

ABSTRACT

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OLIGOMERS

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Porcine reproductive and respiratory syndrome (PRRS) has caused heavily economic losses in the swine industry worldwide and current strategies to control PRRS are inadequate. Previous studies have shown that antisense peptide-conjugated phosphorodiamidate morpholino oligomers (PPMO) can be effective antivirals against PRRS virus (PRRSV). PPMO are structurally similar to DNA with modified backbone and resistant to nuclease. In this study, we characterized the combined effect of PPMO. Two pairs of PPMO combinations were identified to show enhanced inhibitory effect on PRRSV replication in cell culture. The PPMO combination also inhibited replication of heterologous PRRSV strains within the same genotype. Treatment of the cells with the combination reduced PRRSV RNA and protein levels. In transiently transfected cells, the PPMO combination suppressed target mRNA expression, indicating that the suppression was due to their antisense effect. These results suggest potential application of these PPMO combinations for the control of PRRSV infection and spread.

INHIBITION OF PRRSV REPLICATION BY COMBINATION OF ANTISENSE
MORPHOLINO OLIGOMERS

By

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS	ii
TABLE OF CONTENTS.....	iii
LIST OF TABLES.....	v
LIST OF FIGURES.....	vi
LIST OF ABBREVIATION.....	vii
CHAPTER 1: LITERATURE REVIEW.....	1
1.1 Porcine Reproductive and Respiratory Syndrome (PRRS).....	1
1.1.1 Introduction.....	1
1.1.2 Clinical Symptoms.....	2
1.1.3 Pathogenesis.....	4
1.2 Porcine Reproductive and Respiratory Syndrome Virus (PRRSV).....	5
1.2.1 PRRSV Introduction.....	5
1.2.2 Genome Structure, Expression and Replication.....	6
1.2.3 PRRSV Growth Characteristics In Vitro.....	9
1.3 Immune Response to PRRSV Infection.....	12
1.3.1 Humoral Immune Response.....	12
1.3.2 Cellular Mediated Immune Response(CMI).....	15
1.4 PRRSV Antigenic and Genetic Variations.....	16
1.4.1 Antigenic Variations.....	16
1.4.2 Genetic Variations	17
1.5 Infectious cDNA Clones:Reverse Genetics System.....	18
1.6 Diagnosis, Prevention and Control.....	20
1.6.1 Diagnosis.....	20
1.6.2 Prevention and control.....	21
1.6.3 Vaccination.....	23

1.7	Phosphorodiamidate Morpholino Oligomer (PMO) as A Novel Antiviral Agent.....	25
1.8	Research Objectives.....	28
CHAPTER 2: MATERIALS AND METHODS.....		29
2.1	Cells and Viruses.....	29
2.2	Determination of Virus Titer.....	29
2.3	Indirect Immunofluorescence Assay (IFA).....	30
2.4	PPMO Synthesis.....	31
2.5	PPMO Treatment of CRL11171 Cells.....	31
2.6	RNA Isolation.....	32
2.7	Real-Time RT-PCR(Reverse transcription-PCR).....	33
2.8	Plasmid Construction and Cell-Free Luciferase Reporter Assay.....	34
2.9	Statistical Analysis.....	36
CHAPTER 3: RESULTS.....		37
3.1	Anti-PRRSV PPMO.....	37
3.2	Effect of Combination PPMO Treatment on Inhibiting PRRSV Replication..	41
3.3	Effect of Combination PPMO Treatment on PRRSV RNA Synthesis.....	45
3.4	Effect of PPMO Combination Treatment on PRRSV Protein Synthesis.....	49
3.5	Sequence-specific Inhibition of Target mRNA in a Cell-free reporter assay	51
3.6	PPMO Targeting the PRRSV 5' UTR Can Interference with Translation	53
3.7	Inhibition Effect of PPMO Combination Against Heterologous PRRSV Strains.....	57
CHAPTER 4: DISCUSSION.....		61
CHAPTER 5: REFERENCES		69

LIST OF TABLES

Table 3-1: PPMO and their target sites in PRRSV RNA Genome.....	39
Table 3-2: Sequence-Specific Inhibition of Target RNA Translation In A Cell-Free Luciferase Reporter Assay.....	52
Table 3-3: Sequence-specific inhibition of target mRNA translation of a luciferase reporter in CRL11171 Cells.....	56

LIST OF FIGURES

Figure 1-1: Genome Organization of PRRSV.....	8
Figure 1-2: Immune Response after Infection of Pigs with PRRSV.....	13
Figure 1-3: Structure of PPMO.....	27
Figure 3-1: Schematic Illustration of PPMO Target Locations in the PRRSV Genome.....	40
Figure 3-2: PPMO-mediated inhibition of PRRSV Replication in Infected CRL11171 Cells.....	45
Figure 3-3: Effect of combination of PPMO treatment on PRRSV RNA Synthesis.....	48
Figure 3-4: Immunofluorescence assay with N-specific mAbs.....	50
Figure 3-5: Inhibition of target RNA translation by PPMO in a cell-free luciferase reporter assay.....	52
Figure 3-6: Immunofluorescence assay of cells transfected with PRRSV N-plasmid pcDNA3-sg7.....	54
Figure 3-7: Inhibition of target RNA translation by PPMO in a luciferase reporter assay.....	56
Figure 3-8: Cross Strain Inhibition Assay.....	58
Figure 3-9: Alignment of target sequence of PPMO 5UP1, 4P1 and 7P1.....	59

LIST OF ABBREVIATIONS

PRRS:	Porcine Reproductive and Respiratory Syndrome
PRRSV:	Porcine Reproductive and Respiratory Syndrome Virus
PAM:	Porcine Alveolar Macrophage
PPMO:	Peptide-Conjugated Phosphorodiamidate Morpholino Oligomers
PMO:	Phosphorodiamidate Morpholino Oligomers
EAV:	Equine Arthritis Virus
SHFV:	Simian Hemorrhagic Fever Virus
LDV:	Lactate Dehydrogenase Virus
ORF:	Open Reading Frame
MAbs:	Monoclonal Antibodies
NSP:	Nonstructural Protein
ELISA:	Enzyme Linked-Immunosorbent Assay
LV:	Lelystad Virus
GP:	Glycoprotein
RT-PCR:	Reverse Transcriptase-Polymerase Chain Reaction.
UTR:	Untranslated Region
IFA:	Immunofluorescence Assay
TRS:	Transcription Regulatory Sequence
CMI:	Cell-Mediated Immune Response

CHAPTER 1: LITERATURE REVIEW

1.1 Porcine Reproductive and Respiratory Syndrome (PRRS)

1.1.1 Introduction

Porcine reproductive and respiratory syndrome (PRRS) is recognized as one of the most economically important diseases in the swine industry worldwide. The disease is characterized by severe reproductive failure in sows and respiratory disease in young pigs. A clinical outbreak of PRRS was first reported in the United States in 1987 (Keffaber 1989), and subsequently in Europe in 1990 (Wensvoort et al 1991). Initially, due to the unknown etiologic agent, the disease was also referred to as mystery swine disease, swine infertility and respiratory syndrome, porcine epidemic abortion and respiratory syndrome, and blue-eared pig disease (Wensvoort et al., 1991; Terpstra et al., 1991; White M. 1991).

The etiologic agent of PRRS was first isolated on porcine alveolar macrophages by Wensvoort et al in Europe in 1991 and this isolate was designated as Lelystad virus (LV) (Wensvoort et al 1991). In 1992, a similar virus was isolated on an MA104 - derived cell line (CL 2621) by Collins et al in the U.S. and the isolate was ATCC VR-2332, which was shown to reproduce the disease in pigs (Collins et al., 1992; Christopher-Henning et al., 1995). The high transmissibility of PRRS virus (PRRSV) made the disease spread quickly around the world. In 1994, the disease and isolation of PRRSV was officially recognized in 16 countries on three different continents: America,

Europe, and Asia (Nelsen et al., 1999). Currently, only Australia, New Zealand, and Chile are reportedly free from PRRSV infection (Beltran-Alcrudo et al, 2007). The most recent outbreaks occurred in Vietnam and China (Beltran-Alcrudo et al, 2007). In June 2006, a “porcine high fever disease” was reported in China and the disease quickly spread to more than 25 provinces. In early 2007, the causative agent of the ongoing “porcine high fever syndrome” was considered to be an atypical PRRS epidemic. Unlike other previous outbreaks, the PRRSV isolates in China were highly virulent and caused high mortality in adult pigs and pregnant sows (Tian et al, 2007). The PRRS epidemic in China has led to devastating economic impact and has increased pork prices by as much as 85% in that country (Beltran-Alcrudo et al, 2007). The PRRS virus continuously evolves within infected pigs, a characteristic which has important implications for disease transmission, viral virulence, and host immunity among the infected herds. PRRS is the most common and economically important infectious disease in the swine industry worldwide (Albina et al., 1997). In the United States, PRRS is estimated to cause about \$560 million losses per year to the swine industry (Neumann et al 2005).

1.1.2 Clinical Symptoms

PRRSV is highly contagious and causes persistent infection in affected herds worldwide. Pigs are the only species known to be naturally susceptible to PRRSV infection. PRRSV can infect susceptible pigs by a number of routes, including oral, intranasal, intramuscular, intraperitoneal and vaginal (Goyal SM 1993; Prieto et al., 2005). The typical incubation period ranges from days to weeks in natural outbreaks (Rossow at al., 1994; Zimmerman et al., 2006). The clinical illnesses and mortality vary

widely depending on the age, susceptibility of pigs, and virulence of the causative strain of PRRSV (Mengling et al., 1996). The most common clinical signs are reproductive failure of sows and respiratory illness in pigs of all ages. Typical clinical signs of PRRS in pregnant sows include premature farrowing, abortions, increased number of stillborn, mummified and weak-born pigs, and increase in preweaning mortality (Wensvoort et al., 1991; Benfield et al., 1992). Typical clinical signs in young piglets include respiratory disorders, anorexia, lethargy, cutaneous hyperemia, and increased mortality as a result of secondary infections and retarded growth (Wensvoort et al 1991; Wensvoort et al., 1992). Acute PRRSV infection with viremia usually lasts for 4 to 6 weeks, followed by a persistent period in lymphoid tissues that can last for several months before complete resolution of infection (Allende et al., 2000; Johnson et al., 2004; Wills et al., 1997).

The ability to induce persistent infection by PRRSV is an important epidemiological characteristic of PRRSV infection. The virus could persist in the herd for a long time (Stevenson et al., 1993; Bilodeau et al., 1994). Pigs infected with PRRSV can shed and excrete virus via semen, saliva, urine, feces, and nasal and mammary gland secretions for relatively long periods of time (Christopher-Hennings et al., 2001). PRRSV has been detected or isolated in serum up to 210-days post infection (DPI) and semen up to 92 DPI (Swenson et al., 1994; Wills et al, 1997). The PRRSV persistence and virus shedding in infected herds, together with the high degree of antigenic, genetic and pathogenic diversity adds to the complexity of controlling and eliminating this disease in the swine industry worldwide (Bilodeau et al., 1994; Meng et al., 2000).

1.1.3 Pathogenesis

PRRSV infection in pigs consists of an acute infection with viremia lasting for approximately 1 month, followed by a persistent infection of secondary lymphoid tissues that lasts for several months (Murtaugh et al., 2002). The main target for PRRSV infection *in vivo* is porcine alveolar macrophages (PAM) and the virus also replicates in macrophages of different tissues (Collins et al., 1992; Rossow et al., 1995; Sur et al., 1997; Rossow et al., 1998). In PRRSV infection of pigs, alveolar macrophages are the primary target in acute infection, but the virus is also found in macrophages throughout the body, including secondary lymphoid tissues (Lawson et al., 1997). The virus initiates an infection in pigs via entry through nasal epithelial cells, tonsil cells, and alveolar macrophages. PRRSV replicates in these cells, causes viremia, and subsequent rhinitis, pneumonia, myocarditis, vasculitis, encephalitis and lymphadenopathy (Rossow et al., 1996). The virus infects all parts of its host animal, including muscles, blood, organs, and lymphoid tissues (Rossow et al., 1995; Rossow 1996; Rossow et al., 1998). Using *in situ* hybridization and immunocytochemistry, the virus antigen and RNA has been detected in macrophages of multiple tissues, monocytes, endothelial cells, muscle cells, and fibroblast cells (Sur et al., 1997). The virus spreads locally from the initially infected macrophage cells to adjacent cells and its systemic spread occurs through blood and lymphoid fluid (Rossow 1998). PRRSV can cause persistent infection in pigs (Albina et al., 1994; Christopher-Hennings et al 1995). Pigs persistently infected with PRRSV can transmit the virus to naïve pigs by direct or indirect contact (Albina et al., 1994, Christopher-Hennings 2001). Since macrophages are the main antigen presenting cells,

PRRSV replication in macrophages exacerbates the pig's susceptibility to other etiological agents, and reduces the pig's ability to respond to vaccination (Done and Paton, 1999).

1.2 Porcine Reproductive and Respiratory Syndrome Virus (PRRSV)

1.2.1 PRRSV Introduction

PRRSV is a small, enveloped RNA virus with a particle size ranging from 50 to 80 nm (Conzelmann et al., 1993; Meulenbergh et al., 1993a). PRRSV is classified as a member of the family *Arteriviridae* (Meulenbergh et al., 1993a) based on its genome properties, morphology, and virus replication strategy. Viruses in this family also include equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV) of mice, and simian hemorrhagic fever virus (SHFV) (Plagemann et al., 1992; Meulenbergh et al., 1994). PRRSV isolates are more closely related to LDV than to EAV (Meng et al., 1995; Meulenbergh et al., 1993b). One primary feature of viruses in this family is their replication in macrophages, persistent infections in their natural host, and constant antigenic variation in field isolates (Rossow KD, 1998).

The antigenic variation among PRRSV isolates was initially demonstrated between Europe and North American isolates using polyclonal antisera (Nelson et al., 1993) and monoclonal antibodies (Nelson et al., 1993; Drew et al., 1995; Dea et al., 1996; Magar et al., 1997; Zhang et al., 1998). Genomic sequence analyses of the PRRSV isolates from different regions worldwide indicate the existence of two distinct genotypes,

type I European (the prototype is Lelystad virus) and Type II North American (the prototype is ATCC VR-2332) (Meng et al., 1994; Mardassi et al., 1994; Meng et al., 1995). North American and European isolates were found to have about 67% homology at nucleotide sequence level (Meng et al., 1995). Sequence analysis of the isolates from the recent PRRS outbreaks in China indicated that the highly virulent variants belong to the North American genotype but have 30 amino acids deletion in the NSP2 (Tian et al., 2007).

1.2.2 Genome Structure, Expression and Replication

Like other members in *Arteriviridae* family, PRRSV contains a linear, positive-sense, and single-stranded RNA genome. For viruses with a positive-sense viral RNA (+vRNA), +vRNA serve as both genomic RNA and messenger RNA for expression of viral proteins. Thus, the reproduction of the Arterivirus genomic RNA is a combined process of genome replication and mRNA translation. Genomic RNA synthesis is completed through the intermediate full-length negative sense RNA.

The genome of PRRSV is about 15 kb in length and has a cap structure at its 5' end and a poly (A) tail at its 3' end (Meng et al., 1994; Meulenberg et al., 1993a). The genome contains at least nine ORFs (Figure 1-1). The genome organization starts with the 5' untranslated region (UTR), followed by nine open reading frames (ORF1a, ORF1b, ORF2a, ORF2b, and ORFs 3-7) and 3' UTR (Meng et al., 1994; Meulenberg et al., 1993a). ORFs 1a and 1b are located at the 5' end of the genome and comprise about 80% of the genome (Godney et al., 1993; Meulenberg et al., 1993a). ORF1a and ORF1ab

encode viral replicase and expressed from genomic RNA as a polyprotein (Pedersen et al., 1999). The polyprotein is proteolytically processed to at least 13 mature nonstructural proteins (NSPs) by virus-encoded proteases (Meulenberg et al., 1993a; Pedersen et al., 1999). Some of NSPs are known to be involved in virus replication (Meulenberg et al., 1993a), but detailed biological functions of each individual NSP remains to be determined.

The remaining seven smaller ORFs (2, 2a, and 3-7) are located at the 3' end of the genome and encode structural proteins (Meulenberg et al., 1995). ORFs 2, 2a, 3, and 4 encode minor membrane-associated proteins GP2, E, GP3, and GP4 (Meng et al., 1995; Wu et al., 2001). ORFs 5, 6, and 7 encode major envelope glycoprotein (GP5), membrane protein (M) and nucleocapsid protein (N) (Meulenberg et al., 1995; Meng et al., 1995). Recently, a non-glycosylated structural protein was identified and expressed from an ORF2b (Snijder et al., 1999). The small ORF2b is completely embedded within ORF2a (Wu et al., 2001). The proteins from ORFs 2-7 are all translated from subgenomic mRNAs (Figure 1.1). These subgenomic RNAs contain a common leader sequence from the 5' end of viral genome and identical 3' terminal sequence preceding poly-(A) tails (Sawicki et al., 1995; van Marle et al., 1999).

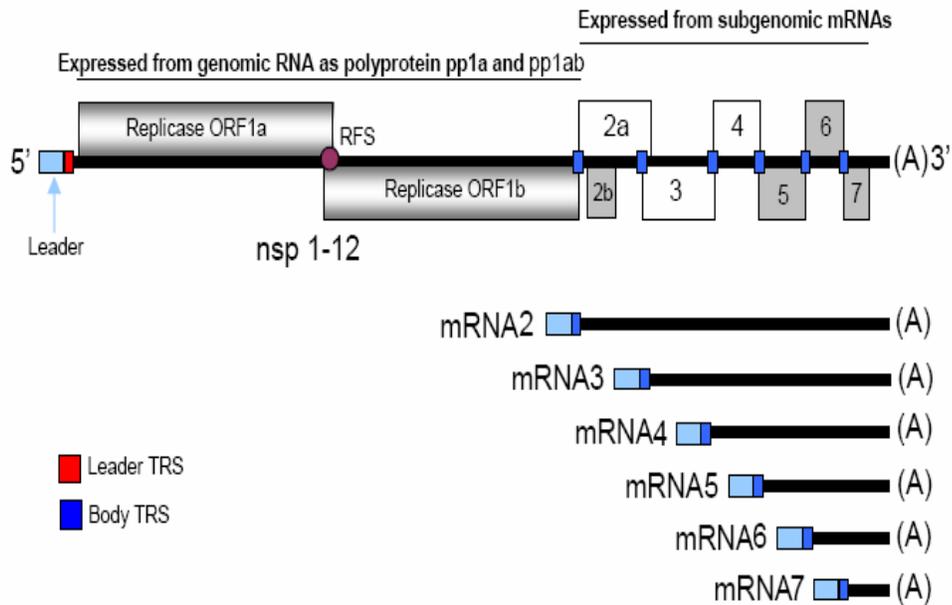


Figure 1-1. Genome organization of PRRSV. The replicase gene, located at the 5' end of the genome and consisting of two overlapping ORFs 1a and 1b, encodes a polyprotein that is cleaved to form 12 non-structural proteins (NSP 1-12). ORFs 2 to 5 encode glycoproteins GP2 to GP5; ORF6 encodes the membrane protein M; and ORF7 encodes the nucleocapsid protein N. The small internal ORF2b is completely embedded within ORF2a. The proteins from ORFs 2-7 are all expressed from a six-nested set of subgenomic mRNAs. These subgenomic RNAs contain a common leader sequence derived (thin blue box) from the 5' end of the viral genome and an identical 3' terminal sequence preceding poly(A) tails. Leader TRS (Transcription regulatory sequence; thick red box); Body TRS (thick blue box); RFS (Ribosomal frameshifting; purple circle)

In PRRSV-infected cells, the virus replication cycle starts with the expression of the replicase gene (ORF1a and ORF1b) from the viral genome RNA. ORF1b translation requires a ribosomal frameshift just before ORF1a translation is terminated. The ORF1a/ORF1b overlap region contains signals that promote this frameshift (Snijder et

al., 1998). At least six to eight subgenomic RNAs are formed (Meulenberg et al., 1993a; Meng et al., 1996b; Conzelman et al., 1993.) All of the subgenomic RNAs have an identical 5'-leader sequence derived from the 5' end of genomic RNA and identical 3' terminal sequence preceding poly-(A) tails of variable length. These co-terminal subgenomic mRNAs are generated through a discontinuous subgenomic mRNA synthesis mechanism that is proposed for coronavirus (van den Born et al., 2005). PRRSV subgenomic RNAs are polycistronic in structure, but it is believed that only the first open reading frame (ORF) of each subgenomic RNA is translated into a viral protein (Meng et al., 1996b).

The entire genomic 5' UTR is present in all subgenomic mRNAs of PRRSV. The length of 5'UTR is 190nt for American PRRSV strains (Nelsen et al., 1999) and 221nt for European PRRSV (Meulenberg et al., 1995). The 3' UTR of North American and European PRRSV strains are 150 and 120 bases, respectively (Meng et al., 1994).

1.2.3 PRRSV Growth Characteristics In Vitro

In vitro, PRRSV can replicate in African green monkey kidney cell MA-104 and cells derived from MA-104, such as CL2621, MARC145, and CRL11171, and porcine alveolar macrophages (PAM) (Yoon et al., 1992, Bautista et al 1993; Kim HS et al., 1993; Meng et al., 1996). CRL11171 and MARC-145 cells, as PRRSV-permissive cell lines, have been extensively used to study the growth characteristics of PRRSV. Previous studies have established that PRRSV replication in cultured CRL11171 or MARC-145 cells follows a complex time-course. PRRSV proteins can be detected by

immunofluorescence assay between 10-20 h post-infection and emergence of cytopathic effect (CPE) usually occurs over the next 1-3 days (Meng et al., 1996). The typical CPE of PRRSV in macrophage and cell lines is characterized by cell rounding and detachment from culture surface. In the multiple –step growth curve, the maximum release of PRRSV virions is about 48-72 h post infection and the maximum virus titers in cell culture are about $10^{6.5}$ - $10^{7.5}$ TCID₅₀/ml for cell-adapted PRRSV strains and about $10^{5.0}$ to $10^{6.0}$ TCID₅₀/ml for their parental wild type VR2332 viruses (Kim WI et al., 2008). Similar maximum virus titers are obtained from one –step growth curve at about 20 to 24 h post-infection (Cafruny WA et al., 2006; Kim WI et al., 2008). In general, the cell adapted vaccine strains grow more efficiently than VR2332 wild-type viruses in CRL11171 and MARC-145 cells (Kim WI et al., 2008). The cell adapted vaccine strains grow fast and produce relatively large plaques with a diameter of more than 4 mm on average, while their parental VR2332 wild type yielded small-sized plaques with a diameter of less than 1.0 mm on average. The difference in growth phenotypic characteristics in MARC-145 cells could be used to differentiate an attenuated vaccine virus from wild type isolates.

The replication cycles of PRRSV in the target cell include the following steps: attachment, entry, genome release, replication, translation, and assembly. After attachment, the PRRSV enters the host cell via the standard endocytosis process. Confocal microscopy showed the labeled PRRSV in clathrin-coated pits (Kreutz et al., 1996). Once inside the cell, the virus-containing endosome releases the virus genome. At present, how the viral genome is released into the cytoplasm remains to be determined. The conformational changes in viral envelop glycoprotein and capsid protein in low pH

condition inside the endosome are speculated to trigger this process. PRRSV is assembled when nucleocapsids bud into the lumen of the smooth endoplasmic reticulum and/or Golgi region. After budding, virions accumulate in vesicles that move to the plasma membrane to release the virus.

Tests using infectious full-length RNA transcript, showed that the virus replication occurred in several non-permissive cell lines but no infectious particles were generated (Meumenburg et al., 1998). This result indicates receptor (s) on the cell surface is required for PRRSV infection. Investigations of PRRSV receptors have identified several putative molecules that can assist the virus attachment, entry and replication in non-permissive cells. Heparan sulfate was identified to be a PRRSV receptor on macrophages and mediate virus attachment, and the viral matrix protein was shown to be a heparan-binding protein, suggesting its potential role as a viral ligand for heparan sulfate (Delputte, P. L et al 2001; Vanderheijden NP et al., 2001). Heparan sulfate as an attachment factor is involved in the binding of PRRSV but is not required for internalization (Delputte PL et al., 2005). Sialoadhesin was also identified as an essential PRRSV receptor that mediates both attachment and internalization on macrophages (Delputte PL et al., 2002; Vanderheijden N et al., 2003; Delputte PL et al., 2004a; Delputte PL et al., 2006). Internalization may be facilitated by sialoadhesin, a 210- or 220- kDa membrane glycoprotein and a sialic acid binding immunoglobulin like lectin (Vanderheijden N et al., 2003). A mAb that blocks PRRSV infection of MARC-145 cells was shown to recognize a complex of cytoskeletal proteins (Kim JK et al., 2006), and an intact cytoskeleton was critical for efficient infection of MARC-145 cells

(Cafruny WL et al., 2006). Simian vimentin was identified to be a part of the PRRSV receptor complex (Kim JK et al., 2006). CD163 is a cellular protein that belongs to the scavenger receptor cysteine-rich superfamily. This cellular protein was shown to be a receptor for PRRSV infection, and transient expression of CD163 in a variety of non-permissive cell lines was sufficient to render these cell lines fully permissive to PRRSV infection and production of progeny virus (Clavert JG et al., 2007). Another cellular protein termed CD151 was also shown to be involved in PRRSV replication and the interaction of viral RNA with CD151 was related to the PRRSV infectivity (Shanmukhappa K et al., 2007).

1.3 Immune Response to PRRSV Infection

1.3.1 Humoral Immune Response

The humoral immune response to PRRSV infection was evaluated by determining PRRSV-specific IgM and IgG antibody levels in serum. Figure 1-2 shows the events of host immune response after PRRSV infection of immune-competent pigs. In general, PRRSV-specific IgM and IgG antibodies were detected approximately 5 to 14 days post infection (Christianson et al., 1992), but the development of virus neutralizing (VN) antibody against PRRSV was delayed to 21 to 28 days after infection (Lopez and Osorio 2004), indicating that the humoral immunity is dysregulated after PRRSV infection (Mulupuri et al., 2008). PRRSV viremia lasts about one month in pigs after the virus infection. Thus the neutralizing antibody has limited role, if any, in the clearance of

viremia. It is not clear what protective immune response is responsible for the clearance of the viremia.

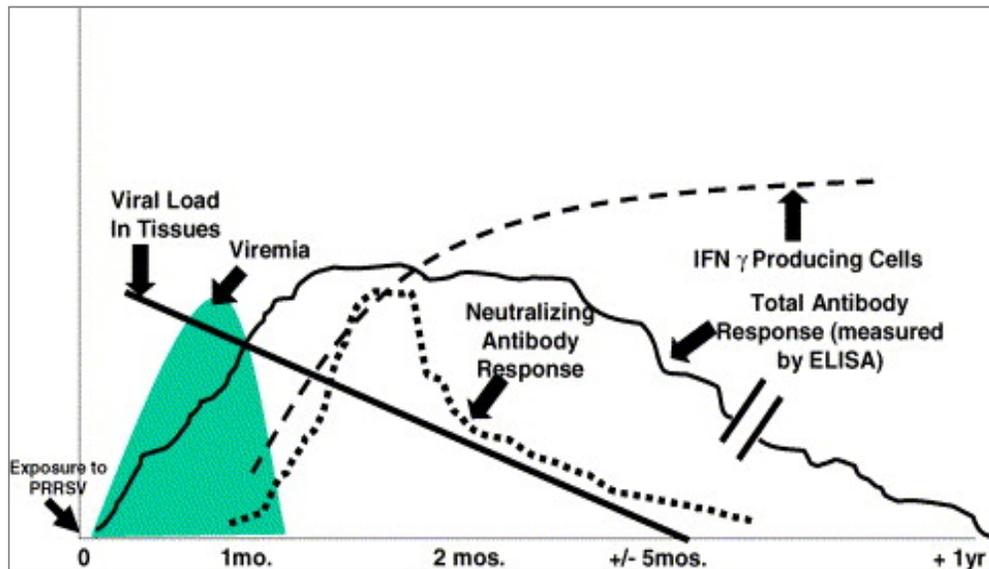


Figure 1-2: Immune response after infection of a pig with porcine reproductive and respiratory syndrome virus. (Adapted from Lopez OJ and Osorio FA, 2004, *Vet Immunology and Immunopathology*, 102: 155-163).

The N protein is highly immunogenic (Christianson et al., 1992; Nelson et al., 1993) and antibody against the N protein developed earlier than antibody to other structural proteins. However, N protein does not have virus neutralizing activity (Nelson et al., 1993; Yoon et al., 1995). *In vitro* expressed N protein appears to be more suitable for diagnostic tests. MAbs directed against the 15 kDa N protein were used to determine the functional epitopes and antigenic variation (Zhang et al., 1998). Antibodies to GP3, GP4, GP5, and M protein were reported to have neutralizing activity, indicating that these proteins are involved in PRRSV attachment or entry to host cells (Loemba et al., 1996; Delputte et al., 2004b). Neutralizing epitopes on GP3, GP4, GP5, and M protein have

been identified using a panel of mAbs (Yang et al., 2000). The mAb to the GP5 was found to be more effective in virus neutralization than monoclonal antibodies to the GP4 (Weiland et al., 1999). GP5, as a major envelope protein and the primary target of neutralizing antibody, is heavily glycosylated in the amino-terminal portion of its ectodomain and forms a hetero-dimer with protein M (Lopez and Osorio 2004). At least two main neutralizing epitopes in the GP5 were mapped to locate at the amino-terminal portion of the protein: the neutralizing epitope A is between amino acid 27 and 31 and the neutralizing epitope B comprises amino acid 37-44 (Ostrowski et al., 2002).

PRRSV antigen specific B-cell response was recently characterized in experimentally infected pigs with a focus on NSP2, N and GP5 proteins (Mulupuri et al., 2008). The IgM response to NSP2 and N protein was detected at day 3, peaked by day 7 and then rapidly decreased to undetectable levels by day 21 (Mulupuri et al., 2008). In contrast, IgM response to GP5 ectodomain epitopes (GP5 3') was minimal and no increase in IgM level was observed within 56 days post-infection. IgM response to GP5 ectodomains (GP5 5') was substantially delayed by 2 weeks, peaked at 21 to 28 days, and even persisted for about 50 days (Mulupuri et al., 2008).

NSP2 specific IgG response was detected at day 7, reached maximum level at about day 35, and remained high thereafter. Similar specific IgG response was observed for N protein, but the anti-N IgG level quickly decreased after day 35. The PRRSV neutralizing IgG response to GP5 3' and GP5 5' was detected at 21 days after infection, peaked at day 35, and remained relative high thereafter (Mulupuri et al., 2008). The

delayed neutralizing IgG response was not correlated with the level of viral antigens, a high level of viremia was present 3 days after infection and remained high for about 2 weeks. Viral antigens were actively produced for an extended period of 3 to 4 weeks. The role of neutralizing antibodies (NA) in protection against PRRSV is under debate. Passive transfer of NA at high dose was observed to confer protection against PRRSV challenge (Osorio et al. 2002). However, other studies showed that the production of NA after an experimental infection or vaccination is low and sporadic with considerable individual variability (Loemba et al., 1996; Meier et al., 2003). Clearance of viremia was also found before a high level of NA developed (Diaz et al., 2005).

1.3.2 Cellular Mediated Immune Response (CMI)

For viral infection, typical CMI response involves antigen-specific activation and expansion of CD4⁺ and CD8⁺ T cells in draining lymph nodes, followed by trafficking specific cytotoxic lymphocytes back to the infection sites to kill the infected cells (Jenkins et al., 2001). PRRSV infections also trigger CMI, but the distribution of PRRSV-specific T cells in peripheral blood varied substantially over time and among animals and the levels of PRRSV specific T-cells and macrophage recruitment in infected pigs were found to be independent of virus load (Xiao et al., 2004). In acute infection, PRRSV-specific (IFN γ -secreting) T cells were detected in PBMC at day 14 p.i, peaked at day 28 p.i. and declined thereafter (Xiao et al., 2004). The antigen-specific T-cell response at sites of infection was highly variable, weak, and independent of the local viral load, appeared transiently at 4 to 8 weeks after infection (Lopez et al., 1999), but became more pronounced based on the IFN- γ response (Meier et al., 2003). Inductions of virus

specific T-lymphocytes response in pigs infected by PRRSV were found mainly directed to the M, GP2, and GP5 protein (Zuckerman et al., 1998).

In addition to the absence of a correlation between viral load and responding T cells in sites of infection, there was no apparent effect of PRRSV on local T-cell populations. The proportions of CD4⁺, CD8⁺, and CD4⁺ CD8⁺ T cells were not altered by PRRSV infection in lung, blood, or lymphoid tissues (Xiao et al., 2004). These results suggest that a weak and irregular CMI response contributes to prolonged PRRSV infection and that PRRSV suppresses T-cell recognition of infected macrophages (Meier et al., 2003; Xiao et al., 2004). Humoral and cell-mediated immunity is induced in due course, and results in clearance of the virus from circulation but not from lymphoid tissues where the infection becomes persistent. Subsequent re-exposure to PRRSV elicits an anamnestic response that is partially to completely protective.

1.4 PRRSV Antigenic and Genetic Variations

1.4.1 Antigenic Variations

The reactivity of mAbs against GP3, GP4, GP5, and N proteins of European and American PRRSV isolates revealed antigenic difference not only between the US and European isolates but also among different European or US isolates (Katz et al., 1995; Nelson et al., 1993; Zhang et al., 1998). Antigenic variation was detected between an isolate and its progeny after *in vivo* passages, indicating a high mutation rate during PRRSV replication in its natural host. Due to a lack of proofreading activity by the RNA

dependent RNA polymerase, like other RNA viruses, PRRSV has high mutation rates during virus replication.

1.4.2 Genetic Variations

Full-length gene sequence analysis of PRRSV isolates has identified genetic variation between North American and European PRRSV isolates on both genes encoding structural proteins (Meng et al 1995; Murtaugh et al., 1995; Nelsen et al., 1999) and non-structural proteins (Allende et al., 1999). The N protein was found to be the most conserved viral protein with 70 to 80% identity between the North American and European isolates (Meng et al 1994; Meng et al, 1995). While GP5 protein is the most variable structural protein with only 51-55% amino acid identity between North American and European isolates. The amino acid sequence homology of ORF2, 3, and 4 was about 76, 72, and 64%, respectively. The NSP2 was found to have the highest variations. The NSP2 of North American isolates is 102 amino acids longer and share only about 30% identity when compared to the European prototype LV strain (Nelsen et al., 1999; Allende et al., 1999). Extensive sequence analysis of multiple isolates from current pandemic in China also confirmed that the highly pathogenic variants belong to the North American genotype but have a discontinuous 30 amino acids deletion in the NSP2 (Tian et al., 2007).

Efforts to identify virulence determinants of PRRSV began by comparing protein sequences between virulent and their cell-adapted avirulent strains. The mutation or deletion in the viral gene(s) may contribute to PRRSV attenuation. However, these

changes may not necessarily represent the true mutation *in vivo*. Many of these mutations may just reflect the necessary adaptation to grow in susceptible cell lines. The development of infectious clones for PRRSV greatly facilitates the understanding of the molecular basis of PRRSV virulence.

1.5 Infectious cDNA Clones: Reverse Genetics System

Reverse genetics is a powerful molecular tool to understand the molecular determinants related to virus attenuation, tissue tropism, and virulence factor(s). Reverse genetics is defined as a process of generating a recombinant virus from a cloned complimentary DNA (cDNA) copy of a viral genome. For viruses with a positive-sense viral RNA (+vRNA), +vRNA is infectious and can serve as mRNA and initiate virus replication using cellular RNA-dependent RNA polymerase (RdRp). Therefore, transfecting full-length +vRNA or DNA plasmids containing cDNA of complete viral genomes into susceptible cells result in the recovery of infectious viruses. This system allows researchers to genetically manipulate the viral genome (Calvert et al., 2002; Nielsen et al., 2003).

For PRRSV, the first infectious clone was established for European Lelystad virus (Meulenberg et al., 1998). Later, infectious clones were also developed for North American prototype VR-2322 strain and other isolates (Calvert et al., 2002, Nielsen et al., 2003; Troung et al., 2004). Transfection of RNA transcripts from the full-length cDNA clone into BHK-21 or MARC-145 cells, infectious viruses were produced and further

propagated in PAMs or MARC-145 cells. The BHK-21 cells were initially used due to the high efficiency in transfection compared to CL2621 cells or other PRRSV susceptible cell lines. Although PRRSV cannot enter BHK-21 cells, once the genomic RNA is introduced into these cells, the virus is produced and can be amplified in a PRRSV permissive cell line (Truong et al., 2004). In addition, direct inoculation of full-length RNA transcript into the pig lymph node and tonsils was demonstrated to produce infectious PRRSV (Key et al., 2007). Using the infectious clone, the N protein dimerization was found to be essential for viral infectivity (Lee et al., 2004). Various deleted mutants of PRRSV at the ORF2, ORF4 or NSP2 genes were also generated from infectious clones and evaluated as potential marker vaccine candidates (Calvert et al., 2003; Welch et al., 2004; Han et al., 2007), or develop recombinant vaccine strains that could offer cross-protection to various genetically diversified PRRSV strains (Yuan et al., 2008) or construction of marker vaccines that contain the specific sequence tag to distinguish the vaccinated pigs from naturally infected ones (Kim W et al., 2008). Using infection clones, various chimeric recombinants between modified live vaccine (MLV) and its parental wild type virus were also produced to identify the corresponding genetic determinants related to virulence and other different phenotypes such as plaque size and cell adaptation (Kim W et al., 2008).

1.6 Diagnosis, Prevention and Control

1.6.1 Diagnosis

Early detection and quick identification of infected herds are the key elements to a PRRS control and eradication program. Active serum surveillance and public awareness programs are valuable in early detection of infection.

A diagnosis of PRRS acute clinical outbreaks in fully susceptible herds is based on the clinical signs and gross pathological findings in affected pigs. However, a confirmed laboratory diagnosis of PRRS is generally considered to have been established when PRRSV is identified in samples submitted from presumed cases of the disease (Mengeling et al 2000; Christopher-Hennings et al 2002). Selection and submission of appropriate samples is the key to a successful diagnosis of PRRSV infection (Christopher-Hennings et al 2002). Laboratory diagnosis can be achieved by detecting PRRSV antigens in affected pigs by immunostaining or viral RNA in situ hybridization and polymerase chain reaction (Sagar, 1993; Prieto and Castro 2005).

Indirect immunofluorescence (IFA), indirect immunoperoxidase monolayer assays (IPMA) and enzyme-linked-immunosorbent assays (ELISA) are the serologic tests used most often to detect and quantitate antibody (Christopher-Hennings et al 2002).

Immunohistochemistry and immunofluorescence are useful tests for detecting PRRSV antigens in tissues obtained from infected pigs (Mengeling et al., 2000). Lung is the tissue of choice for viral antigen and RNA detection. Laboratory diagnosis by virus

isolation is also used but it is time consuming and expensive (Rossow 1998). For a herd of swine that is vaccinated with a modified live PRRSV vaccine, diagnosis is more difficult because the serological test cannot differentiate immune response from vaccination or infection with wild type virus.

The most commonly used test for strain identification is RT-PCR amplification of ORF5 gene, followed by restriction fragment length polymorphism (RFLP) analysis and nucleic acid sequencing (Mengeling et al., 1999, Umthun et al., 1999). ORF4, ORF6, and NSP2 genes can also be used for amplification and digestion (Han et al, 2007, Kim et al., 2008). Base sequencing often involves additional segments of the viral genome in addition to ORF5. Panels of monoclonal antibodies have also been used to provide presumptive strain identification (Yang et al., 2000). Quantitative real time RT-PCR is also commonly used for detection and confirmation North American-like or European-like PRRSV from boar semen and serum samples (Wasilka et al., 2004)

1.6.2 Prevention and Control

Because of severe economic losses associated with PRRS, various attempts are being made to control and eliminate the disease. Vaccination, production management, and biosecurity protocols have not been fully effective (Goyal D., 1993). This was probably due to the features of PRRSV such as persistence of infection, strain variation, transplacental infection, and various routes of excretion and transmission. (Meng et al., 2000). Though not all routes of virus introduction into a naïve herd are completely understood at this time, the primary source of infection is the infected pig. Therefore, it

is critical to routinely isolate and test breeding stock before introducing them to a PRRS-negative herd. Replacement stock to be added to naïve herds should be obtained from known negative sources that carry out a regular schedule of herd monitoring (Prieto and Castro 2005).

One principle of PRRS control is the reduction of virus spread among animals within a herd and between herds (Beltran-Alcrudo et al., 2007). Early removal of infected animals from the herd is an effective way of limiting the spread of the disease. Restriction of replacement animals is very critical for the control PRRS. Quarantine, biosecurity, and movement controls should be imposed on all farms with known or suspected infection. Recently, a model for the control of PRRS that focuses on the elimination of subpopulations was developed (Beltran-Alcrudo et al., 2007). The model includes: (1) understanding the pattern of viral spread through the application of population-based diagnostic strategies; (2) proper containment of replacement stock prior to introduction into PRRS-infected herds; (3) prevention of virus transmission from sow to piglet through breeding-herd stabilization; and (4) control of the virus spread in the nursery or finisher populations through weaned-pig management (Beltran-Alcrudo et al., 2007).

The sanitary precautions that are applied to prevent the spread of most swine infectious diseases must be rigorously used in the case of PRRS. Environmental stress and management are also the factors to be considered when developing preventive programs.

1.6.3 Vaccination

Both inactivated and modified-live attenuated virus vaccines (MLV) for PRRSV are available in the market (Nelson et al., 1997, Beltran-Alcrudo et al., 2007; Mulupuri et al., 2008). *In vivo* challenge studies indicate that the vaccine with the American strain does not protect against infection from European type PRRSV (van Woensel et al., 1998). An attenuated virus vaccine is generally believed to be more effective compared to the killed vaccine. Several studies also establish that vaccination is more effective against a homologous strain than against a heterologous strain (van Woensel et al., 1998). In U.S., the MLV vaccine, Ingelvac PRRS MLV produced by Boehringer Ingelheim, has been used in piglets from 3 weeks of age or in sows and gilts 3-6 weeks prior to breeding.

The MLV vaccine can be of value in preventing and controlling PRRS, but reversion of MLV to virulent phenotype is one of the major safety concerns (Opriessnig et al., 2002). Pigs inoculated with MLV vaccines also shed the vaccine strain which they can transmit from vaccinated pigs to naïve pigs (Mengeling et al., 1998). The persistent shedding and dissemination of the vaccine virus in semen was also demonstrated in boars (Christopher-Hennings et al., 1997). In addition, the occurrence of acute PRRS-like illness was reported in sows after vaccination with a MLV vaccine (Botner et al., 1997; Meng et al., 2000). Transmission and persistence of MLV in the field make it more complex to identify wild-type virus infection both through virology and serology (Zimmerman et al, 2006).

Although vaccines, especially those that are attenuated, have been used for years, there are still some outbreaks of PRRS with the strains whose sequence is nearly identical to the vaccine strain (Opriessnig, Halbur et al. 2002). Occurrence of atypical or acute PRRS-like disease in MLV-vaccinated animals has raised concern about the efficacy and safety of the MLV vaccines (Meng et al., 2000; Meng 2006a). Therefore, alternative approaches to control or inhibit PRRSV infection and transmission are needed.

The best option to control PRRS is to eliminate PRRSV spread from swine farms. The high mutation rate of PRRSV replication and high prevalence of PRRSV infection in swine farms are challenging for the current vaccine that is based on a single strain. The long viremia and late generation of neutralizing antibody add further complexity since the protective immune response in PRRSV-infected pigs is less clear (Fig. 1-2). An efficacious vaccine with broadly protective capability is not available in the near future though it is needed urgently. Under such scenario, novel antiviral drugs to inhibit PRRSV replication are welcome options to complement other strategies for PRRS prevention and control. One of these options would be to use PRRSV-specific and efficacious antiviral compounds like PPMO. PPMO is a novel type of antisense compound, and has been shown to effectively inhibit PRRSV replication *in vitro* (Zhang et al., 2006; Patel et al., 2008). In this study, we evaluated the effect of combining multiple PPMO specific for PRRSV sequence on the inhibition of PRRSV replication in cell culture and defined the mechanism of the inhibition by PPMO combination.

1.7 Phosphorodiamidate Morpholino Oligomers (PMOs) As a Novel Antiviral Agent

The ability of virus-specific antisense oligonucleotides to inhibit viral replication by interfering with translation of viral RNAs was first demonstrated in 1978 (Zamecnik and Stephenson, 1978; Stephenson and Zamecnik 1978). Since then, remarkable progress has been made by modifying oligonucleotides to increase their stability, affinity, and delivery into cells (Kurreck 2003). PMO is a new generation of antisense agents modified to include a phosphorodiamidate linkage and morpholine ring. PMO is structurally similar to single-stranded DNA in that each subunit includes a purine or pyrimidine base (Fig 1-3). In PMO, each base is joined to a novel backbone consisting of one morpholine ring and phosphorodiamidate linkage per subunit (Summerton, 1999; Summerton and Weller, 1997). PMO is uncharged, water-soluble, and highly resistant to nuclease degradation (Hudziak et al., 1996). PMO binds to target mRNA by Watson–Crick base pairing and exerts an antisense effect by preventing access to critical segments of RNA sequence, such as a translation initiation site, through steric blockade. This is a distinctly different process from the RNase H-dependent mechanism induced by the often-used antisense structural type phosphorothioate DNA (Summerton, 1999).

PMO can effectively and specifically block translation of target mRNA *in vitro* and *in vivo* via intravenous, intraperitoneal, subcutaneous, transdermal or oral administration (Arora et al., 2002a; Arora et al., 2002b). In addition, PMO conjugates with short arginine-rich cell penetrating peptides have displayed a higher efficiency in

delivering the PMO into cultured cells (Moulton et al., 2004; Deas et al., 2005). This peptide-conjugated PMO (PPMO) has been found to be fairly stable in cells and human serum for at least 24 h (Youngblood et al., 2007). PPMO sequence-specific antiviral effect in cell culture system have been reported for SARS coronavirus (Neumann et al., 2005), EAV (van den Born et al., 2005), flavivirus (Deas et al., 2005; Kinney et al., 2005), Influenza A virus (Ge et al., 2006), and Kaposi's sarcoma-associated herpesvirus (Zhang et al., 2007). Recently, PPMO has also been applied *in vivo* and shown protection of animals against Ebola Virus (Enterlein et al., 2006), Coxsackievirus B3 (Yuan et al., 2006), and murine coronaviruses (Burrer et al., 2007).

Several PMOs targeted at the 5' terminal region of PRRSV genome have been shown to be effective in inhibiting PRRSV replication in cells in a sequence-specific and dose-dependent manner (Zhang et al., 2006; Patel et al., 2008). PPMO targeted at the translation initiation region of PRRSV ORF6 and ORF7 have also been demonstrated to inhibit PRRSV replication (Patel et al., 2008). Application of these potential PPMO antivirals could yield significant economic benefits to the swine industry, especially for breeding farms, by helping to control and eliminate PRRSV infection.

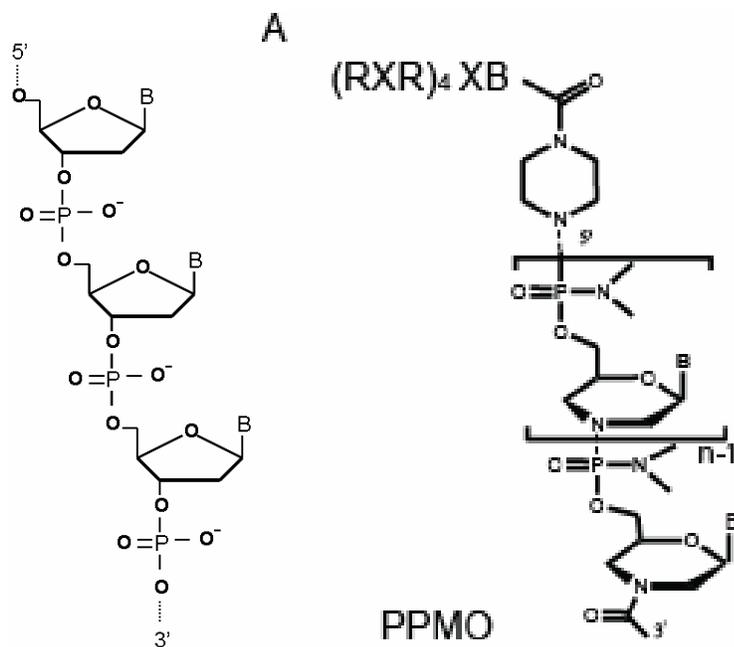


Figure 1-3: Structure of PPMO. A morpholine ring and a phosphoramidate linkage in PMO replace the deoxyribose and phosphodiester linkage of DNA, respectively. “B” represents the bases A, G, C, or T. An arginine-rich peptide (RXR)₄XB is covalently conjugated to the 5' end of PMO.

1.8 Research Objectives

The main goal of this research is to understand the effect of combining multiple PPMO specific for PRRSV sequence on the inhibition of PRRSV replication *in vitro*. The specific objectives are to: (1) Assess the effect of various PPMO combinations in inhibiting PRRSV replication in cell culture, 2) Determine the effect of the PPMO combination in cross strain inhibition against heterologous PRRSV strains *in vitro*, and (3) Define the PPMO inhibition effect at PRRSV RNA level and gene expression (protein level).

CHAPTER 2: MATERIALS AND METHODS

2.1 Cells and Viruses

CRL11171 (ATCC) cell line was grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 2mM L-glutamine. The CRL11171 cells were passaged twice weekly in T-25 flask. For routine viral infection and transfection experiments, a monolayer of CRL11171 cells (\approx 80% confluency) was maintained in DMEM containing 2% FBS and incubated at 37°C in 5% CO₂ after infection or transfection. Trypan blue exclusion method was used to assess the cell viability. The cell numbers were counted using a hemocytometer under an inverted light microscope.

The following PRRSV strains were used in the experiment. ATCC VR2385 (Meng et al., 1996a), and Lelystad (Meulenberg et al., 1993a) were used to inoculate CRL11171 cells at multiplicity of infection (MOI) of 0.5 for PPMO testing. Other PRRSV strains used to test cross strain effect include FL-12, 11604, 16138, 16224B, 17041, 14680, 12773, 13909 and Ingelvac MLV (Patel et al, 2008).

2.2 Determination of Virus Titer

Virus titers were determined from the median end point of tissue culture infectious dose (TCID₅₀). For virus titration, serially diluted virus samples from 10⁻¹ to 10⁻⁶ were added to CRL11171 cells in a 96-well plate. Four replicate wells were used for each dilution. The cultures in 96-well plates were incubated for 48 hours at 37°C after

inoculation, and the TCID₅₀ were obtained by counting the number of wells with cytopathic effect (CPE) microscopically. The typical CPEs in PRRSV-infected CRL11171 cells include cell rounding, clumping and detachment, compared to the control cells. TCID₅₀ value per milliliter was calculated according to the method of Reed and Muench as described previously (Zhang et al., 2006).

2.3 Indirect Immunofluorescence Assay (IFA)

The CRL11171 cells growing on glass coverslips in a 12-well plate were mock infected or infected with PRRSV at an MOI of 0.5, and incubated at 37°C. About 24 hours post-infection, the cells were washed with phosphate-buffered saline (PBS), and then fixed with acetone and methanol (1:1, 1ml) for 15 min at room temperature. The fixed cells were washed once with PBS (pH 7.4) and incubated with 100 µl of a PRRSV N-specific monoclonal antibody at 37°C for 45 min in a humid chamber. Unbound antibody was removed by washing with PBS three times. After final wash, the cells were incubated with 100 µl of 1:100 dilution of fluorescein-conjugated goat anti-mouse IgG fluorescein-isothiocyanate (FITC) conjugate (Sigma, St Louis, MO) for 45 min at 37°C. Then the cells were washed three times to rinse out unbound FITC-conjugated antibody. After final wash, the cells in cover glass were mounted and examined by fluorescence microscopy.

2.4 PPMO Synthesis

PPMO were synthesized by AVI BioPharma Inc (Corvallis, OR), according to the methods described by Summerton and Water in 1997. A random sequence PMO with no homology with the PRRSV or primate mRNA sequence was also included in this study. This control PPMO was called CP1 and used as a control for any non-sequence-specific activity of the PPMO chemistry.

Arginine-rich peptide [NH_2 - (RXR)₄XB-COOH], where X stands for 6-aminohexanoic acids, R stands for arginine, and B stands for β -alanine, was covalently conjugated at 5' end of each PMO (Figure 2-1). The conjugation chemistry, PPMO purification and analysis were also performed at AVI BioPharma Inc according to the procedure detailed in the publications by Abes et al., 2006 and Moulton et al., 2004.

Prior to use in the designed experiments, the lyophilized PPMO was resuspended and diluted with sterilized water to a stock with a final concentration of 2 mM. The stocks were stored at 4°C. The PPMO were further diluted to the working concentrations in DMEM as indicated in the PPMO treatment experiments.

2.5 PPMO Treatment of CRL11171 Cells

The PPMO treatment of CRL11171 cells was performed in 12-well plates as previously described (Zhang et al., 2006). About 5×10^5 cells/well were seeded in a 12-well plate and incubated overnight at 37° C. Prior to the PPMO treatment, confluent

monolayer of CRL11171 cells (~ 90% confluency) in 12-well plate were inoculated with PRRSV (VR2385) at a MOI of 0.5 and incubated at 37° C for 2 hours. After 2-hour infection, the inoculum was removed and the cells were rinsed with serum-free DMEM. PPMO was diluted to the desired concentrations in DMEM and added to the PRRSV-infected cells. Then the cells were incubated at 37°C for 4 hours. DMEM without PPMO was used as a mock treatment control and PPMO CP1 was also included as a negative control. At 4 hours after incubation, the medium was removed from the negative control, mock treatment control, and PPMO-treatment cells. The cells were rinsed with serum-free DMEM and maintenance medium (DMEM supplemented with 2%FBS) was added. The cells were then incubated at 37°C for 48 hours. After incubation, both supernatant and cells were harvested for further analysis.

In addition to PPMO combination treatment, various PRRSV strains were also included following the same inoculation and incubation procedure as described for VR2385 strain. The purpose of the experiment was to evaluate cross strain inhibition effects of PPMO combinations on replication of various PRRSV strains.

2.6 RNA Isolation

Total RNA was extracted from PRRSV-infected CRL11171 cells and PPMO treated cells by TRIzol[®] Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instruction. After removal of the supernatant, the cells in 12-well plate were lysed by addition of 500 µL of TRIzol[®]-Reagent. CP1 or mock- treated cells were also included as controls. For quantitative RT-PCR analysis, the purified RNA was

treated with RNase-free DNase to remove any DNA carry-over during the RNA isolation. The RNA was quantified using μ QuantTM Universal Microplate spectrophotometer (BioTek Instruments, Winooski, Vermont). The RNA was kept in diethyl pyrocarbonate-treated water (DEPC-H₂O) and stored at -80°C for real-time RT-PCR analysis.

2.7 Real-Time RT-PCR (Reverse transcription-PCR)

The viral RNA levels in the PRRSV infected CRL11171 cells and PPMO treated cells were analyzed by quantitative real-time RT-PCR. First, the cDNA synthesis was performed using the random primer and performed in a total of volume of 25 μ l reaction as reported previously (Patel et al., 2008). Typically, extracted RNA sample (\approx 1.0 μ g) was first heat-denatured for 5 min at 75°C in 5 μ l DEPC-treated water in the presence of 100 ng of random primer. The reverse transcription was performed following the addition of 19 μ l of reverse transcription mixture (total 25 μ l). The mixture consisted of buffer 5X 5 μ l, dNTP 2.5 μ l, 10 units of Superscript III Avian Myeloblastosis Virus Reverse Transcriptase (Promega), and 4 units of RNasin ribonuclease inhibitor (Promega). The reverse transcription reaction was conducted for 5 min at 25°C, 60 min at 42°C and 5 min at 85°C on an iCycler system (Bio-Rad Laboratories, Hercules, CA). Afterward, the cDNA was stored at -20°C.

For analysis of the PPMO's inhibition effect at mRNA level, SYBR Green real-time PCR was performed on Chromo 4TM Four-Color Real-Time System (Bio-Rad) by using the specific primers based on the sequence of PRRSV ORF1a gene as following

ORF1-RR-F1: 5'-GTCTG TCCCT AGCAC CTTG-3' and ORF1-RR-R1: 5'-GCCCT CCGCC ATAAA CAC-3'. In order to generate the standard curve for the real-time PCR, a recombinant plasmid containing a fragment of 860 bases from cDNA of the 5' end of the PRRSV genome was used (Patel et al., 2008). Transcripts of β -actin mRNA were used as internal reference to normalize the amount of PRRSV RNA. Critical threshold (Ct) values of the target gene were averaged and normalized to the averaged Ct of β -actin accordingly. Real-time PCR was performed in a total volume of 20 μ L using 96-w microwell plates and a sequence detector. The PCR mixture consists of 5 μ L diluted cDNA, 10 μ L SYBR green PCR Super Mix (Bio-Rad), 1 μ L of 2.5 μ M primers ORF1-RR-F1 and ORF1-RR-R1. The real-time PCR amplification was carried out in the following cycling conditions, 3 min at 95°C, followed by 40 cycles of 10 sec at 95°C and 45 sec at 62°C, and finally, 1 min at 95°C. All RT-PCR was performed in duplicate.

2.8 Plasmid construction and cell-free luciferase reporter assay

The 5'UTR (312nt) of subgenomic RNA7 was PCR-amplified from the cDNA of PRRSV with forward primer 5UF1 (CTAGC TAGCG ACGTA TAGGT GTTGG CTC) and reverse primer SB7-R4 (ACGCG TCGAC CTTCT GCTGC TTGCC GGTG). The PCR product was digested with *Nhe*I and *Sal*I and subcloned into the multiple cloning site of a T7 promoter-containing luciferase reporter vector PciNeoLuc. This cloning effectively replaced the start codon of the luciferase gene with the entire PRRSV 5'UTR of subgenomic RNA7. DNA sequencing was performed to confirm the presence of the desired sequence in the resulting plasmid. Each plasmid DNA was linearized downstream

of the PRRSV sequence-luciferase gene with *NotI*. In vitro transcription was conducted with T7 RiboMAX™ Express Large Scale RNA Production System (Promega) according to the manufacturer's instructions. In vitro translations were carried out by rabbit Reticulocyte Lysate Translation System (Promega) with 2 µg transcribed RNA. The luciferase yield under absence or presence of PPMO treatment was measured by the Bright-Glo™ Luciferase Assay System (Promega) with VICTOR3™ Multilabel Counter (PerkinElmer Life and Analytical Science, Wellesley, MA). Luminescence signal in each test sample was converted to relative percentage of that in mock-treated control reactions and presented as a relative percentage of luciferase yields.

Primers specific to sub-genomic RNA7 excluding the 3'UTR and polyA were designed. *BamHI* restriction enzyme site was introduced into the sense primer 5UF2 (5'-CGCGG ATCCG ACGTA TAGGT GTTGG CTC) and *XbaI* restriction enzyme site was introduced into the antisense primer P7R2 (5'-GATCT AGATC ATGCT GAGGG TGATG). The region of about 500 bp was amplified from the subgenomic RNA7 of PRRSV and cloned into the *BamHI/XbaI* site of pcDNA3 vector. The recombinant plasmid was subjected to DNA sequencing to verify the presence of the PRRSV sequence. CRL11171 cells in 12-well plates were transfected with the plasmid by transfectin (Bio-Rad). At 24 hours after transfection, the cells were fixed with acetone and methanol for IFA as described above in section 2.6.

2.9 Statistical analysis

The significance of differences of viral yield or RNA level between the groups of PPMO-treated cells was assessed by student t-test. A two tailed P value of less than 0.05 was considered significant.

CHAPTER 3: RESULTS

3.1 Anti-PRRSV PPMO

For RNA virus with the (+) sense genome, the efficient replication of the RNA virus requires essential sequence and structural elements in terminal regions of viral genome and antigenome. The terminal regions are recognized by the viral replicase (Panavas et al., 2002; Tortorici et al., 2003). Therefore it is rational to design PPMO targeting these RNA sequences involved in viral replication, subgenomic RNA synthesis and translation. Sequence alignments of PRRSV various strains indicate that the 5'- and 3'-terminus region, as well as the 5' region of ORF1a are highly conserved within each genotype (Zhang et al., 2006, Patel et al., 2008). These conserved regions in PRRSV were speculated to be essential for virus replication (Tan et al., 2001).

Based on these considerations, twelve PPMO against PRRSV were designed (Zhang et al., 2006; Patel et al., 2008). The PPMO sequences and target sites in the PRRSV genome are specified in Table 3-1 and depicted schematically in Figure 3-1. All these PPMO sequences are derived from a virulent strain VR2385 of North American genotype (Zhang et al., 2006; Patel et al., 2008). Of the 12 PPMO, a set of 4 PPMO was designed to target the 5'- and 3'-terminus region. PPMO 5UP1 and 5HP were specific to the 5' end region of PRRSV genome. The 5'-UTR terminal regions of the antigenome

were chosen as PPMO target sites in an attempt to interfere with or block translation of viral RNA replicase. The 5HP is targeted to the predicted hairpin loop structure at the 5'-UTR, which is conserved among different PRRSV isolates. Disruptions of this RNA structure either in the plus- or in the minus-strand region can interfere with the initiation of genomic RNA synthesis. The NSP2 is designed to be complementary to the 3' terminus of negative-sense RNA in an attempt to interfere with the production of positive sense RNA. The 3UP2 is paired to the 3-terminal regions of the genome of PRRSV in an attempt to interfere with the negative strand RNA synthesis and/or block viral RNA replication.

The NSP3 was designed to target the leader transcription regulation sequence (TRS) of negative sense PRRSV RNA, NSP3 binding to the leader TRS is expected to interrupt the production of PRRSV subgenomic mRNA and/or interfere with the translation of the viral subgenomic RNA. The 1bP1, 2P1, 3P1, 4P1, 5P1, 6P1, 7P1 are complementary to the translation initiation region of each open reading frame of PRRSV, ORF1, ORF2, ORF3, ORF4, ORF5, ORF6, ORF7, respectively, to inhibit the translation of these ORFs. Beside the above PRRSV specific PPMO, a random-sequence PPMO, termed CP1, was also included as negative control to monitor the off-target effects and to explore the cellular uptake of the PPMO chemistry.

Table 3-1: PPMO^a and their target sites in PRRSV RNA genome^b

PPMO name	PPMO sequence (5'–3')	Nucleotide position of PPMO target in PRRSV RNA	PPMO orientation	Target region in PRRSV RNA
5UP1	CATAGAGCCAACACCTA TACG	3–23	Antisense	5' terminus of genomic RNA
5HP	GTTTTGGGCTGTGCCAA TGG	54–74	Antisense	5' UTR of genomic RNA
NSP2	GACGTATAGGTGTTGGC TCTAT	1–22	Sense	3' terminus of negative-sense RNA
NSP3	CCTTTAACCATGTCTGG GAT	180–199	Sense	TRS-region
1bP1	CAACTCGAACCCGGAGG GCATG	7982–8003	Antisense	ORF1b ^d
2P1 ^c	CCCATTT <u>CAT</u> CTCAATTC AGGC	11749–11770	Antisense	ORF2 ^d
3P1	ACAGCTATTAAC <u>CA</u> TTG CTG	12379–12398	Antisense	ORF3 ^d
4P1	GGGACGAAG <u>CCAT</u> TCTA GGT	12922–12941	Antisense	ORF4 ^d
5P1	CCCAAC <u>ATA</u> CTTAAACA TTC	13464–13483	Antisense	ORF5 ^d
6P1	GGACGACT <u>CCAT</u> TGTTC CGC	14055–14074	Antisense	ORF6 ^d
7P1	GG <u>CAT</u> ATTTAACAAGGT TTACC	14560–14581	Antisense	ORF7 ^d
3UP2	CCGTGTGGTTCTCGCCA ATTAA	15070–15091	Antisense	3' UTR
CP1	GATATACACAACCCCA ATT	None	None	Random sequence

^a The peptide (RXR)₄XB was conjugated to the 5' end of all the PMO in this study.

^b PMO design was based on PRRSV VR2385 genomic RNA (GenBank accession number [U03040](#), however, sequence of the 5' end of the genome is unpublished).

^c The underlined nucleotides correspond to the AUG translation initiation codons of PRRSV ORFs.

^d Translation initiation regions of PRRSV ORFs.

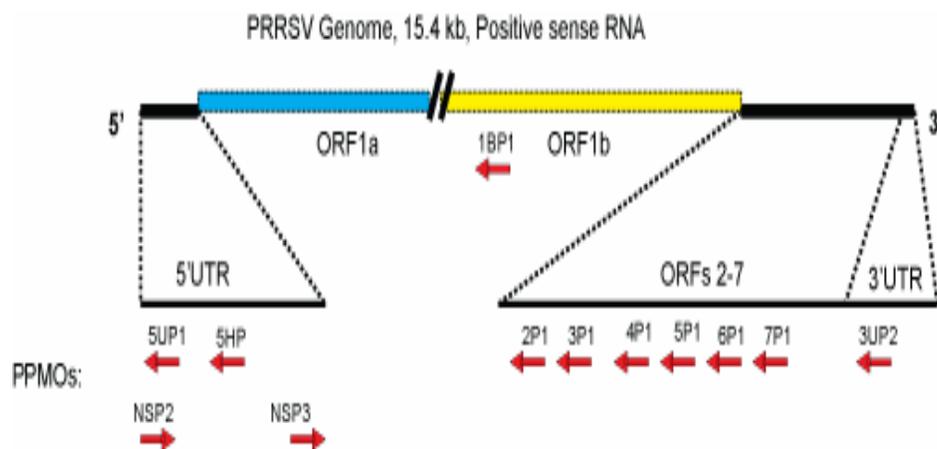


Figure 3-1: Schematic illustration of PPMO target locations in the PRRSV genome.

Positions of PPMO target sites in PRRSV genomic RNA. The arrows indicate the 5' to 3' orientation of the PPMO in relation to the PRRSV RNA genome

Initial evaluation of the individual PPMO indicated that the 5UP1 and 5 HP designed to target the 5'UTR markedly reduced PRRSV replication in cell culture (Zhang et al., 2006; Patel et al., 2008). The inhibition of the two PPMO on PRRSV replication was in a sequence specific and dose dependent manner in the concentration ranging from 2 to 32 μ M (Zhang et al., 2006; Patel et al., 2008). Treatment of cells with individual

5UP1 or 5 HP at the concentration of 16 or 32 μM resulted in more than 4.5 \log_{10} reduction in PRRSV yield, compared to a control PPMO (Zhang et al., 2006; Patel et al., 2008). In addition, in vitro cytotoxicity assay revealed that these PPMO displayed no detectable cytotoxicity effect even at the highest concentration tested (32 μM) (Patel et al., 2008). A moderate reduction of PRRSV replication was also observed for the cells treated with individual 6P1 or 7P1 at a relatively high concentration, but combination of 6P1 and 7P1 was found to be more effective in reducing PRRSV replication compared to 6P1 or 7P1 alone (Patel et al., 2008). The other PPMO, as listed in Table 3-1 and when used alone, were found to have no effect in inhibiting PRRSV replication (Patel et al., 2008). These data indicated that the PPMO designed to base pair with the targets in the genomic 5'UTR are highly effective at the relative low concentration and could be explored as a potent anti-PRRSV compound.

3.2 Effect of combination PPMO treatment on inhibiting PRRSV replication

PPMO 5UP1 was found to be highly effective in inhibiting PRRSV replication in cell culture. Treatment of CRL11171 cell culture with 5UP1 at the final concentration of 2 μM resulted in moderate (about 1.0 \log_{10}) reduction in PRRSV yield (Zhang et al., 2006). To evaluate the effect of PPMO combination on inhibiting PRRSV replication, the PPMO 5UP1 was paired with each of the following individual PPMO, 1bP1, 2P1, 3P1, 4P1, 5P1, 6P1, 7P1, and NSP3 at different concentration range. In the initial experiment, the 5UP1 at the concentration of 2 μM was paired with 16 μM of each of the other PPMO.

A virulent PRRSV strain, VR2385, was used to evaluate the combinatory effect of PPMO pair on inhibiting PRRSV replication in CRL11171 cells. The monolayer cells were inoculated with VR2385 virus at an MOI of 0.5 and incubated for 2 hours. Then cells were treated with various PPMO pairs and concentrations for 4 hours after the virus inoculation. Cells were then observed daily for the CPE development, the supernatant was collected at 48 h p.i., and the virus titers were determined by TCID₅₀ assay. Compared to the virus control group, treatment of CRL11171 cells with 2 μM 5UP1 alone appeared to reduce the yield of PRRSV for 0.5 to 1.0 log₁₀ TCID₅₀/mL (Figure 3-2A), which is in agreement with the previous observation (Zhang et al., 2006). In contrast, treatment of CRL11171 cells with a combination of 2 μM 5UP1 and 16 μM of each PPMO pair resulted in complete inhibition of PRRSV replication, and virus yield was not detectable (Figure 3-2A).

To further assess the combinatory effect of 5UP1 paired with other PPMO, the 5UP1 concentration was reduced from 2 to 0.5 μM and the other PPMO were at 8 μM or 16 μM. CRL11171 cells were treated the same way as performed on 2 μM 5UP1 treatment described above. Titrations of PRRSV from cell culture supernatant collected at 48 h p.i showed that treatment with 5UP1 at the concentration of 0.5 μM did not significantly reduce virus yield compared to virus control or mock treatment (Figure 3-2B). Among all these PPMO pairs tested, a combination of 0.5 μM 5UP1 and 8 μM 4P1 or 7P1 showed more synergistic inhibition effects on PRRSV replication than other PPMO pairs (Figure 3-2B). CRL11171 cells treated with 0.5μM 5UP1 in conjunction

with 8 μM of 4P1 or 7P1 caused a 2 to 3 \log_{10} TCID_{50} reduction in comparison to the mock-treatment or CP1 control (Figure 3-2C). Treatment with 16 μM of 4P1 or 7P1 in combination with 0.5 μM of 5UP1 strongly inhibited PRRSV replication and virus yields were below detection level, while each individual PPMO alone did not show detectable effect at the same concentration tested (Figure 3-2C). This experiment was repeated three times and similar results were observed each time. These results indicated that combination of 5UP1 (0.5 μM) with 4P1 or 7P1 (8 or 16 μM) was more effective in inhibiting PRRSV replication than a constituent individual PPMO of the pairs (Figure 3-2). None of the other PPMO pairs tested showed obvious synergistic effect than that of the individual PPMO in the corresponding group. Therefore, the 5UP1+4P1 and 5UP1+7P1 pairs were selected for further characterization.

Figure 3-2A:

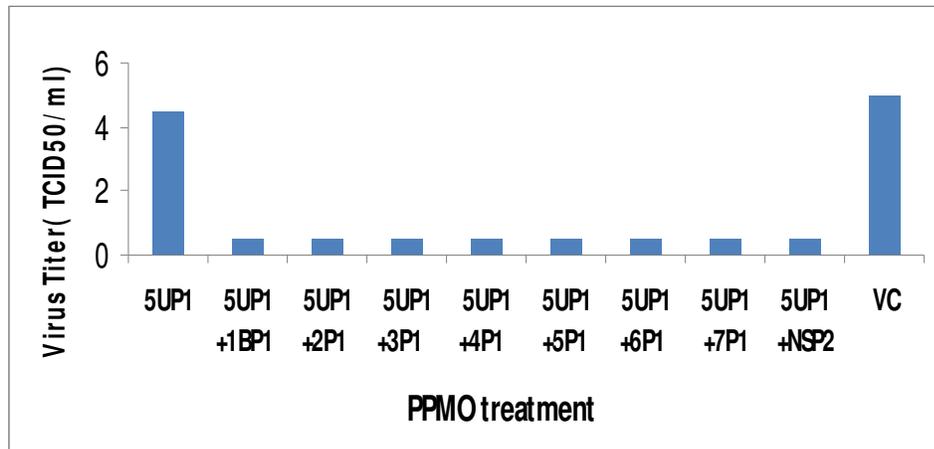


Figure 3-2B:

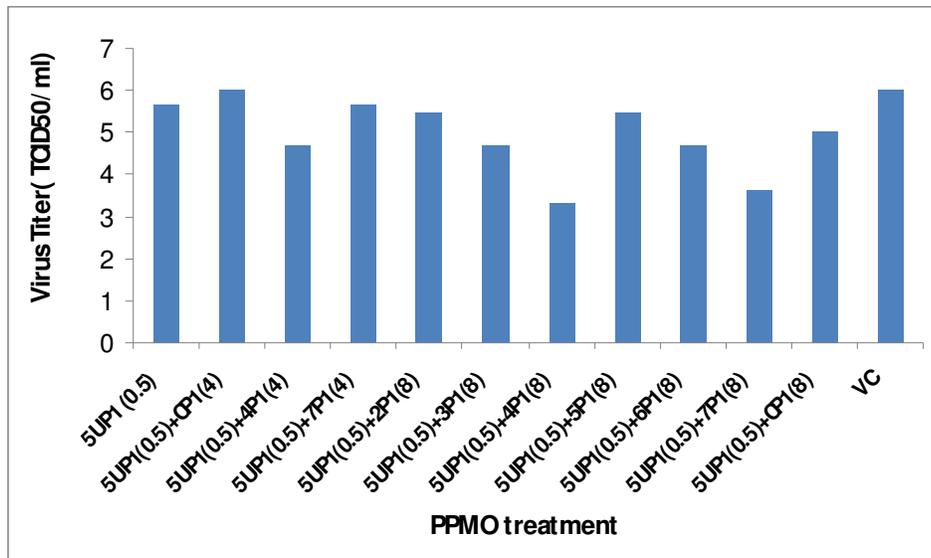


Figure 3-2C:

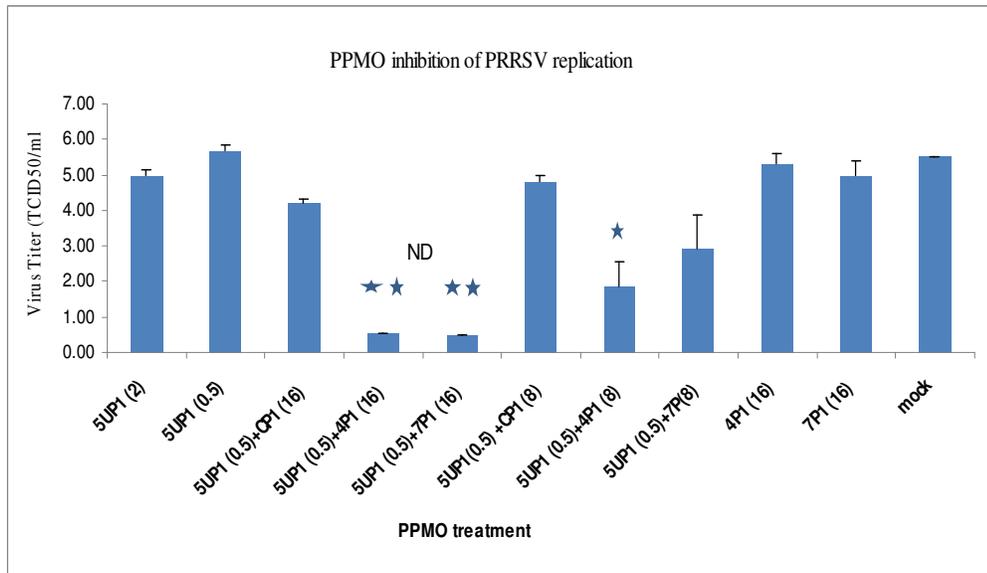


Figure 3-2: PPMO-mediated inhibition of PRRSV replication in infected CRL11171 cells. A. Titration of virus yield from CRL11171 cells after PRRSV infection and indicated PPMO treatment. Virus titer is shown as TCID₅₀ (log₁₀/ml). PPMO 5UP1 was used at 2 μM, and other PPMO used at 16 μM. Cells that were treated with PPMO combination had virus yield below detection level, and a bar is arbitrarily drawn to show the samples in the graph. “VC” indicates cells with virus infection but no PPMO treatment. B. Virus yields from cells treated with combination of PPMO 5UP1 at 0.5 μM and other PPMO used at 4 or 8 μM (in parenthesis). Cells treated with PPMO combination of 5UP1+4P1 or 5UP1+7P1 had better synergistic effect compared with other PPMO combinations at the same concentrations. C. Virus yields from cells treated with PPMO. 5UP1 was used at 0.5 μM and other PPMO used at 8 or 16 μM (in parenthesis). “Mock” sample is virus inoculation with no PPMO treatment. The

significance of difference in viral yields between the treatments: *, $P < 0.05$; **, $P < 0.01$. Cells that were treated with PPMO combination of 5UP1 (0.5 μ M) with 4P1 or 7P1 at 16 μ M had virus yield not detectable in this assay, and a bar is arbitrarily drawn to show the samples in the graph. The experiment was repeated three times and error bars are shown.

3.3 Effect of combination PPMO treatment on PRRSV RNA synthesis

PPMO 5UP1 was specific to the 5' terminal 21 nt of PRRSV genome. This region is known to be critical in the pre-initiation of translation (van der Born et al., 2004) of viral genomic RNA. The 5UP1 binding to its target site interferes with the translation of the viral genomic RNA, and therefore inhibits the translation of PRRSV replicase. Previous work at this lab has demonstrated that treatment of CRL11171 cells with 16 μ M 5UP1 led to a significant reduction in PRRSV minus-strand RNA synthesis as a result of lowered levels of replicase expression (Zhang et al., 2006). To assess the effect of PPMO combination on PRRSV RNA synthesis, CRL11171 cells were inoculated with VR2385 at an MOI of 0.5, treated with PPMO, and harvested at 24 h p.i for RNA isolation. The CRL11171 cells were treated with the two pairs of PPMO combinations, 5UP1 + 4P1 and 5UP1+ 7P1. The 5UP1 PPMO was used at the fixed concentration of 0.5 μ M, while 4P1 or 7P1 was tested at two different concentrations of 8 μ M and 16 μ M. Real-time RT-PCR was performed to measure the levels of PRRSV genomic RNA. The results are summarized in Figure 3-3. PPMO 5UP1 at the concentration of 0.5 μ M had no effect on viral RNA level. PPMO 4P1 or 7P1 at the concentration of 8 or 16 μ M also did not show

inhibition of viral RNA synthesis when compared to the viral RNA level in mock treatment (Figure 3-3), but the combination of 5UP1 (0.5 μ M) with 4P1 (8 μ M) or 7P1 (8 μ M) led to 100 to 1000 fold (2 to 3 logs) reduction of viral RNA level. More reduction in viral RNA level was observed when the concentration of 4P1 or 7P1 increased from 8 μ M to 16 μ M, which led to a RNA copies reduction of 1000 to 10000 fold (Figure 3-3). The results indicate the effect of PPMO combination treatment on the inhibition of PRRSV RNA synthesis.

It was noted that the 5UP1 and CP1 combination also slightly reduced the viral RNA level compared to the mock treatment, but the reduction in viral RNA level was much smaller than combination of 5UP1 and 7P1 or 4P1.

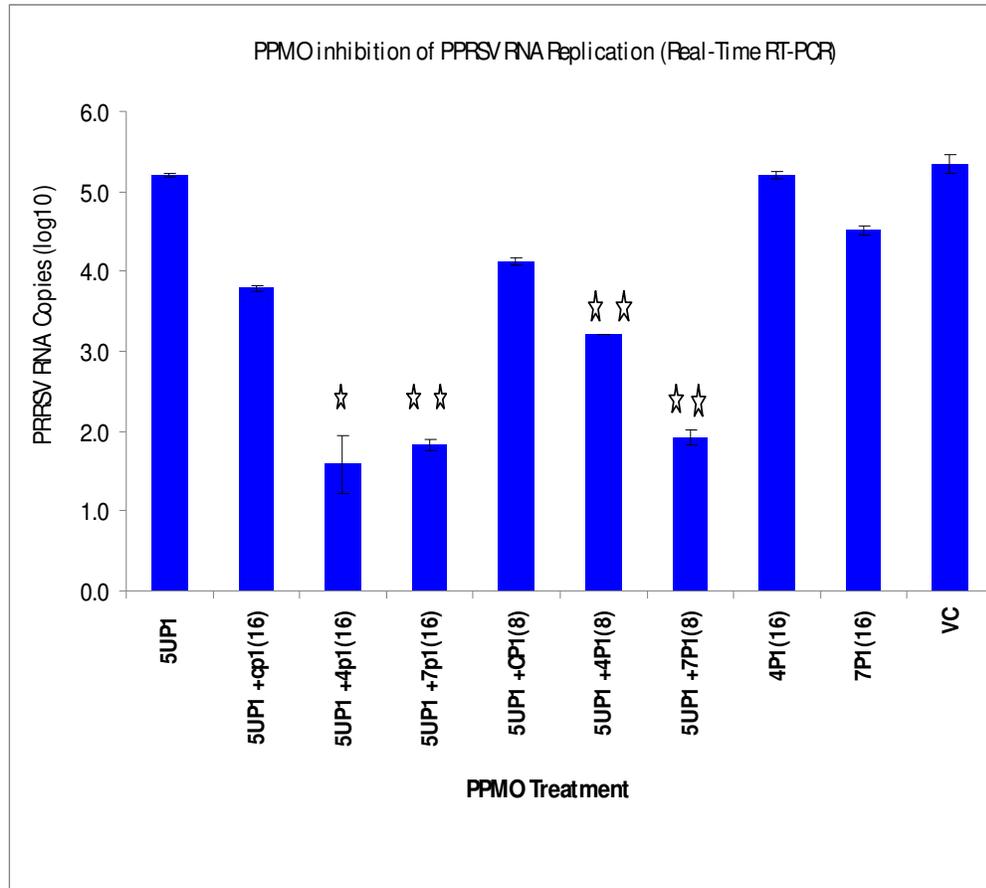


Figure 3-3: Effect of combination of PPMO treatment on PRRSV RNA synthesis.

Quantitation of PRRSV RNA was done by real-time RT-PCR. Treatment of the cells with PPMO combination of 5UP1 (0.5 μ M) with 4P1 or 7P1 at 16 μ M led to significant reduction of PRRSV genomic RNA level. The combination of 5UP1 with 4P1 or 7P1 at 8 μ M also reduce the virus RNA copies 2 to 3 logs compared with the virus control and those PPMO alone. The difference in viral RNA copies between the treatments was significant: *, $P < 0.05$; **, $P < 0.01$. The PRRSV RNA copy numbers were calculated based on a standard curve after normalization with transcript of β -actin.

3.4 Effect of PPMO combination treatment on PRRSV protein synthesis

The effect of treatment with PPMO combination on PRRSV protein synthesis was examined by IFA using a mAb EF11 against the PRRSV N-protein (Zhang et al., 1998). The IFA result is shown in Figure 3-4. Treatment of cells with combination of 5UP1 with 4P1 or 7P1 led to an absence of PRRSV-positive cells. In cells treated with combination of 5UP1 with the control PPMO CP1, fluorescent-positive cells were observed and the percentage was similar to the cells treated with 5UP1 alone. In cells treated with 7P1 or 5UP1 alone, some PRRSV-positive cells were observed, but far fewer than those treated with 4P1 alone or mock treatment control. These results indicate that the combination of 5UP1 with 4P1 or 7P1 inhibited PRRSV N protein expression in VR2385-inoculated cells.

The PPMO 4P1, 7P1 and CP1 were tested at 16 μ M and 5UP1 was at 0.5 μ M. Cell viability assay previously conducted in our lab has demonstrated that individual PPMO or PPMO combination at the concentration of 16 μ M did not have a notable cytotoxicity effect in CRL11171 cells (Patel et al. 2008). Cells treated with 16 μ M 5UP1 PPMO had similar viability to the cells treated with CP1-treated cells and mock treatment control (Zhang et al. 2006). The average relative percentages of cell viability were 97% for 7P1 treated cells. These results indicate that the PPMO was not cytotoxic at 16 μ M under these treatment conditions (Patel et al. 2008). Inhibition of the virus yield and protein synthesis observed in the antiviral experiments above was due to sequence-specific effects but not due to the reduction of the cell viability.

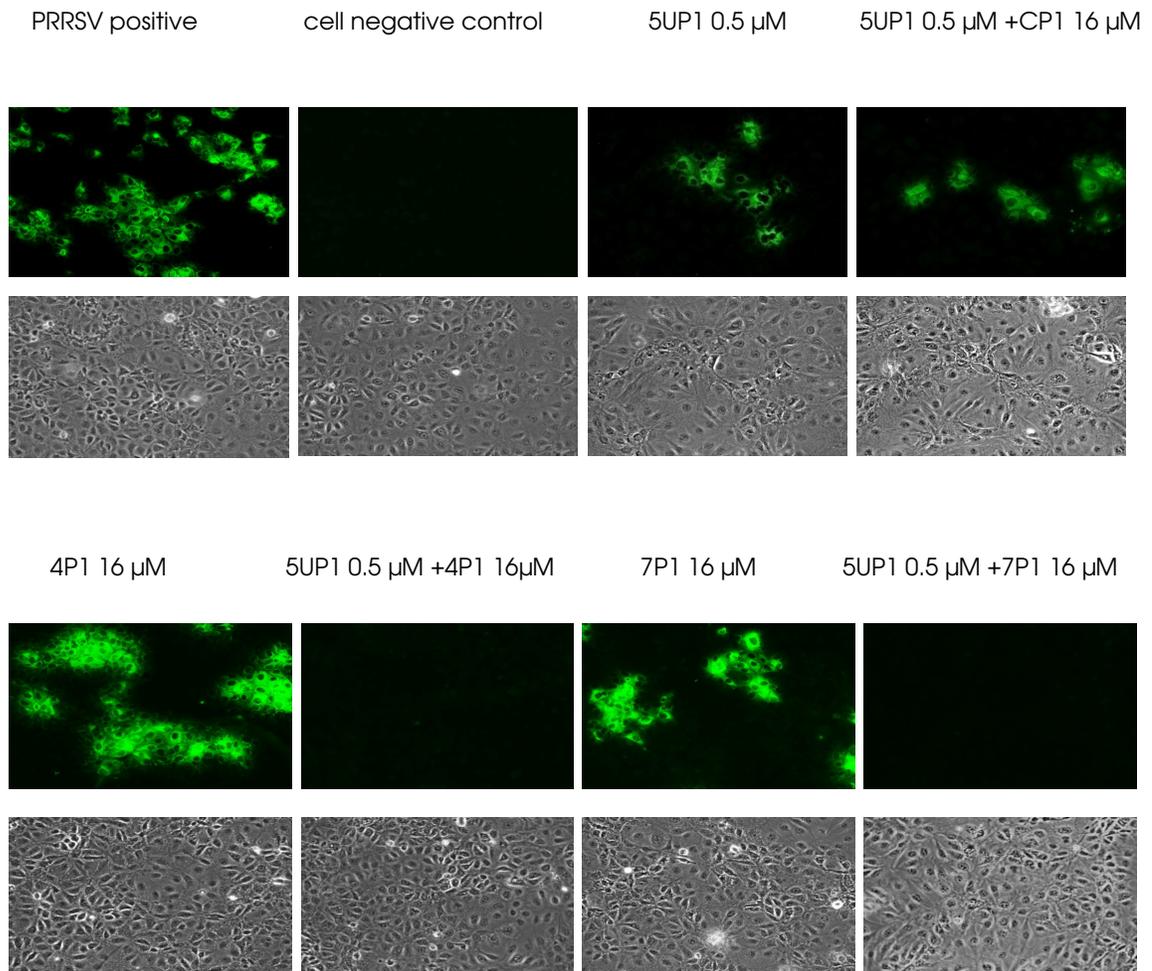


Figure 3-4 Immunofluorescence assay with N-specific mAb. Specific fluorescence was clearly visible at 24 h post PRRSV (VR2385) infection, while no fluorescence is observed in negative control. Treatment with PPMO combination of 5UP1 0.5 μ M+4P1 16 μ M or 5UP1 0.5 μ M+7P1 16 μ M resulted in the reduction of number of PRRSV-positive cells below detection level. The images below the green fluorescence images were taken under phase contrast to show the total number of living cells.

3.5 Sequence-specific inhibition of target mRNA translation in a cell-free reporter assay

To further characterize the PPMO's synergistic anti-PRRSV effect, a cell-free luciferase reporter assay was conducted. The sequence of PRRSV 5'UTR region (312nt) from subgenomic RNA7 was cloned upstream of a luciferase reporter gene in pCiNeoLuc plasmid. In this assay, PPMO binding to its RNA target could inhibit the translation of downstream luciferase coding sequence. Cell-free transcription and translation were conducted to test the luciferase translation in the presence and absence of PPMO. In this test, the cellular uptake of PPMO was avoided and the direct effect of PPMO combination on target mRNA translation was determined.

The results of sequence-specific inhibition of target mRNA translation in a cell-free reporter assay by PPMO or a PPMO combination are shown in Figure 3-5 and summarized in Table 3-2. The 5UP1 at the concentration of 10 nM and 7P1 at the concentration of 50 nM resulted in the expression of luciferase at about 30 to 40% compared to the mock treatment. When 10 nM 5UP1 and 50 nM 7P1 were combined, the luciferase production was down to 16% compared to the mock treatment control, far lower than each of these two PPMO alone. When 5UP1 was combined with control CP1 at the same concentration, no further effect was noticed on the inhibition of luciferase expression. The result demonstrated that the combination PPMO's synergistic effect on inhibition of target mRNA translation was sequence-specific.

Table 3-2: Sequence-specific inhibition of target mRNA translation in a cell-free luciferase reporter assay

PPMO	Concentration	Luciferase production	Relative percentage
5UP1	10 nM	3791	34.6
7P1	50 nM	3474	31.7
5UP1+7P1	10 nM+50 nM	1789.5	16.4
5UP1+CP1	10 nM+50 nM	4421	40.4
None	None	10944	100

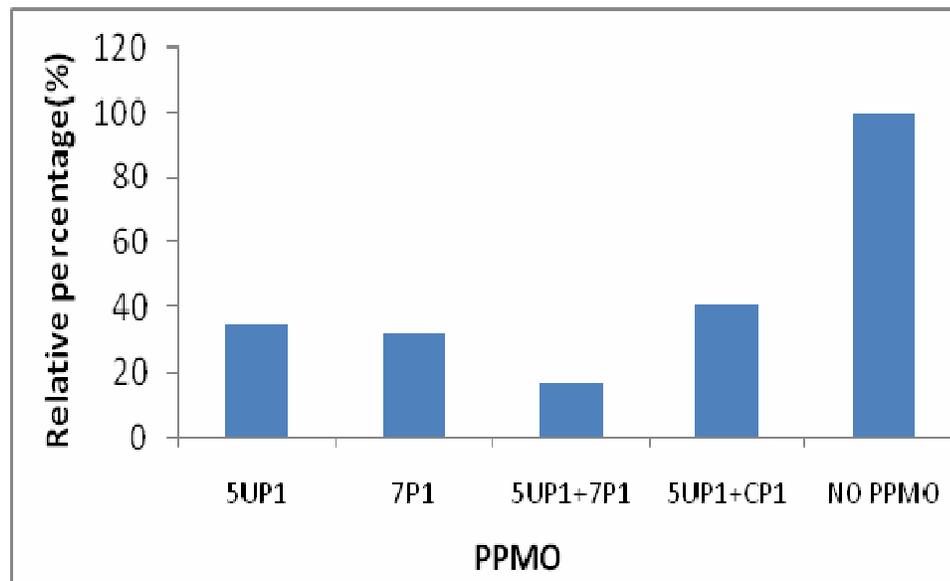


Figure 3-5. Inhibition of target RNA translation by PPMO in a cell-free luciferase reporter assay. Relative percentages of inhibition were calculated in comparison with signal of sample from none PPMO control. Combination of 5UP1 10 nM+7P1 50 nM showed enhanced effect in comparison with 5UP1 and 7P1 alone, while combination of 5UP1+CP1 at the same concentration had no such effect. This is one representative experiment of three repeats.

3.6 PPMO targeting the PRRSV 5' UTR can interfere with translation

PPMO 5UP1 is designed to target the 5'-terminal 21 nt of PRRSV genomic RNA. 5UP1 binding to the target sequence is expected to block the translation of ORF1a and ORF1b, which encode the viral replicases including RNA-dependent RNA polymerase. The viral replicase is also responsible for generation of full-length genomic antisense RNA used for viral RNA replication. To define the mechanism of PMO-mediated inhibition, we have cloned subgenomic RNA7 (ORF7) excluding 3'UTR and polyA into pcDNA3 vector under the mammalian CMV promoter. The resulting recombinant plasmid, pcDNA3-sg7, was transfected into CRL11171 cells to test the effect of PPMO combination on the inhibition of N protein expression. In this case, the target mRNA level is expected to remain the same in the presence or absence of PPMO because of the transient transfection under the same promoter. The PPMO effect on the translation of target mRNA in transfected cells can be determined.

After transfection of pcDNA3-sg7 into cells, the expression of N protein was detected by IFA using a mAb against PRRSV N-protein (Figure 3-6) at 24 h post-transfection. Treatment of the cells with PPMO combination 5UP1 +7P1 inhibited the N-protein expression to below detection level, while 5UP1, 7P1, or combination of 5UP1+CP1 had much less effect on N-protein expression. The relative lower level of N protein expression from pcDNA3-sg7 plasmid in the transfected cells made it difficult to quantitatively evaluate the synergistic effect using the transient expression system by Western blot.

Figure 3-6A

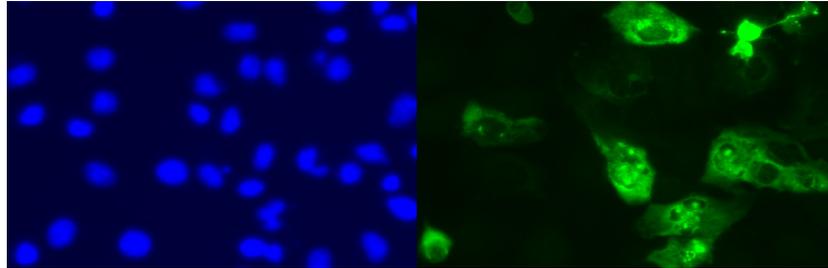


Figure 3-6B

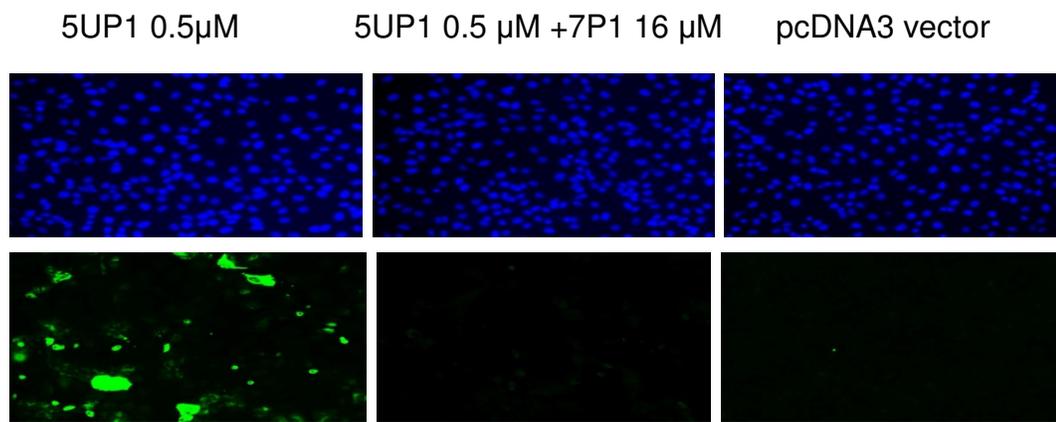


Figure 3-6C

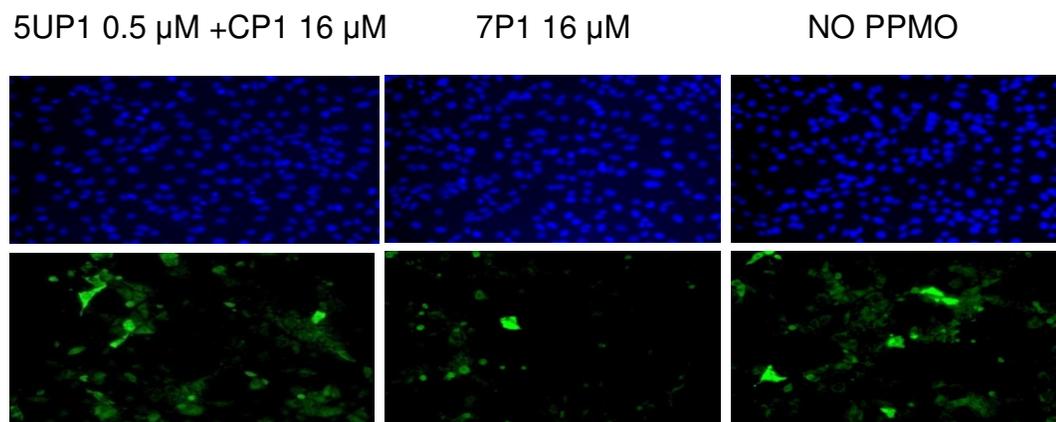


Figure 3-6: Immunofluorescence assay of cells transfected with PRRSV N-plasmid pcDNA3-sg7. A. IFA of the cells with N-specific EF11 monoclonal antibody. The N-protein is expressed mainly in the cytoplasm. B. Effect of PPMO on N-protein expression in the transiently transfected cells. Treatment of the cells with PPMO combination 5UP1 +7P1 inhibited the N-protein expression to below detection level, while 5UP1, 7P1, or combination of 5UP1+CP1 had much less effect on N-protein expression. The image above the green fluorescence images were taken with a DAPI filter to show the total number of living cells.

Since the plasmid pCINeoLuc-5UTR contains 5'UTR sequence of subgenomic RNA7, which has the targets of PPMO 5UP1 and 7P1, this plasmid was also used to transfect into CRL11171 cells. The luciferase production was quantitatively measured and compared between the cells treated with PPMO and the non-treatment control. The result of this analysis is shown in Figure 3-7 and data summarized in Table 3-3. The treatment of CRL11171 cells with combination of 5UP1 (0.5 μ M) +7P1 (16 μ M) resulted in about 30% reduction in luciferase yield in comparison with 5UP1 (0.5 μ M) alone. The combination of 5UP1 (0.5 μ M) +CP1 (16 μ M) did not have further effect compared with 5UP1 (0.5 μ M) alone. This quantitative result further confirmed that combination of 5UP1 0.5 μ M +7P1 16 μ M had enhanced inhibitory effect on the translation of target gene, while 5UP1 +CP1 has no such effect.

Table 3-3: Sequence-specific inhibition of target mRNA translation of a luciferase reporter in CRL11171 cells

PPMO	Luciferase reading	Relative Percentage
No PPMO	3069	100
5UP1	2683	87.4
7P1	3047	99.3
5UP1 +7P1	1816	59.2
5UP1 +CP1	2546	82.9

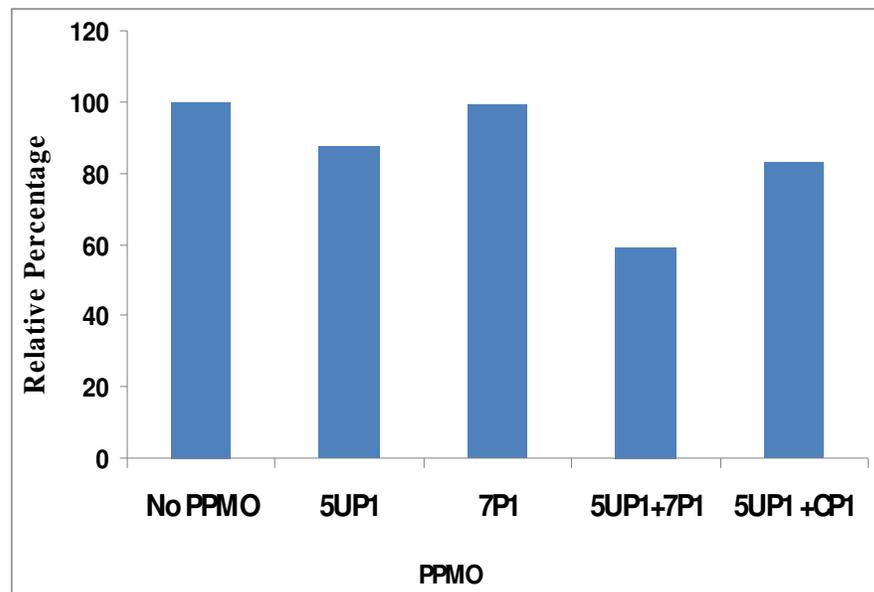


Figure 3-7: Inhibition of target RNA translation by PPMO in a luciferase reporter assay.

PciNeoluc-5UTR plasmid was transfected into CRL11171 cells and luciferase yield was

measured. Relative percentages of luciferase yield were calculated in comparison with signal of none PPMO control. Combination of 5UP1 0.5 μ M +7P1 16 μ M shows enhanced inhibitory effect, while 5UP1 +CP1 has no such effect.

3.7 Inhibitory effect of PPMO combination against heterologous PRRSV strains

The PPMO in this study were designed based on the sequence from the VR2385 strain of North American genotype. To determine the efficacy of combination of 5UP1 with 4P1 or 7P1 against other PRRSV isolates, cross strain inhibition assay was conducted. PRRSV strains FL-12, 16244B, 16138, 11604, 17041, 14680, 12773, 13909, and Lelystad were used in this test. The Lelystad strain is a prototype of the European genotype. Other strains belong to North American PRRSV genotype. The combination of 5UP1 with 4P1 and combination of 5UP1 with 7P1 were tested against each of the PRRSV strains. CRL11171 cells were treated with PPMO after inoculation with the respective viruses. Cells were then observed daily for the CPE development, the supernatant was collected at 48 h p.i., and the virus titers were determined by TCID₅₀ titration assay.

The result for cross strain inhibition assay is shown on Figure 3-8. Virus titration results showed that the combination of 5UP1 and 4P1 and combination of 5UP1 and 7P1 effectively inhibited PRRSV replication of all strains except Lelystad virus. Treatment of PRRSV infected cells with PPMO combinations of 5UP1 at 0.5 μ M with 4P1 or 7P1 at 16 μ M led to virus yields not detectable in this assay. In contrast, replication of the Lelystad virus was not inhibited by any of the PPMO combinations tested. It is not

surprising that the PPMO has little effect on Lelystad virus because the sequence alignment of these PPMO target sites of the PRRSV strains showed that Lelystad virus had low sequence identity with the other strains. In comparison with VR2385, Lelystad has 13 bp mismatch the 5UP1 target site, 5 bp mismatch in 4P1 target site, and 5 bp mismatch the 7P1 target site (Figure 3-9). The results of cross strain inhibition assay further confirmed the sequence-specific inhibition of PPMO on the PRRSV replication and indicates the potential application of the PPMO combination in the field against prevalent heterologous PRRSV isolates.

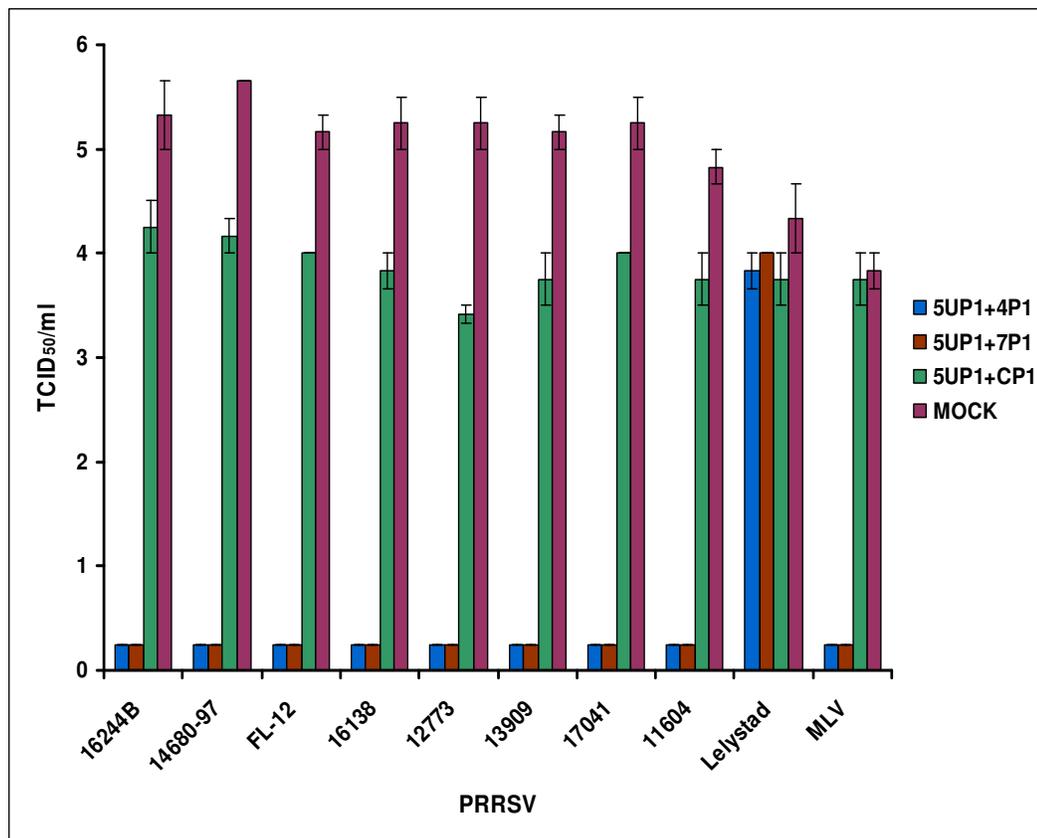


Figure 3-8: Cross strain inhibition assay. A. Virus yield titration shows inhibition of nine North American PRRSV strains by PPMO combination of 5UP1+4P1 or 5UP1+7P1. Lelystad is a prototype of European PRRSV genotype. All other strains are North American PRRSV genotype. “Mock” is virus infection control without PPMO. Treatment of the cells with PPMO combination led to suppression of PRRSV replication of all North American strains, which had virus yields not detectable in this assay, and bars are arbitrarily drawn to show the samples in the graph.

5UP1

PPMO target	c-gtatagggtggtggctctatg
VR 2385	.-.....
11604	.-.....
16138	.-.....
16244b	.c.ggc.....
FL-12	.-.....
MLV	.-.....
Lelystad	tgtgtag...a..cc.c...ca

4P1

PPMO target	acctagaatggctgcgtccc
VR2385
16244bt.....
MLVt.....
FL-12c.....
Lely	.tt.g.....g.a

7P1

```
PPMO target  ggtaaaccttggttaaatatg-cc
VR2385       .....-..
16138       .....c.....-..
11604       .....c.....-..
16244b      .....c.....-..
17041       .....c.....-..
14680       .....c.....-..
12773       .....c.....-..
13909       .....c.....-..
MLV         .....c.....-..
Lely        ...t...c..c..g...g..
```

Figure 3-9: Alignment of target sequence of PPMO 5UP1, 4P1 and 7P1. Sequence analysis identifies nucleotide mismatches between PPMO and their complementary target sites in PRRSV RNA. PRRSV strain names are listed in the first column. “Lely” stands for Lelystad strain. PPMO names are listed above the sequence. VR2385 sequence is used as the reference sequence, as the PPMO were designed against it. For all other sequences, only nucleotides differing from the reference sequence are shown, and identical nucleotides are indicated as “.”. Missing nucleotides are indicated as “-”. The initiation codon ATG of ORFs 4 and 7 is underlined. GenBank accession numbers for PRRSV strains in the alignment are listed in parenthesis: Lelystad (M96262), FL-12 (derived from infectious clone of AY545985), 16244B (AF046869), 11604 (EF523345), Ingelvac MLV (EF484033), and 16138 (EF523346). Nucleotide sequences for other strains in the figure are unpublished.

CHAPTER 4: DISCUSSION

The high prevalence of the PRRS and the limited efficacy of both inactivated and attenuated vaccines have shown the need to search for effective antiviral compounds. PMO, as a novel antisense compound, specifically targets the RNA sequence and displays highly biological stability and low toxicity.

For RNA virus with the positive sense genome, the efficient replication of the RNA virus requires essential sequence and structural elements at the terminal UTRs of the viral genome for viral replication, subgenomic RNA synthesis and translation. The terminal regions are recognized by the viral replicase (Panavas et al., 2002; Tortorici et al., 2003)

The 5' terminal UTR of PRRSV genome was found to contain the most sensitive target for inhibiting virus replication with PPMO (Zhang et al., 2006; Patel et al., 2008). PPMO 5UP1 targeting the 5' terminus of PRRSV genome were found to be highly effective in inhibiting the PRRSV replication in cell culture in a sequence-specific and dose-responsive manner in the concentration ranging from 2 to 32 μM (Zhang et al; 2006; Patel et al.2008). Treatment of cells with individual 5UP1 at the concentration of 16 μM resulted in more than 4.5 \log_{10} reduction in PRRSV yield, compared to a control PPMO (Zhang et al., 2006). Other PPMO designed to target the 3'-terminal region, translation initiation regions of ORFs 2 through 7, and negative-sense PRRSV RNA

(Table 3-1) were found to be less or no effective against PRRSV replication when used individually, even at the relative higher concentration than 5UP1 (Patel et al., 2008).

In this project, we evaluated various combinations of two PPMO on inhibiting PRRSV replication in cell culture. One of the two PPMO in the combination test was 5UP1, and the other one was selected among the PPMO listed in Table 3-1. PPMO 5UP1 was tested at 2 and 0.5 μ M, respectively. To our surprise, the combination of 5UP1 at 2 μ M with all other PPMO tested completely inhibited VR2385 replication to below detection level, while 5UP1 at 2 μ M had a little effect. This result suggests the combinatory effect of two PPMO. PPMO 5UP1 at the concentration of 0.5 μ M was paired with 8 or 16 μ M of each of the following PPMO: 1bP1, 2P1, 3P1, 4P1, 5P1, 6P1, 7P1, and NSP2. Of the total 8 sets of pair combination, we found two sets of combinations, 5UP1+4P1 and 5UP1+7P1, were highly effective in inhibiting the PRRSV replication. Treatment of CRL11171 cells with 0.5 μ M 5UP1 alone had little impact on PRRSV replication, but a combination of 0.5 μ M 5UP1 with 4P1 or 7P1 had a significant synergistic effect on inhibiting PRRSV replication than did either one of the two PPMO alone. Virus titration clearly demonstrated that 5UP1 combined with 4P1 or 7P1 was effective in a dose-responsive manner in inhibiting PRRSV production in infected cells. The reason for the ineffectiveness of the other 6 sets of combination PPMO that targets the initiation site of individual ORF is not clear, but could be due to inaccessibility of PRRSV target sequence or successful PPMO/target-RNA hybrid did not affect PRRSV replication. It is also speculated that GP₄ and N proteins have essential roles in PRRSV replication. However, we do not have data to support this theory since GP₄ or N gene

expression was not monitored and their roles in PRRSV replication are not well understood.

A moderate reduction of PRRSV replication was also observed for the cells treated with individual 6P1 or 7P1 at a relatively high concentration, but combination of 6P1 and 7P1 was found to be more effective in inhibiting PRRSV replication than did either of the two PPMO alone (Patel et al., 2008). A combination of PPMO produced greater efficacy than either PPMO alone against Influenza A virus replication in cell culture (Ge et al., 2006).

Applications of PPMO targeted to the terminal UTRs successfully suppressed virus replication in a sequence-specific manner *in vitro* have been documented for many RNA viruses (Kinney *et al.*, 2005; Deas *et al.*, 2005; Neumann et al., 2005; van den Born et al., 2005; Ge et al., 2006). Recently, PPMO have been also applied *in vivo* and protected animals from challenge with Ebola Virus (Enterlein et al., 2006), Coxsackievirus B3 (Yuan et al., 2006), and murine coronaviruses (Burrer et al., 2007).

To elucidate the mechanism of combination PPMO-mediated inhibition, we conducted quantitative RT-PCR analyses to assess PRRSV RNA level (Figure 3-3). Treatment of cells with the combination of 5UP1 with 4P1 or 7P1 led to a significant reduction of PRRSV genomic RNA level, indicating inhibition of PRRSV RNA synthesis, likely accomplished through blocking translation of the replicase encoded by ORF1a/b. It is not clear whether the presence of 4P1 or 7P1 enhanced the blocking effect of 5UP1

or the combinatory effect of blocking both target sites of the PPMO pair. It is likely that the combinatory blocking effect was responsible for the reduction of PRRSV genomic RNA since combination of 5UP1 with CP1 or other PRRSV PPMO did not have the enhanced effect. It will be interesting to define if the combinatory effect is due to blocking of the two target sites in this assay. An affirmative result will provide a proof of principle for application of the PPMO combination in the field to protect pigs against infection by heterologous PRRSV isolates. A PPMO-associated reduction in PRRSV RNA production by 5UP1 alone at 16 μ M was also observed by RT-PCR in the previous study conducted in this laboratory (Zhang et al., 2006). In addition to evaluating the inhibition at RNA synthesis level, the effect of PPMO combination on PRRSV protein synthesis was also examined by IFA using a mAb against the PRRSV N-protein. The IFA results showed that treatment of cells with 5UP1 combined with 4P1 or 7P1 led to inhibition of viral major N protein synthesis. This result is consistent with the level of PRRSV genomic RNA in previous publications (Patel et al 2008). The reduction of N protein in cells treated with 5UP1+4P1 indicates that PRRSV subgenomic RNA synthesis was reduced.

To further elucidate the mechanism of the inhibition by PPMO combination, a cell-free luciferase/translation assay was performed in the presence or absence of the PPMO combination. This assay avoids PPMO delivery issue in cell culture and directly tests the PPMO combinatory effect. For this assay, only the fragment of PRRSV 5'UTR region (312 nt) from subgenomic RNA was subcloned upstream of a luciferase reporter gene plasmid, as a proof of principle. Concurrent PPMO binding to their RNA target

sites inhibits the translation of downstream luciferase-coding sequence. Our results indicated that 5UP1+7P1 displayed synergistic effect in inhibition of translation of luciferase reporter gene in a sequence specific manner. In this study, the direct effect of PPMO combination on target mRNA translation was determined. The result indicated that the combination PPMO's inhibitory effect was related to their inhibition of target mRNA translation. Since this is a sensitive test, and the concentration of PPMO used was a little high, presence of individual PPMO also showed inhibitory effect. But clearly the PPMO combination offered enhanced effect in reducing luciferase yield.

To examine the effect of PPMO combination on target mRNA translation in cells, a cell culture based translation assay was also performed using transient expression of the ORF7 gene driven by a eukaryotic promoter-cytomegalovirus (CMV) promoter. To do this, we cloned the 5'UTR region and ORF7 gene from subgenomic RNA7 into the pcDNA3 vector and generated the pcDNA3-Sg7 plasmid. Transfection of the pcDNA3-Sg7 into the CRL11171 cells resulted in expression of ORF7 gene (N protein). The expression level of the N-protein from the transient transfection was low in CRL11171 cells, which might be due to the long UTR (312 nt) present before the ORF7 gene or due to low transfection rate. The first speculation may be the reason since we conducted transient transfection of the cells with a GFP reporter plasmid and observed approximately 30% GFP-positive cells. In PRRSV-infected cells, virus-encoded polymerase translates the ORF7 gene from subgenomic RNA efficiently, as shown by intense immunostaining in IFA with N-specific mAb (Fig 3-4). It is likely that the cellular polymerase does not work well on such RNA template. However, the synergistic

effect of PPMO combination on the expression of ORF7 gene was also observed when compared to the cells treated with individual PPMO. Similar inhibition effect on the translation was confirmed by transfecting the luciferase reporter plasmid that contains the PRRSV 5'UTR region cloned into the upstream of the luciferase reporter gene. The advantage of the luciferase reporter system is the quantitative measurement of the luciferase yield, which is preferred to IFA under such scenario due to low rate of N-positive cells in IFA.

These two PPMO pairs were also shown to have an inhibitory effect on several North American PRRSV strains in our cross strain inhibition assay, but had no inhibitory effect on the European PRRSV strain. Sequence alignment showed that 5'UTR region in PRRSV genome is quite conserved across strains in the same genotype. However, the European strain is quite different from the North American strains and shares only 40% sequence identity in 5'UTR and 67% in ORF2-7 with North American strains. This result further proved that the PPMO works in a sequence-specific manner, which is quite a good advantage of PPMO as an antiviral compound compared to small chemical drugs. As the most important swine disease, PRRS causes enormous economic losses in the swine industry. Due to its easy transmission, high variability, and lack of a broadly protective vaccine, the control of this disease is difficult. Currently available vaccines have limited effect, especially when used against the heterogeneous PRRSV strains, which are commonly found in outbreaks (Meng, 2000; Opriessining et al, 2002)

PPMO combination of 5UP1+4P1 and 5UP1+7P1 inhibited replication of ten North American PRRSV strains in our cross-strain inhibition assay. Sequence alignment showed that the region targeted by these PPMO contains highly conserved sequences. In addition, analysis of PRRSV sequences from the GenBank indicates that the complementary sequences of 5UP1 and 7P1 are highly conserved across North American PRRSV strains. Thus, these two antisense PPMO have the potential for application against most North American PRRSV strains. The broad inhibition by the two sets of PPMO combination further confirms that the 5' UTR of PRRSV is a highly productive target. A cell viability assay of PPMO-treated CRL11171 cells detected no cytotoxicity, indicating that the suppression of PRRSV replication observed in the antiviral assays was due to PPMO-specific inhibition of PRRSV molecular events. The absence of PPMO-induced cytotoxicity at effective antiviral concentrations is an important attribute of these compounds, when considering potential *in vivo* applications.

The prevalence of PRRSV and financial losses associated with PRRSV infection in swine herds is high, and current strategies to control PRRS, including the use of commercial vaccines, are inconsistent and generally less than adequate (Meng et al., 2000; Opriessnig et al., 2002). Specific anti-PRRSV drugs are urgently needed to control PRRS disease. In this project, two pairs of PPMO combination (5UP1+ 4P1 and 5UP1+7P1) were found to effectively inhibit the PRRSV replication in cell culture and can be considered potential drug candidates for use in PRRSV control, especially when high variability and mutation rate of PRRSV replication are considered. Further

investigation into the pharmacokinetic, toxicological, and antiviral properties of these PPMO combinations *in vivo* against heterologous PRRSV strains is warranted.

CHAPTER 5: REFERENCES

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