Spring 5-8-2011

Expression of Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) non-structural protein 12 in Escherichia coli

Ariann Boylan
University of Connecticut - Storrs, ariannboylan@gmail.com

*

Honors Thesis

*

"Expression of Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) non-structural protein 12 in *Escherichia coli*"

By: Ariann Boylan
Department of Animal Sciences

College of Agriculture and Natural Resources

University of Connecticut

*

Honor Thesis Advisor:

Dr. Guillermo Risatti

Department of Pathobiology and Veterinary Science

College of Agriculture and Natural Resources

University of Connecticut

2011

Abstract:

Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) is single-stranded, positive-sense RNA virus in the family *Arteviridae,* order Nidovirales. PRRSV began circulating in the U.S swine population in the 1980's and since then becoming the most economically significant disease of swine herds. PRRSV is now disseminated worldwide causing great economic losses to pork industries. PRRSV single-stranded RNA genome is 15 kb in length. The genome encodes 9 open reading frames (ORF1a, ORF1b, ORF2a, ORF2b and ORFs 3 through 7). ORFs 1a and 1b encode for 13 non-structural proteins (nsp) that are suggested to be involved in transcription and viral genome replication. The exact role of non-structural proteins in PRRSV cycle is still unknown. Moreover, there is a limited availability of reagents, such as antibodies against these non-structural proteins, that further limits their study. To overcome that limitation, the gene encoding for non-structural protein 12 (nsp-12) was synthesized using the polymerase chain reaction (PCR) and cloned in-frame into a plasmid vector (pRSET A) to create the plasmid construct pRSETAnsp-12. After transforming *Escherichia coli* with pRSETAnsp-12, individual colonies that grew in the presence of ampicillin were selected, cultured in terrific broth-ampicillin media, and plasmid DNA extracted to evaluate for the presence and fidelity of the nsp-12 gene by means of agarose gel electrophoresis separation and sequencing. DNA plasmids harboring a correct copy of the gene were used to express nsp-12 protein in BL21 competent *Escherichia coli* cells. After growth of BL21 transformants in "Magic media"®, expression of nsp-12 was assessed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) separation followed by Coomassie blue staining and western blot detection using an anti-His monoclonal antibody. PRRSV nsp-12 was then purified by metal

affinity chromatography. Purified nsp-12 protein will be used to produce an anti-nsp-12 polyclonal antibody serum in rabbits for further study the functions of this protein.

Acknowledgements

I would like to thank Dr. Guillermo Risatti for his guidance and instruction throughout my research and during the composition of this thesis, as well as the members of his lab for their help. Special thanks to Kara Rogers and Boris Gavrilov for their assistance and direction, which was vital to the completion of my research. I would also like to thank my family for their support of all of my academic endeavors.

Table of Contents

Need for Research

Porcine Reproductive and Respiratory Syndrome Virus is currently one of the most economically devastating diseases affecting the swine industry. The virus belongs to the family *Arteriviridae* and first appeared in North America in 1987, followed by Western Europe in the 1990s. Porcine Reproductive and Respiratory Virus Syndrome infection induces a wide variety of clinical signs, notably reproductive failure in sows and respiratory disease in swine of all ages. PRRSV genetic determinants of virulence are still poorly understood.

The virus is readily transmitted via the respiratory route and infects primarily cells of the immune system. There are few, if any, effective treatments. While there are two main strains of the virus, considerable genetic variation among individual isolates, when used as vaccines, fail to induce a cross-protective immune response. This genetic variation is one of the main obstacles in producing vaccines that can confer heterologous protection.

The overall goal of the laboratory is to identify PRRSV genetic determinants affecting virus-host interactions and virus virulence. To accomplish this goal and better elucidate the interactions of PRRSV proteins with host cellular proteins, there is a need for reagents that would allow detection of those interactions. Thus, production of reagents such as antibodies to detect specific viral proteins is of paramount importance for these studies. The objective of the work described here was to express and purify PRRSV nsp-12 protein, one of the proteins in the virus replication complex. In turn this purified viral protein will be used for producing antibodies.

Review of the literature

Arteriviruses

Porcine Reproductive and Respiratory Syndrome Virus is a member of the virus family *Arteriviridae*, which also includes Equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV), and simian hemorrhagic fever virus (SHFV). These viruses are distinguished by their structure, ability to manifest in a persistent state of infection, and an overall similar genome organization (Snijder & Meulenberg, 2001). The arteriviruses also have a genome organization and mechanism of protein expression similar to that of the coronaviruses, especially of the replicase genes encoding for the non-structural proteins. Arteriviruses and coronaviruses were grouped into the order Nidovirales based on this correlation, as well as the presence of unique subgenomic (sg) messenger ribonucleic acids (mRNAs) (Snijder & Meulenberg, 2001). Equine arteritis virus was fully sequenced in 1991, and this virus has provided a model for arterivirus research. The viruses are easily inactivated by detergents, high or low pH, and high temperatures.

Arteriviruses virions are enveloped, spherical in shape, small, with a diameter between 40 to 60 nanometers (nm). The surface of the virus is smooth with few notable projections (envelope glycoproteins). The viral nucleocapsid is surrounded by a lipid bilayer envelope. The RNA positive-sense genome, with lengths between 12.7 kilobases (kb) to 15.7 kb, is surrounded by the nucleocapsid, which is situated between a lipid bilayer containing a relatively large number of envelope proteins (Snijder & Meulenberg, 2001). Of these proteins there are usually two structural components and several glycoproteins (Snijder & Meulenberg, 2001).

History

Porcine Reproductive and Respiratory Syndrome first appeared in North America in 1987, and was then found in Western Europe in the 1990s (Albina, 1997). However, retrospective studies revealed that PRRSV had appeared in Canada as early as 1979 and the US in 1985. This information was gathered in the early 1990s when diagnostic assays revealed that PRRSV had spread almost universally to every region in which swine were domesticated. The virus was isolated in the Netherlands and termed the Lelystad Virus, and then in the US where it was called Swine Infertility and Respiratory Syndrome (SIRS) (Zimmerman, 2006).

Clinical signs/epidemiology

Porcine Reproductive and Respiratory Syndrome Virus can induce a variety of clinical signs and presentations in infected swine. The severity and the extent of clinical signs often depend on the physiological state of affected animals, including factors such as age, sex and reproductive status. Furthermore, PRRS sometimes becomes endemic in a herd and infects swine of all ages, and all may exhibit different clinical signs. This makes clinical diagnosis difficult (Zimmerman, 2006). Some of the most common symptoms are fever, anorexia, reproductive failure, blue ears, pneumonia and respiratory disease, and secondary bacterial infection (Rossow, 1998). The initial onset of PRRS in a herd is usually characterized by anorexia in a large percentage of swine of all ages, which can last up to five days. This anorexia spreads slowly throughout the herd as the virus is transmitted, leading to the term “rolling inappetance” (Zimmerman, 2006). Symptoms in sows include spontaneous abortions, late-term abortions (including fresh stillborn piglets as well as mummified piglets), and small, weak offspring

though the sow may not appear sick (Zimmerman, 2006). Boars experience anorexia and reproductive failure due to infection of reproductive organs. The virus can spread to sows through infected semen. Young and grower pigs typically experience anorexia and may show some degree of reduction of average daily weight gain (Zimmerman et al., 2006). PRRSV causes lesions in target organs during replication swine. Consistently these are pneumonia and enlarged lymph nodes, and abortions that usually follow microscopic lesions in lungs and reproductive tissues (Zimmerman, 2006).

Pathogenesis

Porcine Reproductive and Respiratory Syndrome Virus causes disease in a variety of ways by affecting different organ systems in swine. After the virus enters the host, it preferentially infects and productively replicates in alveolar macrophages. Important to note is that PRRSV can also infect and replicate within spermatogenic cells in boars resulting in contamination of semen.

PRRSV enters susceptible host cells via endocytosis, and after productive replication the virus progeny exits infected cells via nucleocapsid budding from cellular membranous structures (i.e: the endoplasmic reticulum or the Golgi apparatus) and subsequent fusion of these vesicles with the cellular membrane (Zimmerman, 2006). After primary replication, a state of viremia is achieved in infected animals. PRRSV can then effectively spread to different organ systems within the host. Replication of PRRSV within host cells often results in cell death via apoptotic pathways that also may affect proximal uninfected cells (Zimmerman, 2006). The effect of apoptosis induced by PRRSV in infected tissues on virus pathogenesis is not clearly understood.

PRRSV infection results in a secretion of cytokines from infected cells, including interferon-gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), and interleukins (IL) 1, 6, 9, 10, and 12. These pro- and anti-inflammatory cytokines trigger the initiation of the host immune response aimed to eliminate PRRSV infection, but can also have negative effects by inducing inflammation of infected organs (Zimmerman, 2006) and even growth retardation (Hollis, 1993). The role of these cytokines in the pathogenesis of PRRSV is not clear.

Porcine Reproductive and Respiratory Syndrome Virus has a marked effect on both infected and uninfected fetuses carried by an infected dam. Rowland, 2010; studied PRRSV replication in fetuses of dams infected late in gestation. The authors observed that both infected and uninfected fetuses displayed signs of PRRSV due to maternal infection, including growth retardation and decreased amniotic fluid. In that study it was observed that the primary site of PRRSV replication in the fetus was the thymus. The lymph nodes of infected fetuses resembled lymph nodes that had been activated by antigens, meaning that the fetuses mounted an immune response to the virus. This observation was supported by the detection of increased cytokine gene expression in infected fetuses, including TNF-α and IFN-γ (Rowland, 2010). The negative impact of this immune response will be discussed later in this thesis.

Although significant progress has been made on understanding the effects of PRRSV infection in swine, the genetic determinants of virulence, attenuation, and host range are yet to be elucidated.

Transmission

One of the primary issues plaguing swine producers is the high transmissibility of PRRSV within and between herds. The virus is readily infectious and is therefore very difficult to prevent the spread of disease. The most prevalent method of transmission is the respiratory route (Snijder & Meulenberg, 2001). Contact between pigs in which nasal secretions, urine or feces are transferred is one way the virus spreads. Since feces are a mode of transmission, fecal matter used in fertilizer can also promote PRRSV spread (Albina, 1997). Aerosolized particles of the virus can also travel over short distances, especially during the winter months (Albina, 1997). PRRSV can be shed via semen in the absence of viremia and in the presence of anti-PRRSV antibodies (Christopher-Hennings, 1995, 2001). The virus most likely infects the reproductive tissue via macrophages and monocytes. Boars can shed virus in semen for up to 90 days (Christopher-Hennings ,2001) post-infection, generating a serious problem for artificial insemination centers.

Kang, 2010; conducted a study investigating the transmissibility of PRRSV from sows to nursing piglets. Evidence from this study confirmed that PRRSV is localized in mammary tissue (in addition to other tissues including the lung, lymph node, spleen liver, and tonsils). However, PRRSV is not found in the epithelial cells of the mammary gland, which would allow the virus to pass directly into the milk. Despite this characteristic, the virus is indeed found in the milk of sows due to infection of the tissues listed above and subsequent release into the milk via immune cells, an indirect pathway. Viral particles and viral antigens are both detected in milk from infected sows,

but it is unclear whether the amount of PRRSV present is sufficient to infect a suckling piglet (Kang, 2010).

Effect of PRRSV on swine growth and development

Porcine Reproductive and Respiratory Syndrome Virus is, as stated earlier, the most economically significant disease currently affecting the swine industry. This is due to the fact that PRRSV impacts negatively on swine fertility and swine growth, which are the foremost factors in the economical gain. PRRSV impacts fetal growth and can lead to abortions and stillbirths and decreases weight gain in pregnant gilts. In grower pigs, economic losses derive from decreased appetite, diminished food intake, and delayed growth of infected swine.

Lewis, 2010; conducted a study investigating the effect of PRRSV infection on pregnant gilts and grower pigs. They found that PRRSV infection decreases the weight gain of both pregnant gilts and growing piglets, in some cases halting weight gain altogether. Furthermore, infection resulted in a decreased litter size in the pregnant gilts. Interestingly, Lewis et al. also found evidence that certain breeds, specifically Landrace, may show some tolerance to infection, as Landrace gilts and growers used in this study did not experience the same amount of weight gain suppression as cross-bred pigs.

As stated earlier, infection with PRRSV results in an immune response from the host, including the release of cytokines. Infected adults *and* fetuses are capable of mounting an immune response to the virus (Rowland, 2010). Evidence suggests that certain cytokines are responsible for the anorexia observed in many diseases (Hollis 1993). For instance, IL-1β and TNF-alpha are both produced in response to PRRSV infection, and both cytokines decrease feed intake due to an effect resembling that of

fibroblast growth factor (FGF). FGF is released after feeding and suppresses appetite by acting as a satiety factor on the hypothalamus, and IL-1β and TNF have a degree of amino acid sequence identity with FGF (Oomura, 1988). In addition, IL-1α has been shown to decrease water and food intake when injected both into the brain and the peritoneum of rats. Futhermore, IL-1 inhibits gastric emptying and acid secretion, which likely compounds its satiety effects (Hollis, 1993). Meanwhile, TNF-alpha causes a variety of metabolic effects, including dehydration, lipolysis, insulin resistance (which would also account for a decrease in appetite), and protein turnover and loss (Hollis, 1993). All of these effects can contribute to a decrease in growth just due to a lack of nutrient intake. It is important to notice that undernourishment also has a marked effect on the somatotropic axis that controls growth. Specifically, undernourishment decreases concentrations of insulin-like growth factors (IGFs), which stimulate growth, and increases concentrations of IGFBPs, which block the action of IGFs. Fetal development is similarly compromised due to the immune response in infected fetuses, but fetuses and neonates are at risk due to the symptoms of the infected dam. As stated earlier, pregnant gilts also exhibit increased anorexia and decreased weight gain, making less energy available for their litters (Lewis, 2010). Infected dams also may pass infection to uninfected neonates through their milk (Kang, 2010), leading to the same sort of growth retardation already explained. The decrease in milk production in infected mothers in response to their decreased IGF concentration is also detrimental to neonatal development as it can contribute to undernourishment, which is already a risk factor for infected neonates.

PRRSV genetic determinants mediating all these effects are not known. Finding those determinants would be critical for rationally design vaccines or other biological tools (e.g. antivirals) aimed to block the above described effects in swine.

Control of PRRSV

There are currently no highly effective control measures for PRRSV infections. The virus replicates primarily in macrophages, and is able to circulate concurrently with the host-produced antibody. This means that the antibody response is not sufficient for clearing the virus from the host (Rossow, 1998). Eventually, the use of antibiotics may contribute to clear secondary bacterial infections that often accompany PRRSV and decrease the severity of the disease (Done, 1996).

There are a number of commercially available vaccines against PRRSV, including live attenuated vaccines (LAVs) and killed virus vaccines. In general, killed vaccines are considered ineffective, although these vaccines may have a limited use in the process of acclimatizing of new herd animals by reducing shedding from vaccinated animals (Zimmerman, 2006) (see below). LAVs tend to be more effective than killed vaccines. These vaccines confer protective immunity that is only effective against homologous strains of the virus while there many different strains of PRRSV circulating within and among herds (Lunney, 2010). This variability among PRRSV isolates leads to poor cross-protection (Zimmerman, 2006).

Ideally, prevention of PRRSV infection originates with high in-farm biosecurity measures. These preventive measures would include screening of new animals for PRRSV exposure, isolation of the entire facility to restrict contact with other animals, people, and equipment; together with disinfection and cleaning measures. It is

important to notice that PRRSV is not highly stable or persistent in the environment, and is eliminated by hot dry conditions. PRRSV cannot survive lipid solvents or detergents (Zimmerman, 2006). Therefore, disinfection is a viable way to prevent infection in a non-infected population. However, due to the prevalence of PRRSV it is not practical to attempt to create a PRRSV-negative herd and maintain it. A large number of herds in the US are already infected with PRRSV and this is an obstacle for locating PRRSV-negative swine.

The most reasonable method for control of PRRSV infection in a herd is to attempt to acclimatize (expose) any incoming animals to PRRSV. Due to the cycling spread of PRRSV throughout a herd, all of the animals in the herd may be at different stages of immunity, and introducing animals that have already developed a protective immunity and stabilized the clinical signs of the disease is a good way to mitigate future losses (Zimmerman, 2006). Introduction of gilts that have developed immunity and are no longer shedding the virus to the breeding herd can help to produce uninfected offspring.

Since vaccination is considered the most effective way to control infectious disease in general, a concerted effort should be made for designing effective vaccines against PRRSV. Understanding mechanisms underlying PRRSV virulence is critical for developing effective control measures against this disease.

PRRSV genome

The genome of arteriviruses consists of a positive strand of RNA which in PRRSV is approximately 15 kb long. The RNA contains a poly-A tail at the 3' end, and the 5' end is capped which protects the RNA from degradation and is essential in the creation of infectious virus. ORFs 1a and 1b are located towards the N-terminal half of the viral genome. These ORFs encode for the RNA replicase complex proteins (Zimmerman, 2006). ORFs 1a and 1b encode for polyproteins that are post-translationally processed by cellular and viral proteases to yield 12 to 13 mature products. In the case of PRRSV nsp-1α, nsp-1β, and mainly nsp-4 are viral proteases that participate in the processing of the polyproteins encoded by ORFs 1a and 1b (Figure 1). This process has been well characterized for the prototype virus in the family *Arteriviridae*, EAV, and it is thought that the same processing takes places in PRRSV. Nsp-4, a serine protease, tends to be highly conserved among arteriviruses suggesting that key functions are maintained by this viral protein across this viral family. Nsp-9, 10, 11, and 12 are encoded by ORF 1b via a ribosomal frameshifting (-1) (Figure 1). Nsp-9 is the RNA-dependent RNA-polymerase and nsp-10 has a helicase motif necessary for functioning in RNA unwinding. Both, nsp-9 and nsp-10 are key proteins in the genome replication process. In EAV nsp-11 possesses an endoribonuclease domain. These proteins control the copy of the virus genome as well as the creation of the subgenomic mRNAs that encode for PRRSV structural proteins (Fang & Snijder, 2010). The function of nsp-12 is still unknown. Figure 2 shows a schematic representation of non-structural proteins encoded by ORFs 1a and 1b.

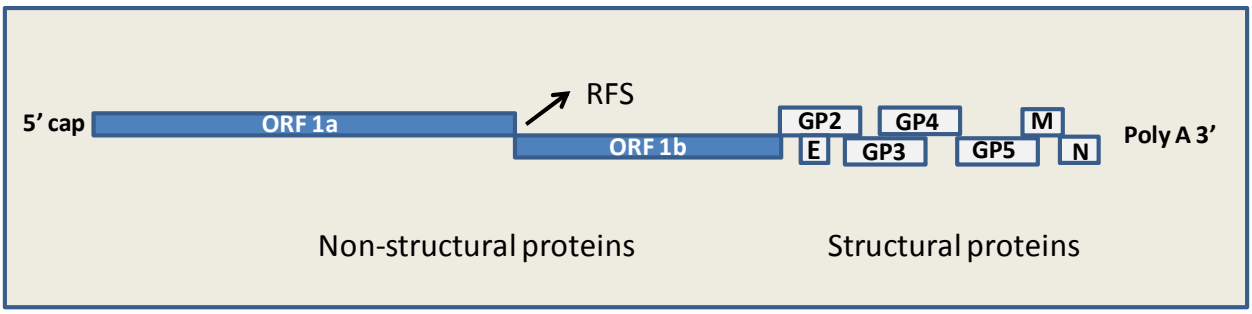


Figure 1: ***schematic representation of PRRSV genome indicating the location of different open reading frames encoding for non-structural and structural proteins. ORF: open reading frame, GP: glycoprotein; E: envelope protein; M: matrix protein; N: nucleocapsid protein; RFS: ribosomal frameshift (Adapted from Fang and Snijder, 2010).***

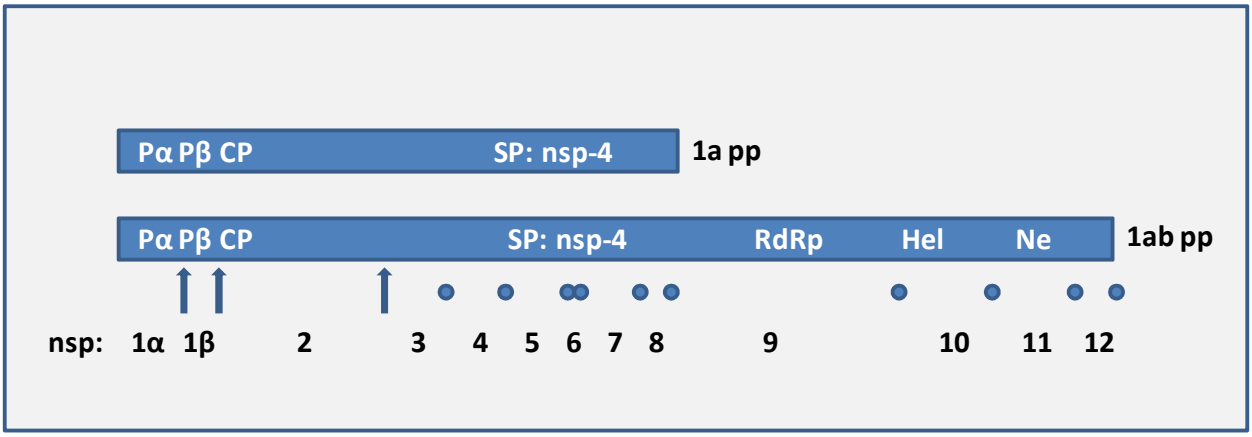


Figure 2: ***schematic representation of polyproteins (pp) encoded by ORFs 1a and 1a/1b of PRRSV. 1ab pp is produced due to a -1 ribosomal frameshift. Arrows indicate sites cleaved by nsp-1α and nsp-1β cysteine proteases (CP) Pα and Pβ. Circles indicate sites cleaved by the viral protease nsp-4 serine protease (SP). nsp: non-structural proteins . nsp-9 is the RNA dependent RNA polymerase (RdRp). nsp-10 helicase (Hel), and nsp-11 endoribonuclease (Ne). (Adapted from Fang and Snijder, 2010).***

The structural proteins of the virus are encoded by ORFs 2 to 7. These ORFs are located towards the 3' end of the genomic RNA (Figure 1). The structural proteins include glycoproteins (GP) GP2, GP3, GP4, and GP5, which are *N*-glycosylated, while structural N protein, the nucleocapsid protein, and M protein, the matrix protein, are not glycosylated. ORF 2 encodes for an additional polypeptide, protein E (envelope), an ion channel protein that participates in the virus entry process. N protein acts to assemble

infectious viral particles. M protein is the transmembrane protein, which is important for assembly of the virion and budding from infected cells. GP5 is the major envelope glycoprotein, which acts in receptor recognition and entry into permissive cells. GP2 and GP4 are minor glycoproteins that are also part of the viral envelope (Zimmerman, 2006) (Figure 3).

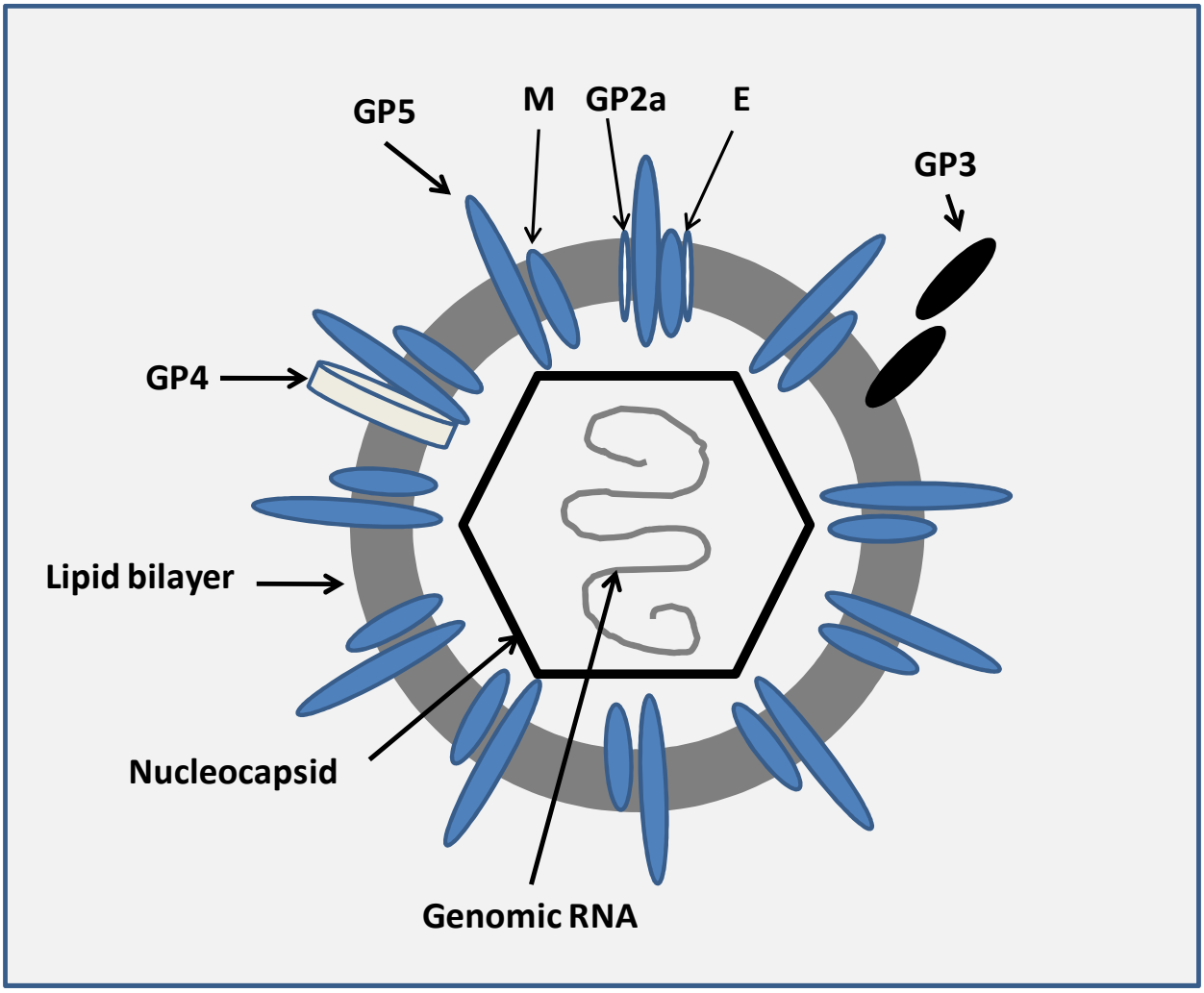


Figure 3: ***Schematic representation of PRRSV virion. GP: glycoproteins, E: envelope proteins, and M: matrix protein.***

Rationale and significance

Assessing the role of PRRSV proteins in the virus life cycle is critical for understanding virus pathogenesis and virus virulence. Interactions between viral and host proteins during virus replication within target cells may result in interference with cellular signaling pathways, ultimately leading to the clinical outcomes observed during PRRSV infection in swine. To accomplish this goal and better elucidate the interactions of PRRSV proteins with host cellular proteins, there is a need for reagents that would allow detection of those interactions. Thus, production of reagents such as antibodies to detect specific viral proteins is of paramount importance for these studies.

Objective

The objective of the work described here was to express and purify PRRSV nsp-12 protein, one of the proteins in the virus replication complex. In turn this purified viral protein will be used for production of antibodies and to study host-virus protein interactions.

Approach

Non-structural protein 12 is encoded by the ORF 1b. nsp-12 is 153 amino acids long (Figure 4). nsp-12 is cleaved by nsp4, which cuts at three sites of pp1ab (Figure 2) to yield nsp9-12. Fang & Snijder, 2010, predicted that nsp-12 is about 152-153 amino acids long, depending on the strain of the virus, and that the terminal residues are Gly followed by Pro/Asn. The function of nsp-12 is currently unknown, and the goal of this research is to express and purify the protein in order to create reagents that will help to identify the role of the protein in the virus life cycle.

GRHFTWYQLXSYASYIRVPVNSTVYLDPCMGPALCNRRVVGSTHWGADLA
VTPYDYGAKIILSSAYHGEMPPGYKILACAEFSLDDPVRYKHTWGFESDTAYLY
EFTGNGEDWEDYNDAFRARQ KGKIYKATATSMRFHFPPGP VIEPTLGLN

***Figure 4:* Amino acid sequence of nsp-12 from PRRSV isolate NVSL 97-7985 used in this work.**

In the present work nsp-12 is expressed fused to a poly-Histidine tag located at the N-terminus. The poly-His tag creates an ionized region in the fusion protein that allows binding of the expressed protein to metals (e.g. nickel or cobalt) to allow purification by metal chromatography. Nsp-12 was synthesized by PCR using specific forward and reverse primers (Table 1) using as target a full-length cDNA copy of PRRSV isolate NVSL 97-7985 (pFL12, kindly provided by Dr Fernando Osorio, University of Nebraska-Lincoln). The amplified nsp-12 gene was inserted in-frame into the pRSETA plasmid (Invitrogen, Carlsbad, CA) using the Infusion cloning system (Clontech, Mountain View, CA), where the expression of the cloned gene is under the control of the T7 promoter. The resulting plasmid pRSETAnsp-12 was used to transform BL21(DE3)pLysS cells (Invitrogen). Transformants were then grown in Magic media (Invitrogen) for maximum expression of nsp-12. Nsp-12 was purified by affinity chromatography using nickel columns, and was detected by SDS-PAGE separation followed by Coomasie blue staining and western blotting.

Procedures and Results

PCR amplification of PRRSV nsp-12:

A PCR was designed to synthesize PRRSV nsp-12 using primers described in table 1. A 50 ul reaction was set up as follows: 50 ng of full-length cDNA PRRSV clone pFL12, 5 µL of 10X Advantage PCR buffer (Clontech), 1 unit of Advantage 2 DNA polymerase (Clontech), 10 mM dNTPs 10 pmol of each forward and reverse primers, and 34.5µL ddH2O. A non-template control reaction was run as negative control. Cycling parameters used were 95℃ for two minutes, followed by 35 cycles of 95℃ for 30 seconds, 55℃ for 45 seconds, and 68℃ for 60 second; finished by a final cycle of 68℃ for 3 min.

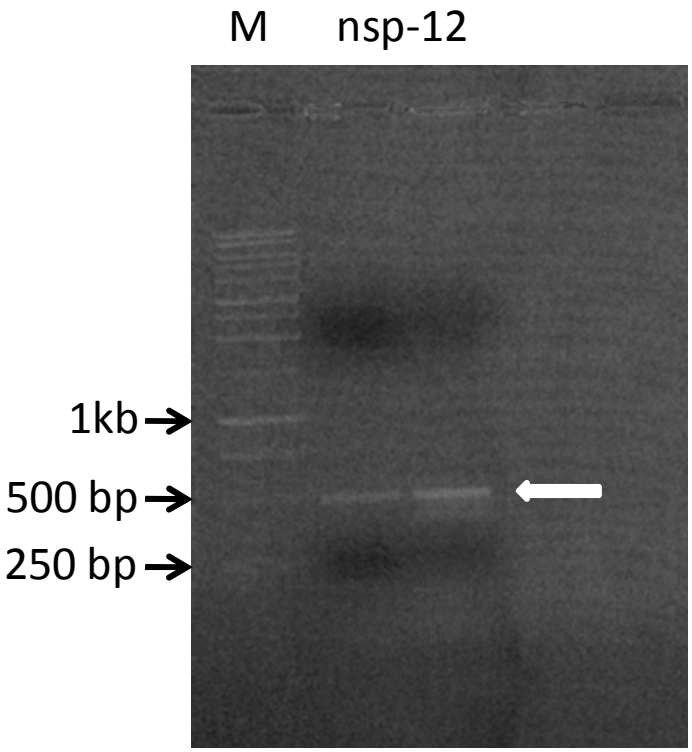
***Table 1:* sequence of the primers used for PCR amplification of PRRSV NVSL 97-7895 nsp-12 gene.**

Primer name	Sequence
nsp-12 _for_infusion	5'-atcgatgg***ggatcc***ggccgccatttcacctggtac-3'
nsp-12 _rev_infusion	5'-gctgcagat***ctcgag***ttaattcaggcctaaagttgg-3'

BamHI (ggatcc) and *XhoI* (ctcgag) site sequences are shown in bold

Purification of amplified nsp-12

Amplicons of nsp-12 were resolved by agarose gel electrophoresis. A 1X-TAE buffer (Tris-acetate-EDTA) 1% w/v agarose containing ethidium bromide was used to separate the amplified DNA. A 1kb DNA ladder (Promega, Madison, WI) was used as molecular size control. Twenty-four µL samples were prepared by mixing 16 µL of ddH2O, 4µL of 6X loading buffer (Promega), and 4 µL of the PCR reactions. Samples were loaded into the gels and DNA was separated by running the sample at 90V for 1 hr (Figure 5).



<u>Figure 5:</u> PRRSV nsp-12 was amplified by PCR and resolved by agar gel electrophoresis in a 1% 1X-TAE buffer (Tris-acetate-EDTA) stained with ethidium bromide. White arrow indicates the presence of an amplicon of 500 bp. M: 1Kb ladder molecular weight marker (Invitrogen).

After separation of the DNA as described above, the nsp-12 amplicon was extracted from the agarose gel using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) according with the instructions by the manufacturers. The agarose gel was removed from the electrophoretic chamber and placed on top of a UV light trans-illuminator to visualize the DNA band. A band of approximately 500 bp was excised from the gel using a razor blade and placed in a 1.5 ml microcentrifuge tube. The band was weighed and 3X volume (e.g. 100 ug = 300 µl of buffer) of Buffer QG was added to the tube. The tube was placed at 50℃ in a heating block and incubated until the agarose was completely dissolved (~10 min). Then, 200 µL of isopropanol were added to the tube, mixed, and centrifuged for 1 min at 14,000 rpm in a bench-top

microcentrifuge (Eppendorf, Hauppauge, NY). The entire volume of the sample was transferred to a QIAquick column and centrifuged for 1 min at 14,000 rpm. The flow-through was discarded and 500 µL of Buffer QG were added to the column, followed by centrifugation for 1 min at 14,000 rpm. The flow-through was discarded and the column washed once by adding 750 µL of Buffer PE followed by centrifugation for 1 min at 14,000 rpm. The flow-through was discarded and the column centrifuged again for 1 min at 14,000 rpm to remove residual ethanol. DNA was eluted from the columns using 50 µl of Buffer EB and centrifuged for 1 min at 14,000 rpm. Recovered DNA was ready for cloning into pRSET A (Invitrogen) (Figure 6).

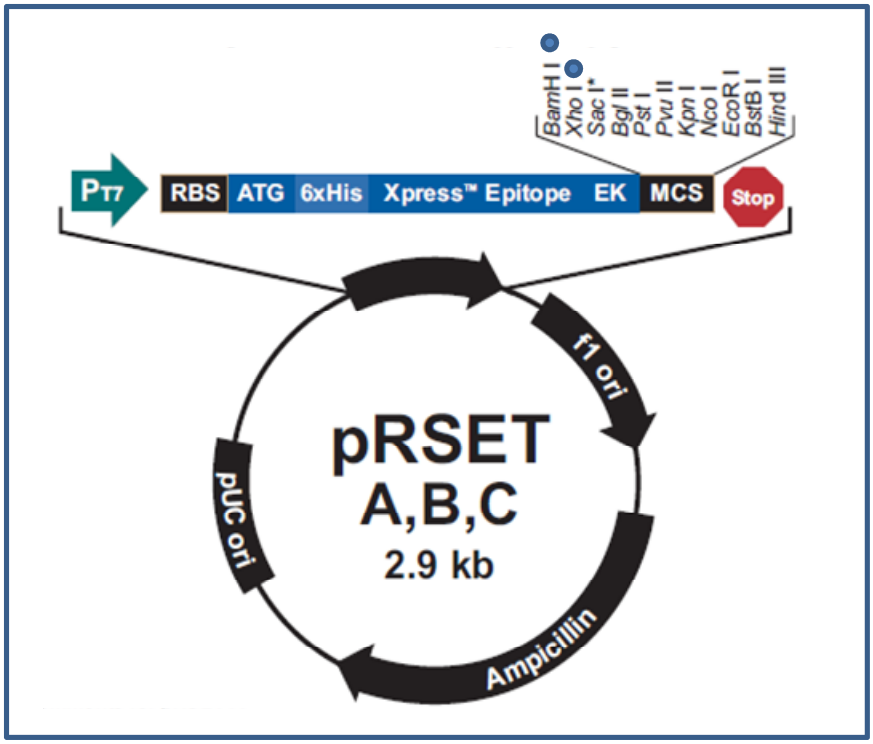


Figure 6: ***Invitrogen's pRSET diagram. PT7: T7 promoter. RBS: ribosomal binding site. ATG: codon encoding for methionine. 6xHis: histidine tag. Xpress Eptope: synthetic epitope tag. EK: enterokinase cleavage site. MCS: multiple cloning site. Stop: stop codon. Blue circles denote the restriction endonucleases used here.***

Cloning of nsp-12 gene into pRSET A vector

For cloning nsp-12 into pRSET A the In-Fusion® PCR Cloning System (Clontech) was used. This system allows the ends of the amplified DNA (i.e. nsp-12) to "fuse" to the homologous ends of a linearized vector. The 3' and 5' regions of homology were generated by adding 15 bp extensions to both PCR primers (Table 1) that precisely match the ends of linearized pRSET A. The linearized vector was combined with nsp-12 amplicon and the In-Fusion® enzyme included in the kit converts the double-stranded extensions into single-stranded DNA and fuses these regions to the corresponding ends of the linearized pRSET A.

The pRSET A plasmid(2.9 Kb) was digested with restriction endonucleases *Bam*HI and *Xho*I in a 20 µl reaction. The reaction was set as follows: 10 µl of pRSET A (390 ng/ul), 2 µl of 10X NEB buffer 2 (New England Biolabs, Ipswich, MA), 0.5 µl of 100X BSA, 1 µl of *Bam*HI enzyme (20,000U/mL) (New England Biolabs), 1 µl of *Xho*I enzyme (20,000U/mL) (New England Biolabs), and 5.5 µl ddH2O. The reaction was then incubated at 37℃ in a water bath for 2 hr. After digestion, pRSET A linearized DNA was resolved in a 1% agarose-TAE-ethidium bromide gel, and a 2.9 Kb band excised and purified from the gel using QIAquick Gel Extraction Kit (Qiagen) as described above.

After purification of the digested vector (pRSET A), a ligation reaction was set up using the In-Fusion® PCR Cloning System (Clontech). The purified insert and vector were mixed in a 2:1 ratio using the following equation:

$$[insert] = \frac{[vector]x\ size\ of\ insert(bp)}{size\ of\ vector(bp)}x2$$

The reaction was set as follows: 4µL 5X In-fusion Reaction Buffer, 2µL In-fusion enzyme, 7µL vector, 2µL insert, 5µL dH2O, and 30µL TE Buffer. The reaction was incubated for 30 min at 25^0C. After incubation the In-fusion reaction was used to transform chemically competent TOP10 *E.coli* cells (Invitrogen) via heat-shock transformation. The In-fusion reaction was added to the cells and incubated on ice for 30 minutes, for 40 seconds at 42℃, followed by incubation on ice for 2 min. To recover the cells 250 µL of SOC media (Invitrogen) was added to the cells and incubated at 37℃ with shaking for 1 hour. Transformed cells were then plated in terrific broth agar plates containing ampicillin and incubated overnight at 37℃.

Recovery of pRSETAnsp-12

Four ampicillin resistant colonies were picked from the plates and transferred to 5mL terrific broth ampicillin media. The cultures were incubated at 37℃ with shaking overnight. Growth of bacteria was detected by observing an increased turbidity of the culture media. The bacterial culture was then spun down in bench-top centrifuge at 4000rpm for 15 minutes to produce a cell pellet. Supernatant was discarded and plasmid DNA was extracted from the cells using the QIASpin Miniprep Kit (Qiagen) according with the instructions provided by the manufacturer. The cell pellet was resuspended in 250µL of Buffer P1 (kept at 4^0C before use), this is a cell lysis buffer that contains RNAase. The suspension was then transferred to a microcentrifuge tube and 250µL of Buffer P2 (high pH buffer) were and mixed thoroughly by inverting the tube 4-6 times. After mixing, 350µL of Buffer N3 (low pH buffer) were added and rapidly and thoroughly mixed. The mixture was then centrifuged for 10 min at 14,000rpm to separate cellular debris from the aqueous phase where plasmid DNA is in solution. The

supernatant was then applied to the QIAPrep spin columns by decanting and the tube was centrifuged for 30 sec at maximum speed. The flow-through was discarded and the spin column was washed by adding 0.75mL of Buffer PE containing ethanol and centrifuged again for 30 sec at maximum speed. The flow-through was discarded and the tube was centrifuged again for 1 min at maximum speed to remove the residual wash buffer. Plasmid DNA was recovered by placing the column on top of a clean 1.5 mL microcentrifuge tube, adding 50 µL Buffer EB (elution buffer) to the center of the column, and spinning 1 min at maximum speed.

Analysis of recovered plasmid DNA

Recovered plasmids were digested with restriction endonucleases *BamHI* and *XhoI* to determine if they contained an insert of the proper size (nsp-12, 500 bp). The restriction reaction was set as follows: 5µL of each extracted plasmid DNA, 2µL of 10X NEB Buffer 2 (New England Biolabs), 0.5mL BSA (100X), 1µL *BamHI* (20,000U/mL), 1µL *Xho1* (20,000U/mL), and 10.5µL of ddH_2O. This mixture was incubated at 37℃ in water bath for 2 hr. After digestion, DNA was resolved in a 1% agarose-TAE-ethidium bromide gel as described above (Figure 7).

To assess the fidelity of the cloned gene, plasmid DNA showing the expected restriction pattern were submitted for sequencing (Genewiz, South Plainfield, NJ). Obtained sequences were analyzed using the Bioedit software.

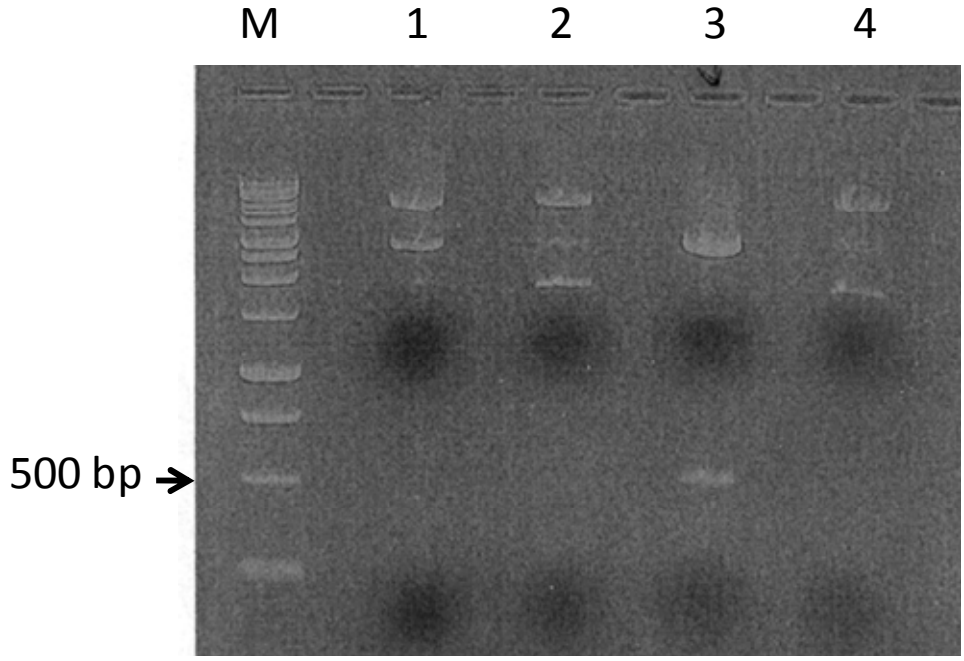


Figure 7: ***Restriction analysis (BamHI and XhoI digestion) of plasmids DNA extracted from isolated E.coli colonies. Clone number 3 was excised by both restriction enzymes and produced two definite bands of 2.9 kb (top band) and 500 bp (bottom band). DNA was resolved by agar gel electrophoresis in a 1% 1X-TAE buffer (Tris-acetate-EDTA) stained with ethidium bromide. M: 1 Kb ladder molecular weight marker (Invitrogen).***

Scaling up PRSETAnsp12 plasmid concentration

Once fidelity of the inserted nsp-12 gene into pRSET A was determined, the concentration of plasmid DNA was increased by using the Hispeed Plasmid Maxi Kit (Qiagen). *E.coli* harboring pRSETAnsp-12 was cultured in 150 ml of terrific broth media containing ampicillin overnight at 37℃ with shaking. After incubation the culture was centrifuged at 4,000 rpm for 15 min. Supernatant was decanted and the bacterial pellet was resuspended in 10mL of Buffer P1 (kept at 4^0C) incubating for 5 minutes at room temperature. After thorough mixing by vortexing to resuspend the bacterial pellet, 10 ml of buffer P2 were added and mixed by repeated inversions of the flask. To neutralize

the activity of buffer P2, 10 ml of Buffer P3 were added, mixed gently by inversion. The mixture was transferred to QIAfilter cartridge and incubated at room temperature for 10 minutes. In the meantime a HISpeed-tip 500 column was equilibrated by adding 10mL Buffer QBT and allowed to drain for 10 min. The content of the QIAfilter cartridge was transferred to the equilibrated a HISpeed-tip 500 column for binding of the DNA to the silica filter. After the flow through was discarded the column was washed one with 60 ml of Buffer QC. DNA was then recovered by eluting with 15 ml of Buffer QF. Plasmid DNA was then precipitated by adding 10.5 ml (0.7 volumes) of isopropanol and incubating at room temperature for 5 min. The eluate/isopropanol mixture was run through a QIAprecipitator and flow-through was discarded. The QIAprecipitator was then washed twice with 2 ml of 70% ethanol and DNA recovered by adding 1ml of Buffer TE. Recovered DNA was then submitted again for sequencing (Genewiz).

Expression of PRRSV nsp-12

E. coli BL21(DE3)pLysS chemically competent cells (Invitrogen) were transformed with pRSETAnsp-12 as previously described in this work. Two colonies were picked from the terrific broth agar ampicillin plates and transferred to 5mL of terrific broth containing 50 µg/ml of ampicillin and 35 µg/ml of chloramphenicol and grown with shaking overnight at 37℃. Chloramphenicol selects for maintenance of the pLysS plasmid required for T7 lysozyme expression and ampicillin selects for the pRSET A plasmid. After incubation, 150 µL of the culture were then added to 150 mL of Invitrogen's Magic Media. The culture was incubated for 18 hr at 37^0C, after which it was centrifuged at 4,000rpm for 30 min and the supernatant was discarded. The pellet containing expressed nsp-12 was kept at -20℃ prior to lysis.

To extract nsp-12, BL21 cells pellet was thawed on ice and 20mL of CellLytic B buffer (Sigma, St Louis, MO) containing 100µL of protease inhibitor (Sigma) was added per 1 g of cells. The pellet was resuspended by pipetting and vortexing, and incubated at 37℃ for 15 min with shaking. The lysate was then transferred to 1.5 ml microcentrifuge tubes and centrifuged for 30 min at 13,200rpm. Supernatant was collected and stored at -20℃.

To determine nsp-12 expression a NuPAGE® Novex 12% Bis-Tris Gel (Invitrogen) was run. Samples were run under reducing conditions by mixing 2.5 µL of NuPAGE® LDS Sample Buffer (4X), 1µL of NuPAGE® Reducing Agent (10X), and 6.5 µL protein lysate. Separation of proteins was done in 1X SDS Running Buffer (50 ml 20X NuPAGE® MES SDS Running Buffer in 950 ml of ddH2O). A molecular size marker was run next to the cell lysates (Novex® Sharp Pre-stained Protein Standard, Invitrogen). The gel was run at 200V for 35 min. After separation the gel was transfer to a clean tray and stained with Coomasie blue (SimplyBlue Self Stain, Invitrogen) (Figure 8).

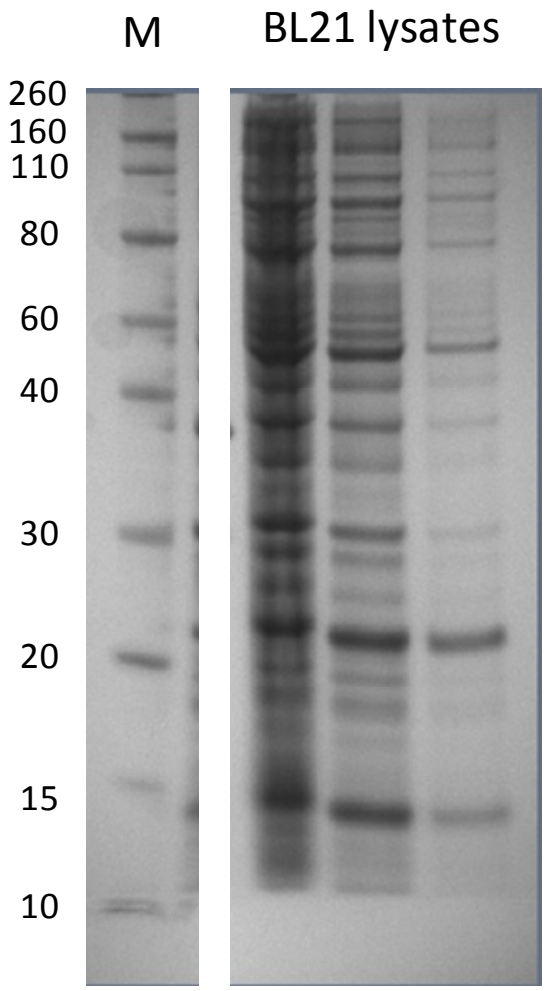


Figure 8. ***BL21 cell protein lysates were separated by SDS-PAGE using a NuPAGE® Novex 12% Bis-Tris Gel (Invitrogen) and stained with SimplyBlue Self Stain (Invitrogen). M: molecular size marker expressed in kDa.***

Detection of nsp-12 by western immunoblots

A SDS-PAGE was set and run under the conditions described above. After the run, proteins were transferred to a PVDF (polyvinylidene difluoride) membrane (Invitrogen). To perform the transfer, the gel is layered next to the PVDF membrane and placed in a voltage gradient perpendicular to the gel (X Cell II Blot Module, Invitrogen). Negatively charged molecules (i.e. proteins) will migrate out from the gel,

move towards the positive electrode, and get deposited on the membrane. The transfer buffer for reducing conditions was set by combining 50 mL of NuPAGE® Transfer Buffer (20X), 1 mL NuPAGE® Antioxidant, Methanol* 100 mL, and 849 ml of ddH2O. Before transferring the PVDF membrane was activated in methanol for 30 seconds and then transferred to 100 ml of transfer buffer. The transfer was run for 70 min at 30V, 170mVa using the X Cell II Blot Module (Invitrogen).

After transfer the PVDF membrane was washed three times for 5 minutes with ddH2O. The membrane was then blocked for 90 minutes in blocking buffer (WesternBreeze Chemiluminescent immunodetection kit, Invitrogen) containing 5mL water, 2mL Blocker/Diluent A, and 3mL Blocker/Diluent B. After blocking, the membrane was washed with ddH2O twice for 5 min. The membrane was incubated with the primary antibody, an anti-His tag monoclonal antibody diluted 1:2000 in a buffer containing 7mL of ddH2O, 2mL of Blocker/Diluent A, and 1mL Blocker/Diluent B for 60 min at room temperature. The membrane was then washed three times with antibody wash buffer containing 10mL of antibody wash, and 150mL of ddH_2O, for 5 min at room temperature. After washing, the membrane was incubated with the secondary anti-mouse antibody in a buffer containing 10 ml of secondary antibody solution and 4 µL of goat anti-mouse alkaline phosphatase conjugated antibody for 35 min. The membrane was washed twice for 5 min with ddH2O to remove unbound secondary antibody. Using a clean pipette 2.5 ml of alkaline phosphatase Chemiluminescent substrate were placed on top of the PVDF membrane and the reaction was allowed to proceed for 5 min. Excess substrate was removed with a paper filter without allowing the membrane to dry out. The membrane was then covered with a transparency and exposed to a Kodak X-

OMAT AR film for several minutes. The substrate-enzyme reaction releases light that is captured by the X-ray film (see below).

Purification of nsp-12

Chemically competent One Shot BL21 (DE3) pLysS *E. coli* (Invitrogen) cells were transformed with 10 ng of pRSETAnsp-12 plasmid encoding PRRSV nsp-12, cultured in MagicMedia (Invitrogen) for 21 hours at 37℃ with shaking, and harvested by centrifugation at 4,000 rpm for 15 minutes. Cell pellets were then treated with CelLytic buffer (Sigma Aldrich, St. Louis, MO) according to the manufacturer's large scale extraction protocol. For His-tagged protein purification, cell lysates were applied to HisPur Cobalt Spin Columns (Thermo Fisher Scientific, Rockford, IL). Briefly, the 3mL capacity spin columns were equilibrated at room temperature and protein lysates were thawed on ice for 30 min before purification. The bottom plug at the bottom of the column and storage buffer was allowed to drain out by gravity. The spin column was equilibrated by adding two volumes of resin bed in 6 mL of Equilibration/Wash Buffer with the cap at the bottom of the column in place. The column was allowed to sit for 4 min before use. After this equilibration step, columns were inverted a few times and the Equilibration/Wash Buffer was drained from the columns for 4 min. To purify nsp-12, the protein lysate was mixed with an equal volume of Equilibration/Wash Buffer and vortexed. The bottom of the column was capped and the protein lysate was applied to the spin column. The spin column was inverted several times to mix and incubated on ice for 80 min under vigorous shaking. During this incubation, the spin column was shaken vigorously by hand every 2 to 3 min to ensure that resins do not form a packed pellet. The spin column was then placed on the top a 15 ml centrifuge tube and drained

by removing the bottom cap and loosening the top cap. The flow through was collected, labeled Collection 1 and kept on ice. The resin in the column was washed with 2 volumes of Equilibration/Wash Buffer and incubated for 4 min. The flow-through was collected in a new 15 ml centrifuge tube and labeled Wash 1. The process was repeated two more times and Wash 2 and Wash 3 were collected. His-tagged nsp-12 was eluted from the resin by adding one resin-bed volume of Elution Buffer and incubating for 4 min. After incubation the flow-through was collected in 15 ml centrifuge tube and labeled Elution 1. The same process was repeated two more times and Elutions 2 and 3 were collected. All fractions were stored at -20^{0}C. PRRSV nsp-12 has a molecular size of 17 kDa. The protein purified here was expressed as a His-tagged fusion protein with a molecular mass of 21 kDa (Figure 9).

MRGSHHHHHHGMASMTGGQQMGRDLYDDDDKDRWGSGRHFTWYQL
XSYASYIRVPVNSTVYLDPCMGPALCNRRVVGSTHWGADLAVTPYDYGAKII
LSSAYHGEMPPGYKILACAEFSLDDPVRYKHTWGFESDTAYLYEFTGNGEDW
EDYNDAFRARQ KGKIYKATATSMRFHFPPGP VIEPTLGLN

***Figure 9:* Amino acid sequence of PRRSV nasp-12 fusion protein expressed in this work. In red is the fused sequence that includes 6x-His, Express® epitope, and enterokinase cleavage site.**

Collected fractions were analyzed on a 10% NuPage Novex Bis-Tris gel (Invitrogen) using a discontinuous SDS-PAGE system as described above (Figure 10). Western blots were performed using PVDF membranes (Invitrogen) and a His-Tag antibody (Novagen, EMD Biosciences, Madison, WI) as described above. Reactions were developed using alkaline phosphatase labeled goat anti-mouse IgG antibody with the WesternBreeze Chemiluminescent Detection Kit (Invitrogen). The purified proteins

were desalted twice using 7K MWCO Zebra Spin Desalting Columns (Thermo Fisher Scientific). Protein concentration was determined by BCA Protein Assay (Thermo Fisher Scientific) against known standards at OD of 595 nm using NanoDrop instrument (Thermo Scientific NanoDrop Products, Wilmington, DE) (Figure 11).

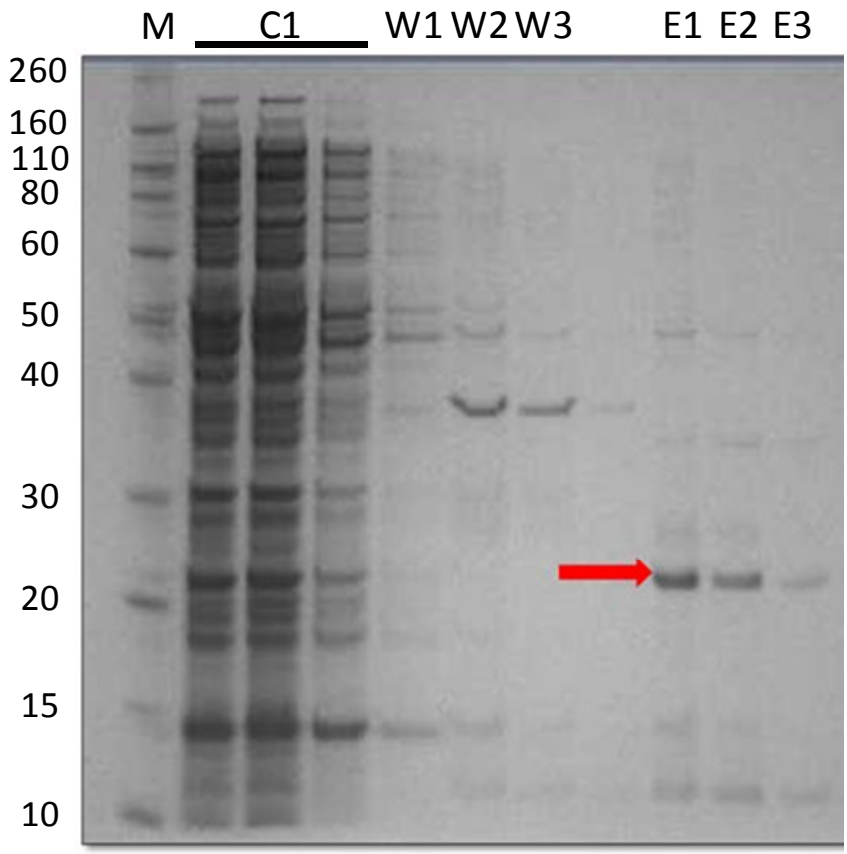


Figure 10. ***Purification of His-tagged PRRSV nsp-12. SDS-PAGE electrophoresis stained with Simple Blue (invitrogen). M: molecular size markers expressed in kDa. C1: collection 1. W: washes. E: ellutions. Arrow indicates nsp-12 His-tagged fusion with a molecular size of 21 kDa.***

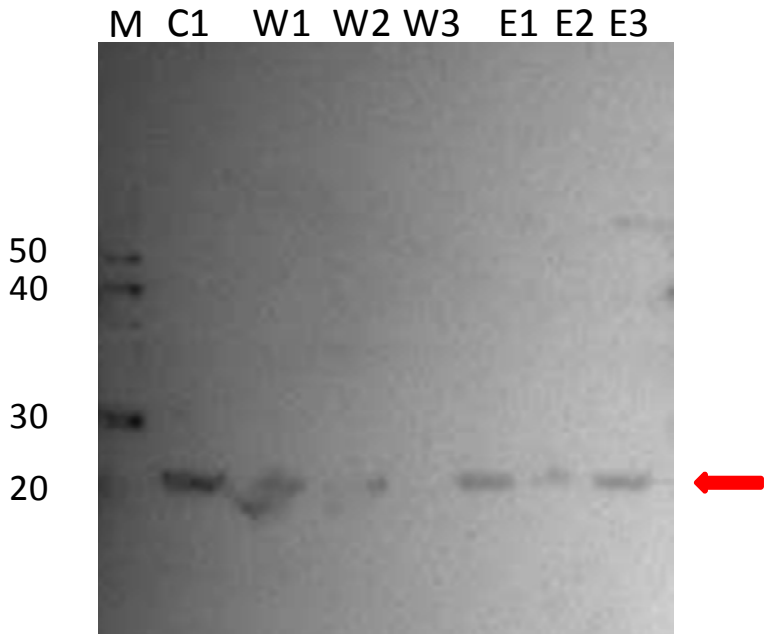


Figure 11. ***Westen immunoblot showing His-tagged nsp-12. M: molecular size markers expressed in kDa. C1: collection 1. W: washes. E: ellutions. Arrow indicates nsp-12 His-tagged fusion with a molecular size of 21 kDa.***

Summary

Nsp-12 from PRRSV isolate NVSL 97-7985 was synthesized by PCR amplification, cloned into *E. coli* expression vector pRSET A, and expressed in *E. coli* BL21 (DE3) pLysS. PRRSV nsp-12 was purified by affinity chromatography and its purity verified by SDS-PAGE and Western blot. A variety of molecular techniques were used and successfully adjusted during the course of this work, including DNA amplification, protein expression, protein separation, and protein purification. Overall the objective of this work was fulfilled.

PRRSV nsp-12 will be used for production of antibodies. These antibodies against nsp-12 are critical for studies such as protein-protein interactions between viral and host proteins. This work was highly significant and will make attaining those goals possible.

Outlook

Porcine Reproductive and Respiratory Syndrome Virus is an extremely prevalent and economically significant disease for the swine industry. Although much is known about Equine Arteritis Virus the prototype virus in the family *Arteriviridae* there is limited information about the biology of PRRSV. One of the more limiting aspects in the fight against PRRSV is that many of the functions of the viral proteins are unknown. The advantage of purifying these proteins is to create reagents that can be used in research such as, determining the function of nsp-12 in PRRSV cycle. With the production of an antibody to PRRSV nsp-12, it may soon be possible to discover functions such as, which cellular proteins interact with nsp-12 during infection. It is hypothesized that nsp-12 may be involved in host infection and/or virus replication, and the availability of a reagent that binds specifically to nsp-12 can pave the way for future experiments in identification and possibly blocking of the protein's function. Furthermore, the successful purification of this protein may serve as a protocol for the purification of other little-researched PRRSV proteins.

Literature Cited

Albina, E. 1997. Epidemiology of porcine reproductive and respiratory syndrome (PRRS): an overview. Veterinary Microbiology 55(1-4), 309-16.

Christopher-Hennings J, Nelson E, Hines R, Nelson J, Swenson S, Zimmerman J, et al. 1995. Persistence of porcine reproductive and respiratory syndrome virus in serum and semen of adult boars. Journal of Veterinary Diagnostic Investigations ;7:456–64.

Christopher-Hennings J, Holler L, Benfield D, Nelson E. 2001. Detection and duration of porcine reproductive and respiratory syndrome virus in semen, serum, peripheral blood mononuclear cells, and tissues from Yorkshire, Hampshire and Landrace boars. Journal of Veterinary Diagnostic Investigations;13:133–42.

Done SH, Paton DJ, White MEC. 1996. Porcine reproductive and respiratory syndrome (PRRS): A review, with emphasis on pathological, virological and diagnostic aspects. British Veterinary Journal 152(2):153-174

Fang Y, Snijder EJ. 2010. The PRRSV Replicase: Exploring the multifunctionality of an intriguing set of nonstructural proteins. Virus Research 154:61-76.

Hollis GR, editor. 1993. Growth of the Pig. Wallingford: Cab International. 224p.

Kang I, Ha Y, Kim D, Oh Y, Cho K, Lee B, Lim J, Kim S, Kwon B, Chae C. 2010. Localization of porcine reproductive and respiratory syndrome virus in mammary glands of experimentally infected sows. Research in Veterinary Science 88(2):304-306.

Lewis CRG, Ait-Ali T, Wilson A, Westcott DG, Frossard JP, Naidu B, Mellencamp MA, Torremorell M, Drew T, Bishop SC, Archibald AL. 2010. Effects of porcine

reproductive and respiratory syndrome virus infection on the performance of pregnant gilts and growing pigs. Animal Production Science 50(9): 890-896.

Lunney JK, Benfield DA, Rowland RR. 2010. Porcine reproductive and respiratory syndrome virus: An update on an emerging and re-emerging viral disease of swine. Virus Research 154 (1-2):1-6.

Meulenberg JJ, Bende RJ, Pol JM, Wensevoort G, Moormann RJ, 1995. Nucleocapsid protein N of Lelystad virus: expression by recombinant baculovirus, immunological properties, and suitability for detection ofserumantibodies. Clinical Diagnostic Laboratory Immunology. 2: 652–656.

Oomura, Y. 1988. Chemical and neuronal control of feeding motivation. Physiology and Behavior 44(4-5), 555-60.

Rossow KD. 1998. Porcine Reproductive and Respiratory Syndrome. Veterinary Pathology 35(1):1-20.

Rowland RR. 2010. The interaction between PRRSV and the late gestation pig fetus. Virus Research 154 (1-2):114-122.

Snijder EJ, Meulenberg JJM. 2001. Arteriviruses. In *Fields Virology*. Knipe DM, Howley PM. editors. 4th ed, vol. 1. Philadelphia: Lippincott Williams & Wilkins. p 1205-1220.

Zimmerman J, Benfield DA, Murtaugh MP, Osorio F, Stevenson GW, Torremorell M. 2006. Respiratory syndrome virus (porcine arterivirus). In: *Diseases of swine*. Straw BE, Zimmerman JJ, D'Allaire S, and others, editors. 9th ed. Ames, Iowa: Blackwell Publishing. p 387-417.