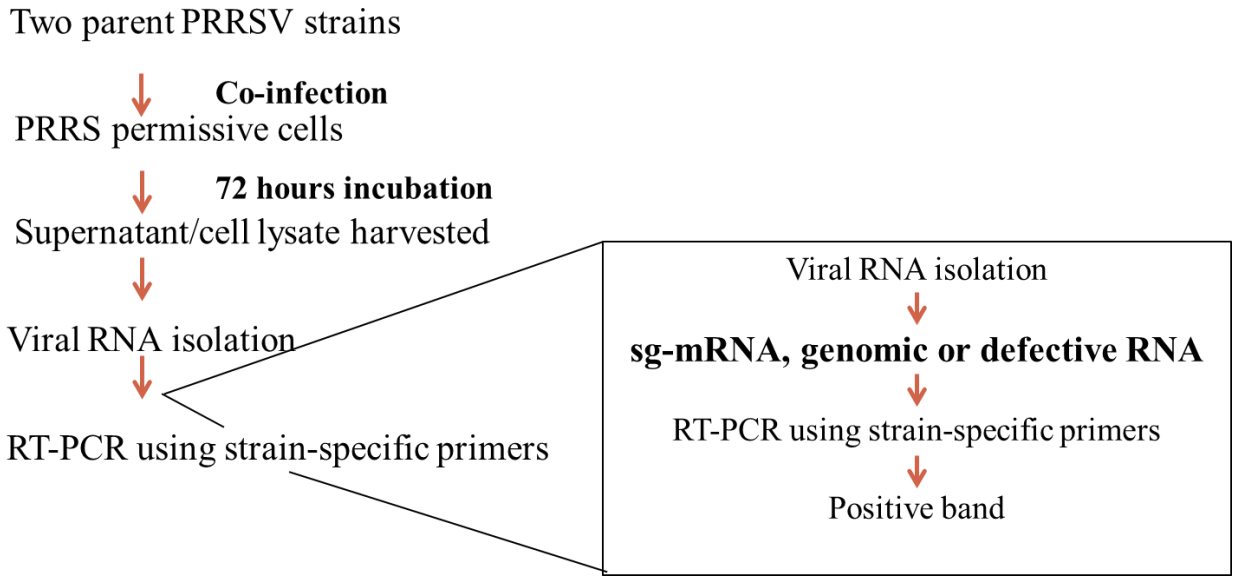
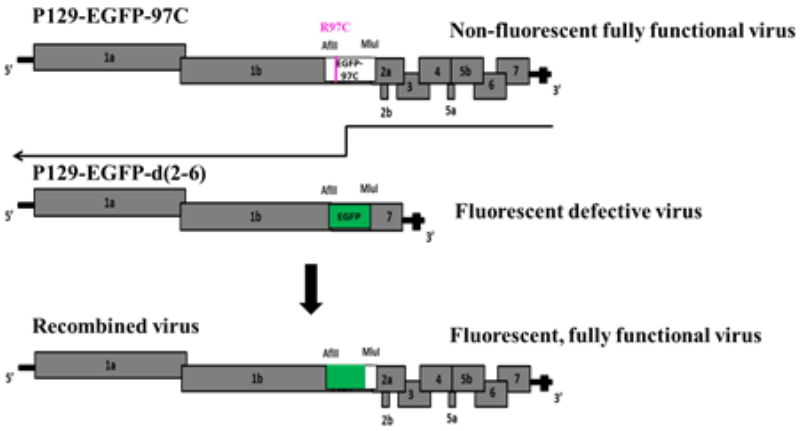


Figure 3-6 Study design: *in-vitro* model for recombination

A. Approach for new *in-vitro* model



B. Experimental design for new *in-vitro* model

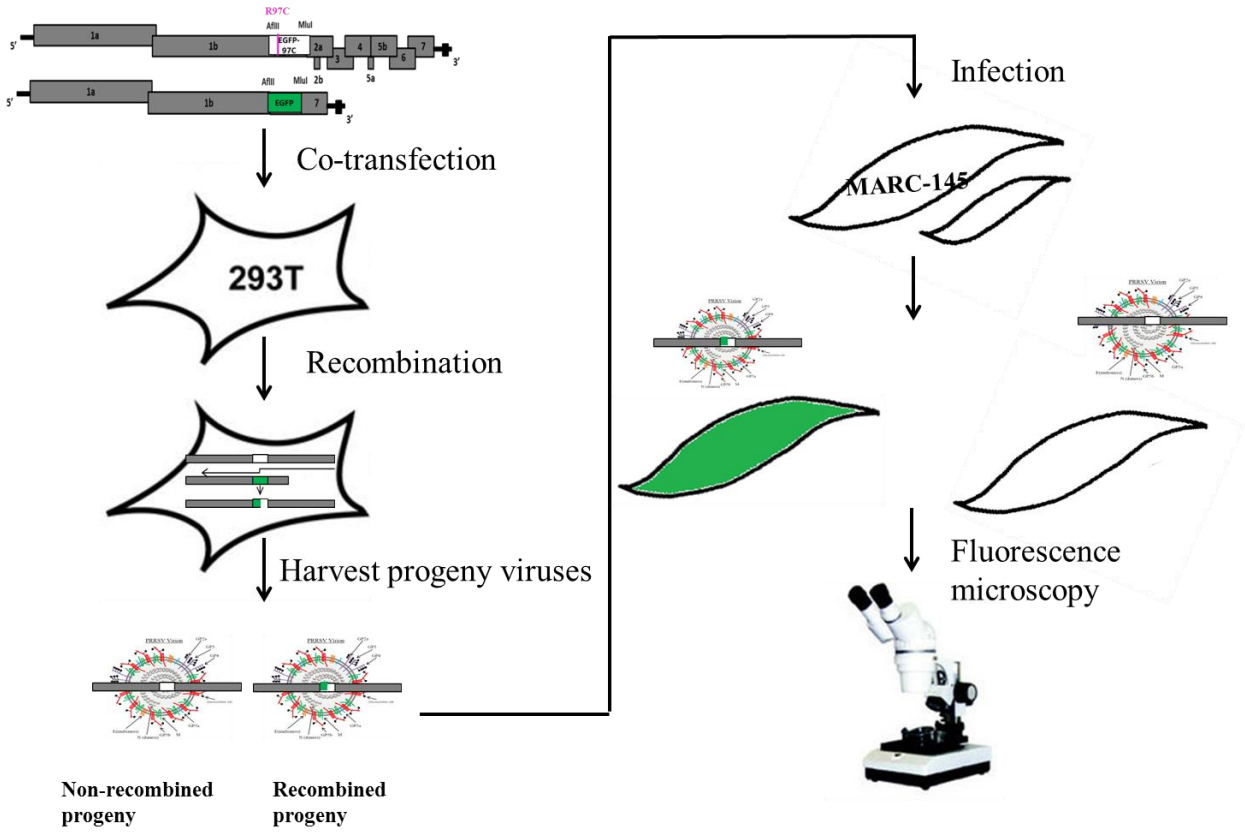


Figure 3-7 Recombination within EGFP during negative or positive strand synthesis.
The figure shows a single recombination event within a 426 nucleotide region, which will replace the 97 cysteine mutation with arginine to yield a full-length fluorescent virus. Recombination occurs in HEK cells transfected with defective and full-length PRRSV infectious clone plasmids. The supernatant is used to infect MARC-145 cells. Successful recombination is detected by the presence of green fluorescence.

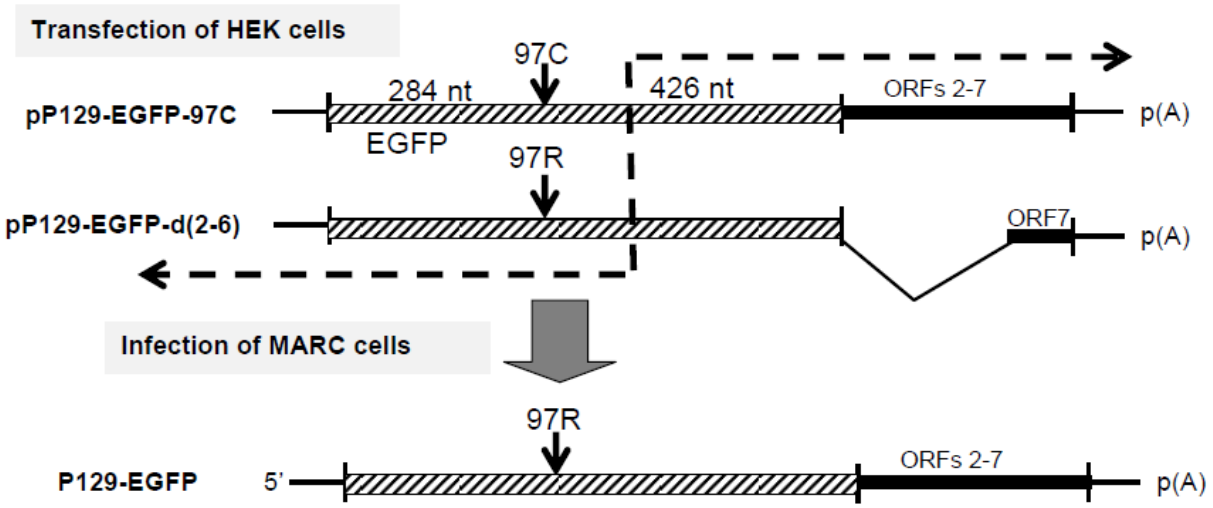


Figure 3-8 Recombination between P129-EGFP-97C and P129-EGFP-d(2-6).

Plasmids were co-transfected into HEK cells. At 48 hrs after transfection, the supernatants were transferred to MARC cells. Cells were stained for N protein at 48 hr after transfection/infection.

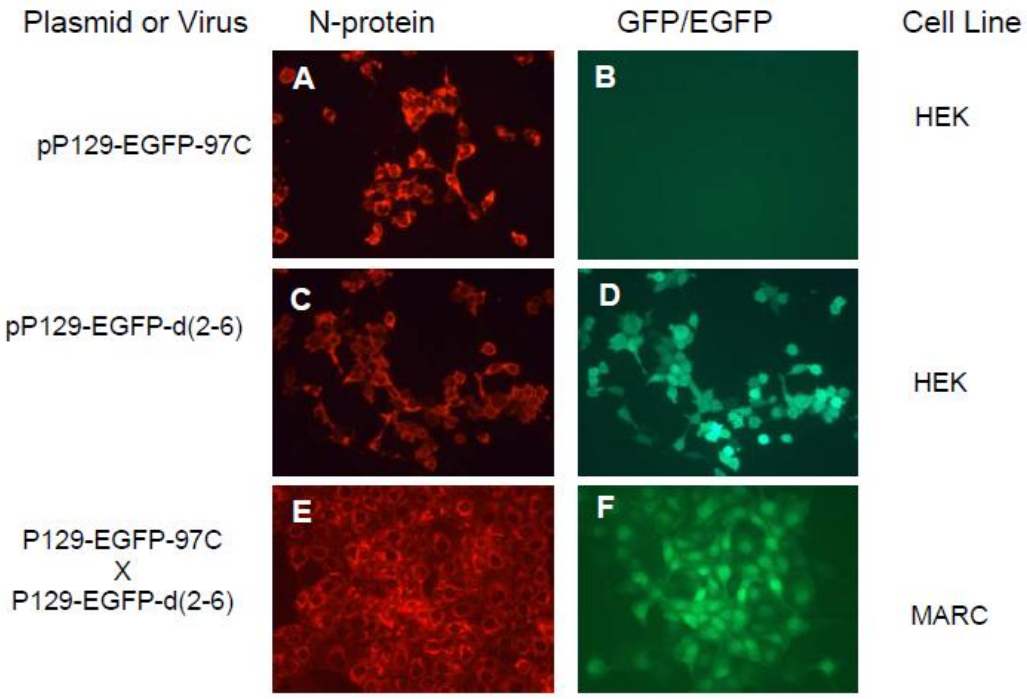


Figure 3-9 Recombination between P129-EGFP-97C, P129-GFP-d(2-6) and P129-GFPm-d(2-6).

Individual plasmids were transfected into HEK cells and 48 hr after transfection, media transferred to MARC-145 cells. Cells were fixed and stained for the presence of N protein at 48 hr after transfection or infection.

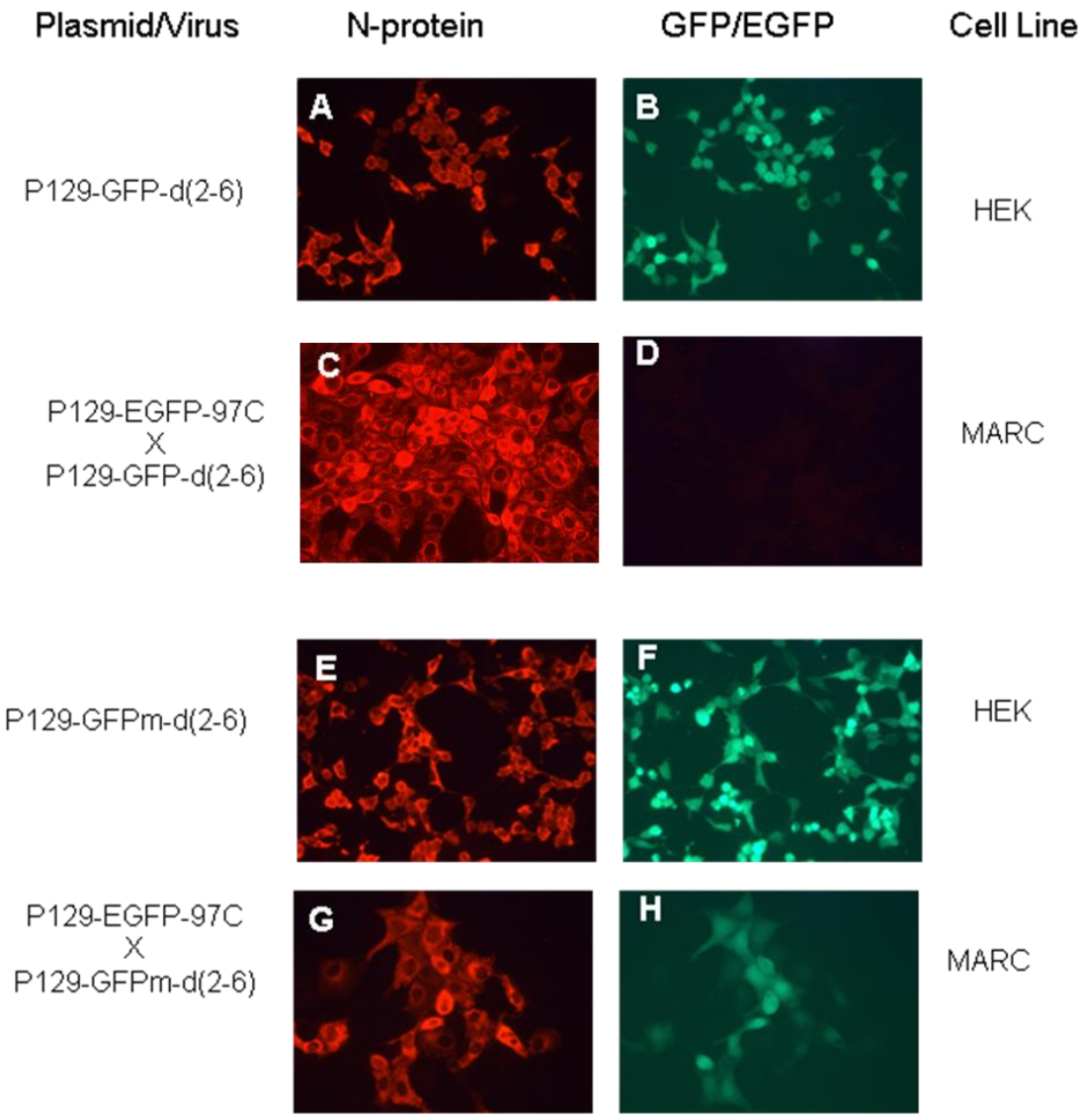


Figure 3-10 Single crossover event between P129-EGFP-97C and P129-GFPm-d(2-6) to yield a fluorescent virus.

The upper portion of the figure shows sequences for the parent viruses: EGFP-97C, GFPm and the recombined virus. The shaded region identifies the site of recombination. The lower figure shows the location of the recombination site.

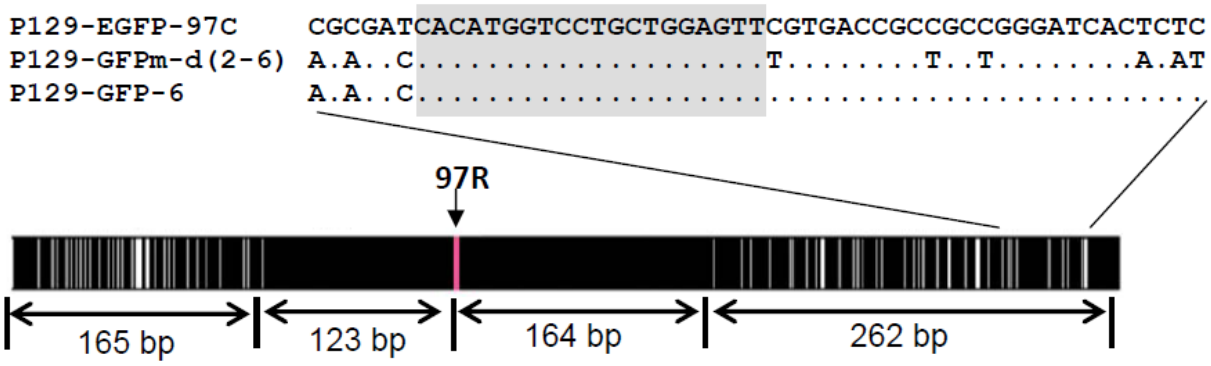


Figure 3-11 Control experiments for the dual transfection and infection studies for recombination performed in this study.

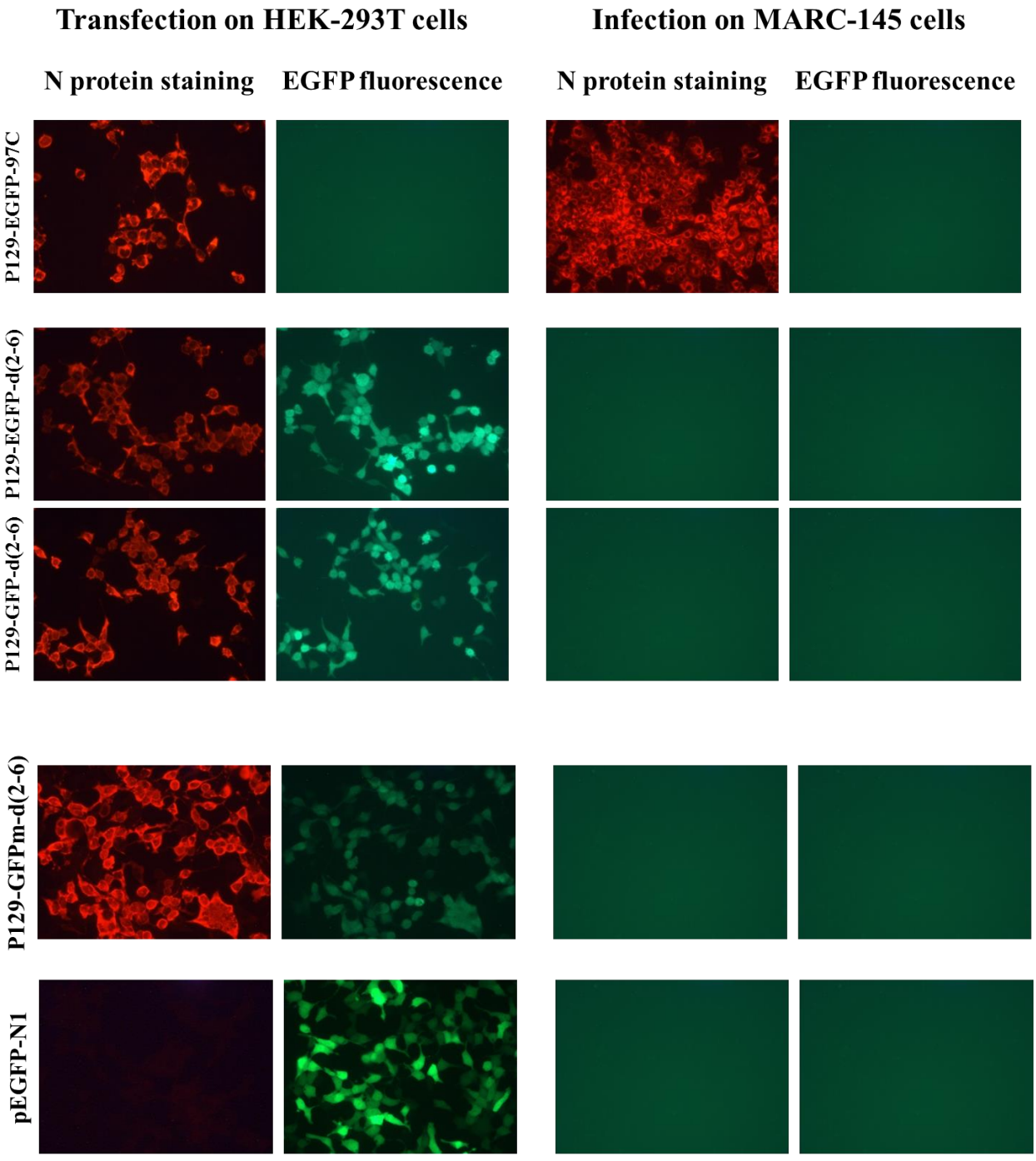


Figure 3-12 Sequence comparison of marker region in parent and recombined viruses

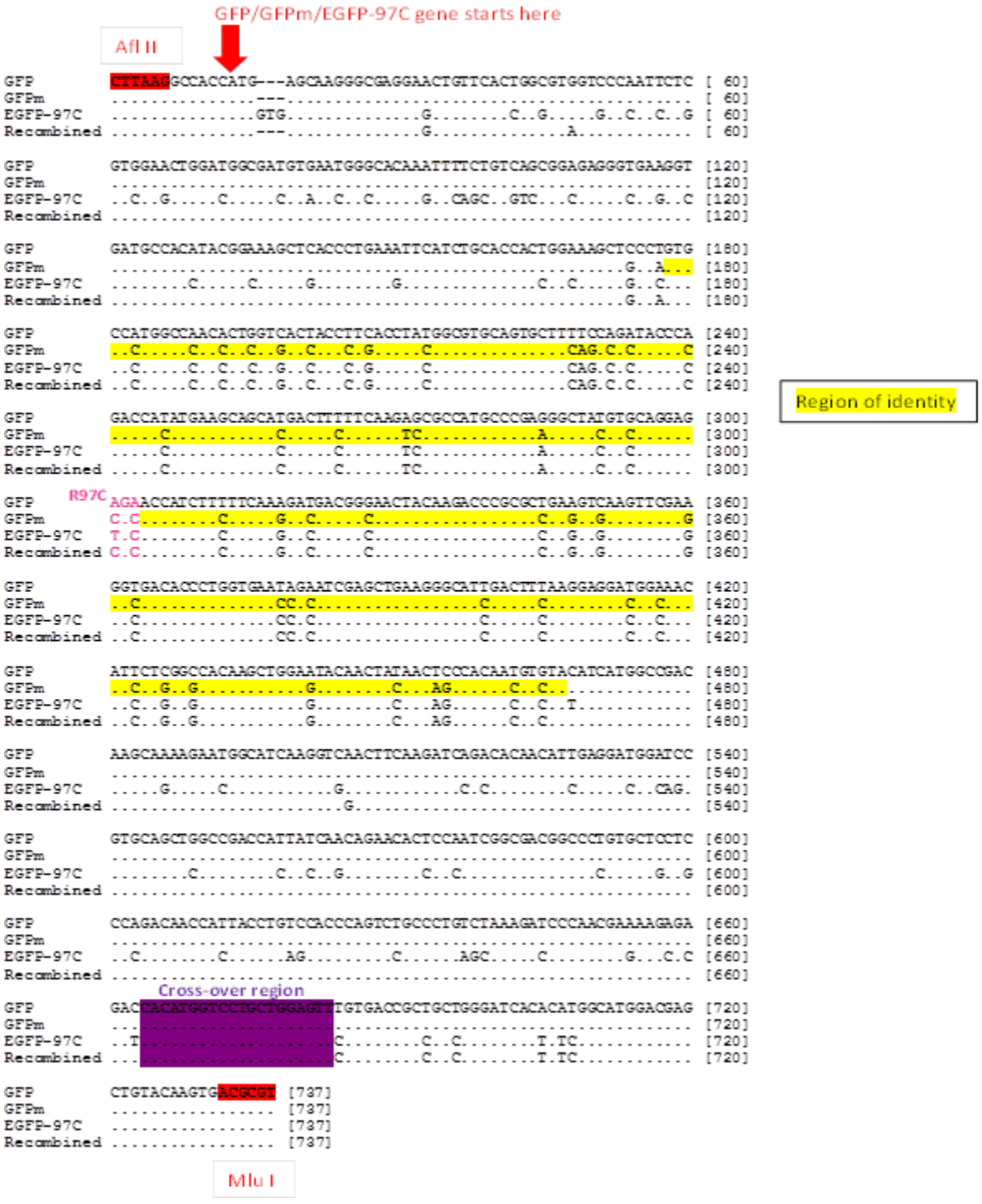


Table 3-1 Frequency of recombinant viruses produced after co-transfection

Sample with recombined viruses was titrated (serial 1:10) on MARC cells

↓

End-points: CPE for Parent virus and fluorescence for Recombined virus

↓

$TCID_{50}$ per ml calculated by Spearman-Karber method

↓

$$\text{Recombination Frequency} = \frac{TCID_{50}/\text{ml of Recombined Virus}}{TCID_{50}/\text{ml of Parent Virus}} \times 100\%$$

↓

Virus	$TCID_{50}$ per ml
Parent EGFP-97C (non-green)	1.5×10^5
Recombined virus (green)	5×10^2

Recombination frequency is 0.3%. This is in the range of previous studies (0.1-10%)

Chapter 4 - Conclusion and application

PRRS has become one of the most challenging diseases to the global swine industry from the time of its initial identification in the early 1990s. Extensive research and elimination programs have been undertaken to control the spread and losses caused by PRRSV. During the last 23 years, our understanding of this disease has increased tremendously in the areas of the virus biology, the factors that influence immunity and the genetic heterogeneity of the virus. Efficacies of the modified live and inactivated vaccines have not been found to be promising for control of the disease. Novel vaccine strategies are being developed to cope with the increasing economic burden caused by this disease globally (Huang and Meng, 2010). The vast genetic and antigenic heterogeneity in PRRSV are observed in the inter-genotype, intra-genotype and quasispecies diversity that have a major influence on vaccine efficacy. Point mutations and recombination drive the rapid divergence of this virus. Recombination is a less investigated source for genetic diversity in PRRSV. However, recombination results in greater evolutionary jumps as compared to point mutations. The research presented in this dissertation focused on understanding the molecular and cellular features of PRRSV recombination.

Recombination in positive stranded RNA viruses has been most widely accepted to occur by a template-switch model (Lai, 1992, p. 199). The concepts of this model and several structural signals that influence this mechanism have been studied for RNA viruses that infect animals, plants and bacteria. These aspects have been discussed in detail in Chapter 2. A detailed literature review about recombination in PRRS virus showed that there are currently two methods to study recombination:

(i) An *in-vitro* method that uses differential PCR amplification followed by sequencing of these amplicons to detect cross-over.

(ii) An *in-silico* method that uses a range of different bioinformatic tools to analyze sequence datasets to detect recombination hotspots, cross-over patterns, and influence of nucleotide sequence and RNA secondary structure on recombination. The sequences used in these analyses are either from isolated viruses or PCR amplification products.

As discussed in Chapter 2 there are several constraints for a recombination event to result in a biologically relevant recombined virus. However there are reports that indicate recombination to be responsible for emergence of a new Western Equine encephalitis virus (Hahn et al., 1988) and for large-scale Chinese PRRSV outbreaks (Shi et al., 2013). Therefore while studying recombination it is important to focus on features of recombination events that result in viable viruses that are 'fit' to compete with the existing strains of parent viruses. In other words, detection of viable virus is important because such an observation would provide substantial amount of information regarding occurrence and significance of recombination events. The most critical limitation of currently available PCR detection methods is the lack of knowledge regarding the viability of the virus carrying the recombined region. This is because PCR amplification can use genomic, subgenomic or defective RNA strands as templates. Therefore the primary objective of this research was to develop a simple *in-vitro* model system that would generate viable recombined viruses. To demonstrate the usefulness of this system, recombination frequency was compared to previous data in literature. This *in-vitro* system creates quantitative estimates of parameters to predict the occurrence of recombination. To demonstrate this property the system was applied to elucidate how the degree of nucleotide sequence homology influences cross-over. Another application of this system was made to gain insight in to the localization of recombination event in a PRRSV infected cell.

Future applications of the *in-vitro* system

The experiments in this project showed that GFPm gene was synthesized and cross-over was studied in a specific 164 bp region. In a similar manner, a number of modifications involving synonymous and non-synonymous mutations can be made in EGFP/GFP genes to study influence of various nucleotide features on the mechanism of cross-over. The incorporation of GFP gene as the target for recombination creates the opportunity to easily make mutations in this gene as it is non-essential for virus replication. One of the important features of this system is the absence of selection pressure. This will help simulate recombination events in the absence of artificial selective conditions.

One of the implications in a previous study was that recombination is favored when short homologous stretches are flanked by longer regions of high degree of identity (van Vugt et al., 2001). The copy-choice mechanism proposed by Lai et al., 1991 also suggests that longer stretches of sequence alignment would enable the nascent RNA transcript to bind and orient the RNA polymerase complex to continue synthesis after switching templates (Lai, 1992). Our study also showed that increasing the length of identical stretches promotes recombination. Similarly the effect of flanking region homology on recombination can be determined by sequentially incorporating ORF2 to ORF5 in the infectious clone P129-EGFP-d(2-6). The influence of the increased flanking region homology should be evident on the measured recombination frequency. Variable rates of recombination between the 5' and 3' proximal regions were observed in case of Equine Arteritis Virus (EAV). It was demonstrated that the 5' end of the genome had about 100-fold lower recombination rate as compared with the 3' structural region (Molenkamp et al., 2000a). Similarly, the recombination frequency in the spike gene in the coronavirus MHV was three times greater than that in the replicase gene (Fu and Baric, 1992). A similar analysis can be performed for the PRRSV genome using the *in-vitro* system. For this purpose recombination in the non-structural (ORF1) and structural (ORF2-7) regions can be compared. Two different studies have shown the occurrence of recombination in Nsp2 region of ORF1a which is at the 5' end of the genome. In the in-vivo study done by Dan Liu et al 2011 about 27% recombinants were found to have cross-over in the nsp2 region and 14 different cross-over sites were detected. Phylogenetic analysis of the field strain Em2007 from China showed that this strain was a recombinant between a field and vaccine strain with 2 cross-over sites in Nsp2 region (Li et al., 2009a). Nsp2 is of interest in the study of virus evolution and pathogenesis (Han et al., 2006; Shen et al., 2000) as it is the largest non-structural protein and is multi-functional in nature. Nsp2 has a N-terminal cysteine protease domain that is essential for the processing of pp1a encoded by ORF 1, possesses de-ubiquitinating and immunomodulatory functions (Fang and Snijder, 2010). However, despite the critical functional roles of this protein it is the most variable region in the entire PRRSV genome. Natural deletions and insertions are frequently observed in this region. Insertion of EGFP gene in the Nsp2 region in the defective virus and EGFP-97C gene in the full length infectious virus will give insight into the occurrence of recombination in the Nsp2 region. Comparison of the frequency of recombination in this experiment and with EGFP inserted between ORF1 and ORF2 would give an estimate of

frequency of recombination at the 5' and 3' end of the PRRSV genome. The in-vitro system will not only improve our basic understanding of PRRS virus biology but also can be applied to investigate other RNA viruses.

Several studies have shown strong evidence of RNA structure to influence the location of recombination hotspots and recombination frequency (Dedepsidis et al., 2010; Simon-Loriere et al., 2010). The *in-vitro* system developed in this research can be used to study this aspect of recombination. Comparison of maps of RNA secondary structure of EGFP, GFP and GFPm could possibly contribute to some insights regarding the preference of 3' 20 bp region for cross-over. The *in-vitro* system will enable predictions for successful recombination events based on a better understanding of nucleotide sequence features that influence cross-over. These predictions include recombination between field viruses, between genotypes and, between field and vaccine strains. In case of DIVA (differentiating infected from vaccinated animals) vaccine strategy, this system can be applied to study if DIVA markers will be eliminated from the vaccine strain or acquired by the field strains by recombination. Recombination in the DIVA marker region is a significant problem because it can lead to failure of this vaccination approach. Isolation of viable recombined viruses resulting from cross-over events between vaccine and field strain have been detected in a number of studies from Britain and China (Frossard et al., 2013; Li et al., 2009a; Wenhui et al., 2012). MLV vaccine is used extensively in the field for the control of PRRS and hence the potential for recombination with field strains is a significant concern. The infectious clones P129-EGFP-97C and P129-EGFP-d(2-6) can be used as backbone viruses where non-structural and/or structural genes of vaccine strain and particular field strains can be incorporated to predict the chances of recombination among them. Such data will also be useful for designing multi-strain vaccines for PRRSV control. Recombination can lead to genetic shift resulting in viruses with new phenotypic features that may lead to increased pathogenesis. This *in-vitro* system can be applied to predict emergence of novel variants of the virus arising due to such genetic shifts.

Reference

1. Dedepsidis, E., Kyriakopoulou, Z., Pliaka, V., Markoulatos, P., 2010. Correlation between recombination junctions and RNA secondary structure elements in poliovirus Sabin strains. Virus Genes 41, 181–191.
2. Fang, Y., Snijder, E.J., 2010. The PRRSV replicase: exploring the multifunctionality of an intriguing set of nonstructural proteins. Virus Res. 154, 61–76.
3. Frossard, J.-P., Hughes, G.J., Westcott, D.G., Naidu, B., Williamson, S., Woodger, N.G.A., Steinbach, F., Drew, T.W., 2013. Porcine reproductive and respiratory syndrome virus: Genetic diversity of recent British isolates. Vet. Microbiol. 162, 507–518.
4. Fu, K., Baric, R.S., 1992. Evidence for variable rates of recombination in the MHV genome. Virology 189, 88–102.
5. Hahn, C.S., Lustig, S., Strauss, E.G., Strauss, J.H., 1988. Western equine encephalitis virus is a recombinant virus. Proc. Natl. Acad. Sci. 85, 5997–6001.
6. Han, J., Wang, Y., Faaberg, K.S., 2006. Complete genome analysis of RFLP 184 isolates of porcine reproductive and respiratory syndrome virus. Virus Res. 122, 175–182.
7. Huang, Y.W., Meng, X.J., 2010. Novel strategies and approaches to develop the next generation of vaccines against porcine reproductive and respiratory syndrome virus (PRRSV). Virus Res. 154, 141–149.
8. Lai, M.M., 1992. RNA recombination in animal and plant viruses. Microbiol. Rev. 56, 61–79.
9. Li, B., Fang, L., Xu, Z., Liu, S., Gao, J., Jiang, Y., Chen, H., Xiao, S., 2009. Recombination in Vaccine and Circulating Strains of Porcine Reproductive and Respiratory Syndrome Viruses. Emerg. Infect. Dis. 15, 2032–2035.
10. Molenkamp, R., Greve, S., Spaan, W.J., Snijder, E.J., 2000. Efficient homologous RNA recombination and requirement for an open reading frame during replication of equine arteritis virus defective interfering RNAs. J. Virol. 74, 9062–9070.
11. Shen, S., Kwang, J., Liu, W., Liu, D.X., 2000. Determination of the complete nucleotide sequence of a vaccine strain of porcine reproductive and respiratory syndrome virus and identification of the Nsp2 gene with a unique insertion. Arch. Virol. 145, 871–883.
12. Shi, M., Holmes, E.C., Brar, M.S., Leung, F.C.-C., 2013. Recombination Is Associated with an Outbreak of Novel Highly Pathogenic Porcine Reproductive and Respiratory Syndrome Viruses in China. J. Virol. 87, 10904–10907.
13. Simon-Loriere, E., Martin, D.P., Weeks, K.M., Negroni, M., 2010. RNA Structures Facilitate Recombination-Mediated Gene Swapping in HIV-1. J. Virol. 84, 12675–12682.
14. Van Vugt, J.J., Storgaard, T., Oleksiewicz, M.B., Bøtner, A., 2001. High frequency RNA recombination in porcine reproductive and respiratory syndrome virus occurs preferentially between parental sequences with high similarity. J. Gen. Virol. 82, 2615–2620.
15. Wenhui, L., Zhongyan, W., Guanqun, Z., Zhili, L., JingYun, M., Qingmei, X., Baoli, S., Yingzuo, B., 2012. Complete Genome Sequence of a Novel Variant Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) Strain: Evidence for Recombination between Vaccine and Wild-Type PRRSV Strains. J. Virol. 86, 9543–9543.