

Spring 5-8-2011

Expression of the Porcine Reproductive and Respiratory Syndrome Virus non-structural protein 3 (NSP 3) in Escherichia coli

Lidia Beka

University of Connecticut - Storrs, lidiabeka1@gmail.com

Follow this and additional works at: http://digitalcommons.uconn.edu/srhonors_theses



Part of the [Cell Biology Commons](#), and the [Molecular Biology Commons](#)

Recommended Citation

Beka, Lidia, "Expression of the Porcine Reproductive and Respiratory Syndrome Virus non-structural protein 3 (NSP 3) in Escherichia coli" (2011). *Honors Scholar Theses*. Paper 202.
http://digitalcommons.uconn.edu/srhonors_theses/202

This Article is brought to you for free and open access by the Honors Scholar Program at DigitalCommons@UConn. It has been accepted for inclusion in Honors Scholar Theses by an authorized administrator of DigitalCommons@UConn. For more information, please contact digitalcommons@uconn.edu.



University of Connecticut

Honors Thesis

“Expression of the Porcine Reproductive and Respiratory Syndrome

Virus non-structural protein 3 (NSP 3) in *Escherichia coli*”

By: Lidia Beka

Department of Molecular and Cell Biology

College of Liberal Arts and Sciences

University of Connecticut

Honors Thesis Advisor

Dr. Guillermo R. Risatti

Department of Pathobiology and Veterinary Science

College of Agriculture and Natural Resources

University of Connecticut

Honors Advisor

Dr. James Cole

Department of Molecular and Cell Biology

University of Connecticut

Spring 2011

Abstract

Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) is single-stranded, positive-sense RNA virus in the family *Arteriviridae*, order Nidovirales. PRRSV is the most economically significant viral infection of swine herds in the United States. The single-stranded RNA genome is 15 kb in length and 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 coding for non-structural protein 3 (nsp-3) was synthesized using the polymerase chain reaction (PCR) and cloned in-frame into a plasmid vector to create a construct named pRSETA-nsp3. After transforming *Escherichia coli* with pRSETA-nsp3, individual colonies that grew in the presence of ampicillin were selected. These colonies were cultured in terrific broth-ampicillin media. Then, plasmid DNA was extracted to evaluate for the presence and fidelity of the nsp-3 gene by means of agarose gel electrophoresis separation and sequencing. DNA plasmids harboring a correct copy of the gene were used to express nsp-3 protein in BL21 competent *Escherichia coli* cells. After growth of BL21 transformants in "Magic media"®, expression of nsp-3 was assessed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) separation followed by Coomassie blue staining and western blot detection using an anti His-tag monoclonal antibody. PRRSV nsp-3 was then purified by metal affinity

chromatography. Purified nsp-3 protein will be used to produce an anti nsp-3 polyclonal antibody serum in rabbits for further study of the functions of this protein.

TABLE OF CONTENTS

1. Chapter 1: Porcine Reproductive and Respiratory Syndrome	6
1.1. Introduction	7
1.2. The economic significance of PRRS	8
1.3. Clinical manifestations of PRRS	10
1.4. Transmission of PRRS	12
2. Chapter 2: Control of PRRSV infections	14
2.1. PRRSV Vaccines	15
2.2. Herd Management	16
3. Chapter 3: Porcine Reproductive and Respiratory Syndrome Virus	17
3.1. The Virion	18
3.2. Genetic diversity among PRRSV types	19
3.3. PRRSV genome	19
3.4. PRRSV structural proteins	21
3.5. PRRSV nonstructural proteins	22
4. Chapter 4: Rationale and significance, objective, and approach	24
4.1. Rationale and significance	25
4.2. Objective	25
4.3. Approach	25
5. Chapter 5: Procedures and results	27
5.1. Synthesis of PRRSV NSP 3 gene by polymerase chain reaction (PCR)	28
5.2. Purification of amplified NSP 3 gene	28
5.3. Cloning of NSP 3 gene into pRSETA vector	30
5.4. Ligation reaction	32

5.5. Purification of pRSETAnsp3 vector.....	32
5.6. Maxiprep of selected pRSETAnsp3 vector.....	34
5.7. Expression of PRRSV NSP 3 in <i>E. coli</i> BL21 cells.....	35
5.8. Purification of NSP 3.....	38
5.9. Detection of NSP 3 by western immunoblot.....	40
Summary.....	43
References.....	44

Chapter 1

Porcine Reproductive and Respiratory Syndrome

1.1 Introduction

Porcine reproductive and respiratory syndrome virus is an arterivirus and the etiological agent of porcine reproductive and respiratory syndrome (PRRS). This syndrome is a disease of great interest especially with respect to the United States and European pork industries. PRRS syndrome has been described as “the most economically important infectious disease of swine production” (Music and Gagnon, 2010). It was first discovered in the United States in 1987 and then in the Netherlands in the following years. The main symptoms include severe reproductive failure in gilts and sows, and respiratory problems in pigs of any age group. This very serious disease has caused many economic and production obstacles, costing swine industries almost \$600 million/year in the United States (Neumann et al. 2005).

The virus can establish a chronic infection in swine macrophages *in vitro* and as studies show, it causes cell induced apoptosis or necrosis as well as chronic PRRSV infection (Cafruny et al., 2006). This virus began to emerge in North America in 1987 and was first identified in 1992 by Collins et al. In Europe, the virus was detected in 1990 and was first identified in the Netherlands by Wensvoort et al. in 1991 (Wensvoort et al, 1991).

Because PRRSV belongs to the family of *Arteriviridae*, the viral particles are characterized by a spherical shape with a diameter of 40-60 nm and are enveloped. Consistent with other arteriviruses such as Equine Arteritis Virus (EAV), PRRSV has the ability to establish chronic infection in swine host cells, with macrophages being the main target cells. Thus, PRRSV causes a debilitating illness in swine. This virus can manufacture its own structural and nonstructural proteins to administer its

functions. The nonstructural proteins allow the virus to replicate within permissive cells of the host. When this occurs repeatedly, cell after cell is destroyed in the host organism and its immune system is heavily burdened. Therefore over time, the host organism is at increasingly higher risk. The symptoms of PRRS range from none to acute hemorrhagic fever and abortion (Collins et al., 1992).

1.2 The economic significance of PRRS

PRRSV has played a significant role in the US swine industry in the modern day. A case study done by Neumann et al. 2005 from the Office of Swine Health Information and Research of the National Pork Board, shows the analytical results of the impact on the United States economy (Table 1). PRRSV affected and unaffected farms were compared in health and productivity, considering parameters such as farrowing rate (the rate of parturition in the sow), pigs weaned per litter, and pigs weaned/sowed per year. In that study, the number of affected litters and the duration of the PRRSV outbreak were recorded and analyzed against data obtained from farms not affected by PRRSV to calculate the total cost of the disease per farm. Utilizing national estimates of PRRSV prevalence among swine herds, the total annual economic impact of the disease was estimated for the US pork industry.

It has been reported that there was high variability among affected farms in terms of duration of PRRSV outbreaks. While a certain proportion of affected farms had a markedly clear period of occurrence of the disease, usually ending with implementation of immunological control measures, other farms suffered from chronic exposure to PRRSV. Farrowing rates in PRRSV-affected farms tend to

decrease by a mean of 13.76%, with a negative impact on productivity of 16.43%. The effect of the viral infection on the number of pigs weaned/sowed showed a drastic decrease of 2.3 to 11.2 pigs per year. Analysis of mortality rates of nursery-age pigs in two PRRSV infected farms showed a mean increase of 10.65%, although with relatively large variability. For pigs in the grower-finisher phase of production, the amount of feed necessary to sustain these animals increased, translating to a mean loss of feed efficiency of 7.57%. Furthermore, there was a substantial increase in the mortality rate up to 6.05% in this category of animals.

Variable	Value
Projected annual costs for breeding-farrowing phase	
Reduced No. of pigs weaned/litter	
No. of breeding females	6 million
Percentage of breeding females in affected herd	15%
No. of litters affected	0.9 million
Reduced revenue/affected litter	\$45.00
Total reduced revenue for reduced No. of pigs weaned per/litter	\$40.50 million
Reduced farrowing rate	
Increased fixed cost/affected litter	\$13.75
No. of litters affected	0.9 million
Increased fixed costs for reduced farrowing rate	\$12.38 million
Increased variable costs/affected litter	\$15.41
No. of litters affected	0.9 million
Increased variable costs for reduced farrowing rate	\$13.87 million
Total increased cost for reduced farrowing rate	\$26.25 million
Total increased cost for breeding-farrowing phase	\$66.75 million
Projected annual cost for nursery phase	
No. of nursery pigs annually	104.16 million
Percentage of pigs in affected nurseries	32.16%
No. of affected nursery pigs	33.50 million
Increased cost/pig in affected nurseries	\$6.01
Total increased cost for nursery phase	\$201.34 million
Projected annual cost for grower-finisher phase	
No. of grower-finisher pigs annually	100 million
Percentage of pigs in affected grower-finishers	38.10%
No. of affected grower-finisher pigs	38.1 million
Increased cost/pig in affected grower-finishers	\$7.67
Total increased cost for grower-finisher phase	\$292.23 million
Total annual cost of PRRS to US swine producers	\$560.32 million

Table 1: Projected annual cost of PRRS to US swine producers as described by Neumann, 2005).

Through an economic lens, these data prove quite staggering, with a total reduction of revenue of \$45 per litter. A cost increase of \$3.58 per nursery-phase pig and \$3.23 per grower-finisher phase pigs was calculated. To US swine producers as a whole, the total increased cost of PRRSV-affected swine, is \$560.32 million/year. This is based on a sum of calculated increased costs for PRRSV affected pigs of the nursery, breeding-farrowing, and grower-finisher phases. The total cost attributed to classical swine fever in 1961 was \$364.09 million after inflation adjustment. The relatively higher cost corresponding to PRRSV today demonstrates that PRRSV is a successful, and thus important, pathogen.

1.3 Clinical manifestation of PRRS

PRRS exhibits a number of distinct clinical manifestations with respect to two discriminatory pathological classes: reproductive failure and respiratory ailments. This syndrome is characterized by “late-term abortions, an increased number of stillbirths, mummified and weak-born pigs,” and “non-specific lymphomononuclear interstitial pneumonitis,” which is a type of inflammation of the lungs (Music and Gagnon, 2010). Other features of the disease include “anorexia, fever, lethargy, pneumonia, agalactia, red/blue discoloration of the ears and vulva, subcutaneous and hind limb edema, delayed return to estrus after weaning, and in rare cases, death” (Done and Paton, 1995; Rossow, 1998; Terpstra , 1991).

The degree of respiratory ailment varies with age, but is most severe in young swine. Neonatal pigs are affected up to 100% with mortality. Live born pigs infected with PRRSV *in utero* may exhibit clinical signs of edema in the periorbital region and eyelids. Shaking, diarrhea, and neurological signs can also be observed. Weaning

pigs show failure to thrive, lethargy, and fever. The disease also renders young pigs vulnerable to concurrent infections, as its compromised immune system may succumb to secondary bacterial or viral pathogens, such as *Streptococcus suis* type 2, swine influenza virus, and porcine respiratory coronavirus. In fact, pneumonia is not an uncommon clinical sign in weaning pigs as well.

In the post-weaning phase, there is a body temperature increase ranging from 1 to 3 degrees C above normal which can last up to four days after infection (Knox, 2005). In a more severe infection PRRSV lesions are consistent with enlarged lymph nodes and interstitial pneumonia including lungs that fail to recoil. The most documented occurrence of PRRSV-induced lesions are a result of interstitial pneumonia, a form of lung disease characterized by scar tissue present on both lungs, and in the case of neonatal pigs, leaked alveolar exudate with large quantities of necrotic debris and immune cells. Other less common lesions can occur in lymphoid tissue and are marked by hypertrophy, necrosis, and cystic spaces between lymph nodes. Finishing pigs, which are pigs in a higher weight class fattened for slaughter, and boars are less likely to express any clinical signs and a blood test may be the only means to determine infection (Done and Paton, 1995; Rossow, 1998).

The reproductive phase of the disease generally causes abortions 2 to 3 weeks post-infection, if the pig is in the second half of the gestation period. Another clinical occurrence is premature delivery, usually of a mixture of live and dead fetuses. There is an increase in the number of stillborn pigs as well as mummified fetuses. Fetal mummification is the shriveling of a retained unborn offspring in the uterus. Reproductively, the sow is also affected with decreased capacity to produce

milk. Males may demonstrate low quantity or abnormal sperm cells. If a female mates with an infected male, she may become infected and fail to conceive (Knox, 2005).

The severity of the disease depends on a number of factors. Virus virulence varies with different PRRSV isolates. For example, in a study by Halbur et al, 5-week old pigs of the same breed and cesarean-derived, were infected with 9 different PRRSV isolates of the North American strain. The researchers discovered that there were major differences in pathogenicity among the isolates (Halbur et al, 1996). In addition to genetic differences that give rise to varying pathogenicities of different PRRSV isolates, another factor of severity seems to be the swine breed. To illustrate, a study done by Vincent et al in 2006 shows significant differences in clinical signs of two genetically distinct commercial lines of pigs that were infected with a known highly virulent strain of PRRSV (VR-2385) (Vincent et al., 2006). Other factors include age of the pig and the occurrence of other secondary infections that may have compromised the immune system.

1.4 Transmission of PRRS

Transmission seems to increase with proximity of the animals as the main means of virus spread is via aerosols. This suggests that even pigs that are not in direct contact but are still housed together in close conditions are still susceptible infection. In fact, “aerosol transmission from farm to farm across miles is suspected, but as of yet, has not been proven” (Knox, 2005). Once it enters the respiratory tract, PRRSV will replicate and disseminate to multiple organs where it mainly targets macrophages, especially in the lungs and bronchiolar epithelial cells, as well

as arteriolar endothelial cells and monocytes. Virus is also shed in urine, feces, and semen of infected boars as well as a variety of other secretions such as saliva, nasal fluid, serum and mammary gland secretions. PRRSV has the ability to cross the placenta and so, the fetuses that have contracted the virus *in utero* and survive are major concerns for farmers as they can be carriers after birth (Knox, 2005).

Indirect transmission is also possible and mainly results from common farming practices like non-sterile use of needles and practices that generate aerosols. Insects might be another method of transfer (Otake et al., 2003). The virus has also been isolated on clothing, saliva, and fingernail washings of personnel in direct contact with infected animals, and as a particular study shows, they were able to transmit the virus to non-infected animals (Knox, 2005). However, there is no evidence supporting transmission of PRRSV via rodents or birds (Knox, 2005).

Chapter 2

Control of PRRSV infections

2.1 PRRSV vaccines

Currently, there are live and killed vaccines against PRRSV. However, these vaccines are not highly effective, and are probably affected by different factors, including viral loads of the affected herds, virulence of circulating PRRSV, concurrent illnesses, or genetic predispositions of the pigs (Knox, 2005). Current vaccinations mainly rely on the use of live attenuated vaccines (LAV). The first LAV against PRRSV was developed in 1994 via attenuation of the virus by successive passages in a permissive monkey kidney derived cell line (MARC-145) (Kim, 1993). These LAVs were able to confer protection against homologous virus but failed to protect swine against heterologous PRRSV strains (Plana-Duran et al, 1997). Although LAVs do not confer an efficacious protection against PRRSV and do not confer a long lasting immunity, they reduce the occurrence of fever, abortions, and transmission of the virus (Kimman, 2009). Killed PRRSV vaccines are even less efficacious than LAVs particularly in their capability to reduce virus transmission. The efficacy of killed vaccines might be improved by the use of more effective adjuvants (Linghua et al, 2007).

Besides their efficacy, a major concern with LAVs is their safety. Live viruses, attenuated or virulent, have the ability to cross the placenta and cause *in utero* infections of fetuses, so their use in pregnant sows is counter indicated. Furthermore, LAVs persist in vaccinated animals and may revert to a pathogenic phenotype as observed after large-scale vaccination of Danish swine herds (Botner, 1997; Nielsen, 2001). Also, it has been suggested that PRRSV can exist as a quasi-species leading to decreased efficacy of vaccines due to the appearances of novel variants in infected animals. In addition to this, multiple variants of PRRSV can exist

on farms in different individuals at the same time (Music and Gagnon, 2010). More conclusive studies done on the PRRSV proteins, nonstructural in particular, may provide better insight into the modification of a live-attenuated virus that would benefit swine health.

Experimentally, other types of vaccines against PRRSV have been designed such as DNA vaccines. DNA vaccines using ORF 5 induced high levels of neutralizing antibodies (Barfoed et al, 2004), suggesting promising results. More recently, small interference RNAs (siRNAs) have been tested as antiviral tools. siRNA targeting ORF 1a-b and ORF 7 inhibited PRRSV growth *in vitro* (Patel et al, 2008). Although this anti-viral method has proven effective, it is still only in an *in vitro* stage. Clearly, live attenuated PRRS vaccines of improved safety, efficacy, and utility are needed. Better understanding of viral genetic determinants critical for viral virulence and host range, including those which allow current LAVs to efficiently infect and spread in susceptible cells and tissues and persist in the pig, will permit rational design of improved PRRS LAVs.

Formatted: Normal,
Justified, Indent: First
line: 0"

2.2 Herd management

Other key methods for controlling PRRSV spread within and among farms are based on herd management. These non-vaccination based methods include several approaches such as prevention of contact between newly introduced pigs and an established herd until outbreaks of infection subside, disinfection of newly introduced animals, isolation of infected animals, ensuring use of non-infected semen when practicing artificial insemination, and establish biosecurity methods to avoid indirect transmission of the virus (Knox, 2005).

Chapter 3

Porcine Reproductive and Respiratory Syndrome Virus

3.1 The Virion

PRRSV is an enveloped virus of spherical shape. Virions harbor a single-stranded positive sense RNA viral genome of 15 kb in length, capped at the 5' end and polyadenylated at the 3' end. As a member of the family *Arteriviridae* and the genus *Arterivirus*, it is very similar to EAV and Simian Hemorrhagic Fever Virus (SHFV) (Figure 1).

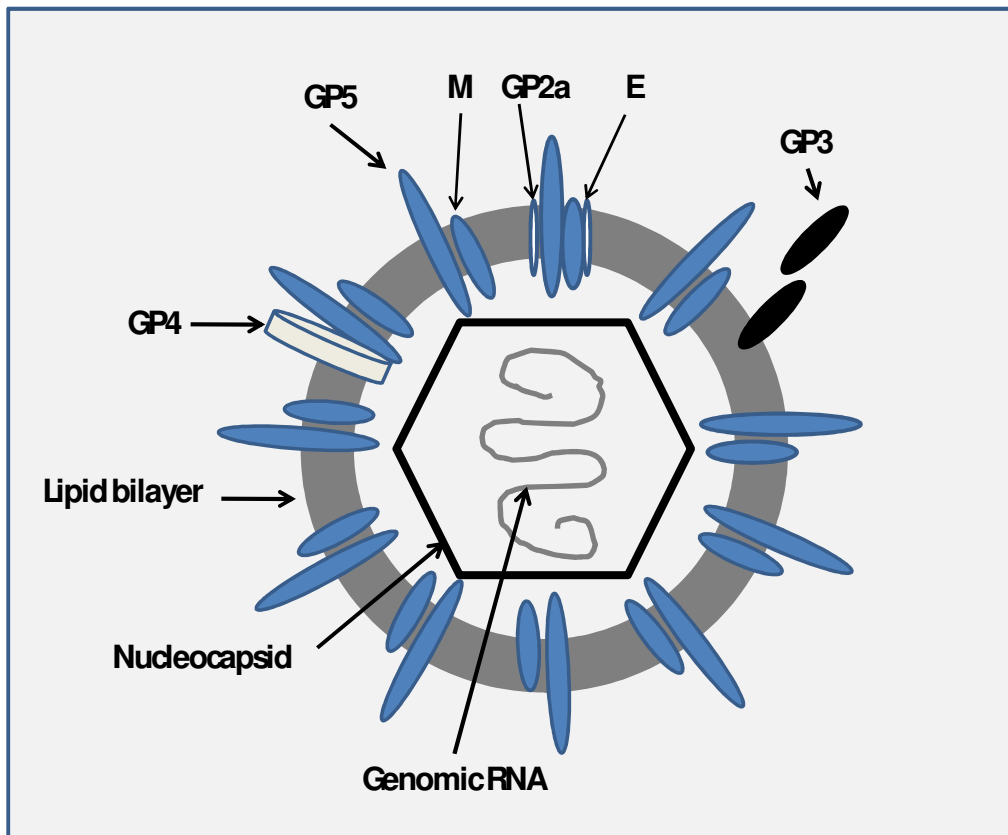


Figure 1: Schematic representation of PRRSV virion. GP: glycoproteins, E: envelope proteins, and M: matrix protein.

The viral envelope (lipid bilayer) can be maintained only within a certain range of environmental conditions. Outside from its natural host (i.e. swine), PRRSV remains stable in a -70 to -20°C temperature range. PRRSV can survive in a 6.5 to

7.5 pH range. Some detergents have proved effective at disrupting the viral envelope (Music and Gagnon, 2010).

3.2 Genetic diversity among PRRSV types

There are at least two distinct genotypes recognized for this heterogeneous virus, one endemic to Europe (Type 1) and one endemic to North America (Type 2). Although the two genotypes share many similarities, such as genome organization, they are distinguished genetically (Music and Gagnon, 2010). PRRSV isolates, from both the North American and European types, vary in antigenic capacity. There are similarities at the level of amino acid sequence ranging from 96-100% within Type 2 North American PRRSV, but when compared to the European isolates, identities range between 57-81% (Meng et al, 1995).

3.3 PRRSV genome

The PRRSV genome encodes for nine open reading frames (ORFs) (Figure 2). Structural proteins are encoded for by open reading frames (ORFs) 2 to 7, while the non-structural proteins are encoded by ORF1a and ORF1b. All ORFs are expressed through the production of a nested set of subgenomic 3' coterminal mRNAs.

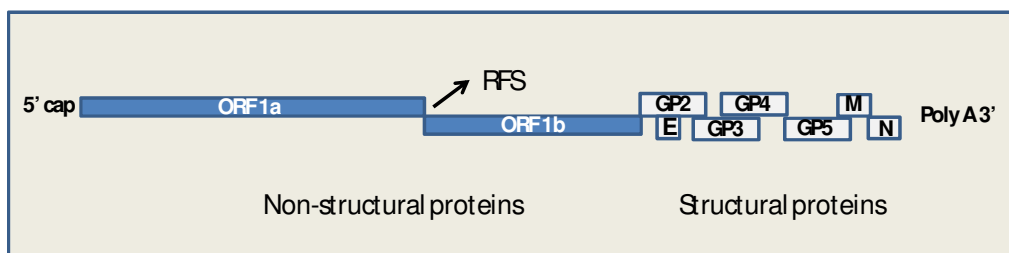


Figure 2: schematic representation of PRRSV genome indicating the location of different open reading frames encoding for non-structural and structural proteins. ORF: open reading frame, GP: glycoprotein; E: envelope protein; M: matrix protein; N: nucleocapsid protein; RFS: ribosomal frameshift (Adapted from Fang and Snijder, 2010).

ORF1a and ORF1b encode for the proteins (n=12) that compose the PRRSV replication complex (Figure 3). These ORFs constitute two-thirds of the entire viral genome. ORF1a and ORF1b encode polyproteins 1a and 1ab that are post-translationally processed by viral and cellular proteases. While all the nonstructural and structural proteins of PRRSV are critical for viral replication, nonstructural proteins 9 to 12 are directly involved in viral transcription and replication.

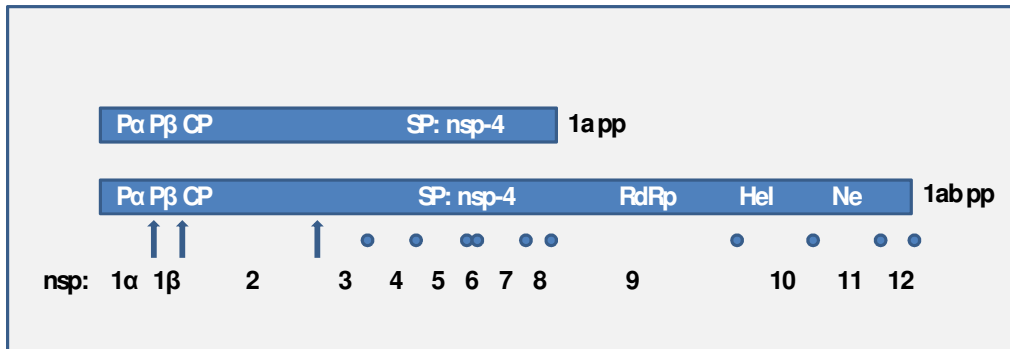


Figure 3: schematic representation of polyproteins (pp) encoded by ORFs 1a and 1a/1b of PRRSV. 1ab pp is produced due to a -1 ribosomal frameshift. Arrows indicate sites cleaved by nsp-1 α and nsp-1 β cysteine proteases (CP) P α and P β . Circles indicate sites cleaved by the viral protease nsp-4 serine 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).

ORF2a, 3, and 4 encode for minor structural *N*-glycosylated proteins GP2a, GP3, and GP4. ORF 5 encodes for GP5, the major PRRSV glycoprotein. ORF 6 encodes for the membrane protein M, and ORF 7 encodes for the nucleocapsid protein N.

3.4 PRRSV structural proteins

The main target cells of PRRSV are mature, well-differentiated cells of the monocyte-macrophage lineage. In particular, PRRSV infects porcine alveolar macrophages (PAMs) and interstitial macrophages of other tissues including heart, thymus, spleen, Peyer's patches, hepatic sinusoids, renal medullary interstitium, and adrenal gland (Music and Gagnon, 2010). PRRSV RNA and nucleocapsid proteins have also been found in other tissues such as testicular germ cells, thymus cells, endothelial heart cells, bronchiolar epithelial cells, interstitial, alveolar, and intravascular macrophages, and even dendritic cells of the spleen and Peyer's patches.

Entry of PRRSV into target cells is via receptor-mediated endocytosis. PRRSV receptors on PAMs were identified to be heparan sulphate and sialoadhesion molecules that allow virus binding and internalization. CD163 is a molecule expressed only on monocyte-derived host cells that mediates PRRSV entry (Van Gorp, 2008). High expression of CD163 has been shown to correlate with increased susceptibility to PRRSV infection. Furthermore, non-permissive cells transiently expressing both CD163 and sialoadhesion molecules cause a significant increase in production and propagation of PRRSV (10 to 100 times greater) than cells transiently expressing only CD163 (Music and Gagnon, 2010).

PRRSV N (nucleocapsid) protein is a multifunctional protein that is important in virus pathogenesis. N protein is highly immunogenic and can be phosphorylated in PRRSV-infected cells and synthesized even in the absence of other viral constituents. Mutational analysis of N suggests that the C-terminus of the protein is critical to the synthesis of conformational epitopes and the binding of N-specific

monoclonal antibodies (Music and Gagnon, 2010). The membrane envelope protein M, together with major glycoprotein envelope protein GP5, is involved in the process of PRRSV assembly, budding, and virus infectivity (Music and Gagnon, 2010).

These two proteins form heterodimers in infected cells (Mardassi et al., 1996). Refer to Figure 1.

GP5 is the most abundant of the glycoproteins present in the virion envelope (Figure 1) and for that reason is called the major envelope glycoprotein, whereas the GP2, GP3 and GP4 are called minor envelope glycoproteins as they are present in lesser amounts than GP5 (Das, 2011). The GP2, GP3 and GP4 interact among themselves and GP5 interacts with both GP4 and M protein (Das et al., 2010; Mardassi et al., 1996). These interactions are required for assembly of the infectious virus (Wissink et al., 2005). GP2 and GP4 proteins have been shown to specifically interact with the CD163 molecule (Das et al., 2010), a receptor for PRRSV entry (Calvert et al., 2007; Van Gorp et al., 2008).

3.5 PRRSV nonstructural proteins

Understanding the PRRSV life cycle in detail, including the role of nonstructural proteins, is critical to learning more about its pathology in the host. Once PRRSV enters the target cells, its replication cycle begins with the release of the genome into the host cell cytoplasm. The replicase genes located in ORF 1a and ORF 1b, are expressed as polyproteins pp1a or pp1ab that are translated after ribosomal frameshifting (-1) (Figure 3). Although not well-understood, pp1a is most likely cleaved at 8 different sites to form 9 nonstructural proteins, NSP1 α , NSP1 β , and NSP2 to 8. These proteins are responsible for a number of intracellular

interactions including proteolytic activities mediated mainly by NSP4 that result in further processing of the polyproteins. NSP 9 to 12, are proteins derived from the cleavage of pp1ab. These proteins seem to have a greater involvement in PRRSV transcription and replication. NSP 9 is the RNA dependent RNA polymerase (RdRp), NSP 10 has helicase activity (Hel), and NSP 11 possesses endoribonuclease (Ne) activity.

Chapter 4

Rationale and Significance, Objective, and Approach

4.1 Rationale and Significance

It is essential to identify PRRSV mechanisms involved in induction of disease, generalization of infection, tissue tropism, host range, and modulation/suppression of host immune response in order to rationally engineer PRRS biological control tools. Assessing the roles 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 obtain this knowledge on PRRSV virulence 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.

4.2 Objective

The objective of this study was to express and purify PRRSV nsp-3, encoded by ORF1a and part of the viral replication complex. Purified nsp-3 will then be used for producing reagents such as antibodies. Today, these reagents have a limited availability and hamper key studies.

4.3 Approach

Non-structural protein 3, encoded by ORF 1a, is 231 amino acids long (Figure 4) with a predicted molecular weight of 24 kDa (kilo-daltons). Nsp-3 is cleaved by nsp-4 (Figure 3).

```

GGPHLIAALHVACSMALHMLAGVYVTVAVGSCGTGTNDPWCTNPFA
VPGYGPGSLCTSRLCISQHGTLPLTALVAGFGLQEIALVVLIFVSIIG
MAHRLSCKADMLCVLLAIASYVWVPLTWLLCVFPCWLRWFSLHPLT
ILWLVFFLMSVNMPSGILTVVLLVALWLLGRYTNVVGLVTPYDIHHYT
NGPRGVAALATAPDGTYLAAVRRRAALTGRTVLFTPSQLGSLE

```

Figure 4: Amino acid sequence of NSP 3 from PRRSV isolate NVSL 97-7985 used in this work.

The function of NSP 3 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 NSP 3 in the virus life cycle. In the present work, NSP 3 was expressed fused to a poly-Histidine tag (6xHis) 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 3 was synthesized by PCR using specific forward and reverse primers and a full-length cDNA copy of PRRSV isolate NVSL 97-7985 as a target (pFL12, kindly provided by Dr Fernando Osorio, University of Nebraska-Lincoln). The amplified NSP 3 gene was inserted in-frame into the pRSET A plasmid (Invitrogen, Carlsbad, CA) using the T4 DNA ligase (New England Biolabs, Ipswich, MA) where the expression of the cloned gene is under the control of the T7 promoter. The resulting plasmid pRSETAnsp-3 was used to transform BL21(DE3)pLysS cells (Invitrogen). Transformants were then grown in Magic® media (Invitrogen) for maximum expression of NSP 3. NSP 3 was then purified by affinity chromatography using nickel columns (Thermo Scientific, Waltham, MA) and was detected by SDS-PAGE separation followed by Coomassie blue staining and western blotting.

Deleted: ¶

Chapter 5

Procedures and Results

5.1 Synthesis of PRRSV NSP3 gene by Polymerase Chain Reaction (PCR)

The purpose of this first step in the process of NSP3 expression is to produce a high number of copies of the gene. A PCR was designed to synthesize PRRSV NSP 3 using primers described in table 2. Primers included sequences for *Bam*HI (ggatcc) and *Xho*I (ctcgag) restriction sites. 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, Mountain View, CA), 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°C for two minutes, followed by 35 cycles of 95°C for 30 seconds, 55°C for 45 seconds, and 68°C for 60 second; finished by a final cycle of 68°C for 3 min.

Deleted:

Formatted: Subscript

Table 2: sequence of the primers used for PCR amplification of PRRSV NVSL 97-7895 (pFL12 full length cDNA copy) NSP 3 gene. Underlined BamHI and XhoI restriction sites included in the sequences of NSP3_For and NSP_3Rev primers, respectively.

Primer name	Sequence
NSP 3_For	5'-atggatccggaggcccgacctcattg-3'
NSP 3_Rev	5'-atctcgagctcaagaaggaccaccaagctg-3'

5.2 Purification of amplified NSP 3 gene

The 693 bp amplicon of NSP 3 was 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 (Invitrogen) 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).

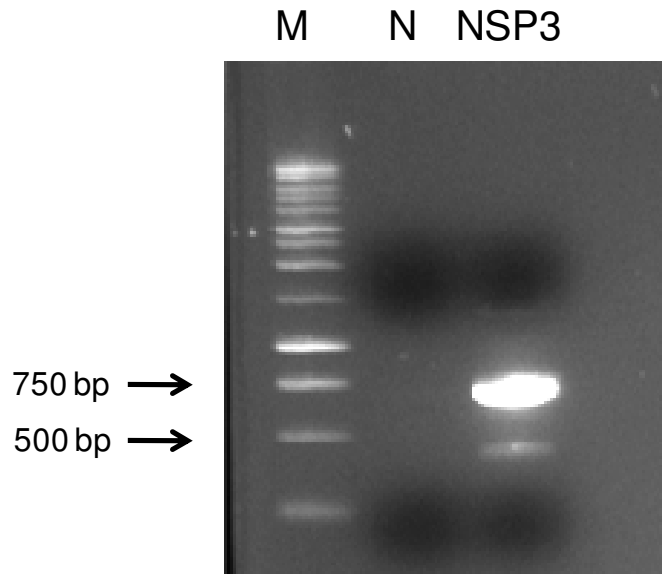


Figure 5: PRRSV NSP 3 was amplified by PCR and resolved by agar gel electrophoresis in a 1% 1X-TAE buffer (Tris-acetate-EDTA) stained with ethidium bromide. A band of 693 bp was detected. M: 1Kb ladder molecular weight marker (Invitrogen), N: non-template control.

After separation of the DNA as described above, the NSP 3 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. The band of approximately 700 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 μ l of buffer) of Buffer QG was added to the tube. The tube was placed at 50°C in a heating block and incubated until the agarose was completely dissolved (~10 min). Then, 200 μ L 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).

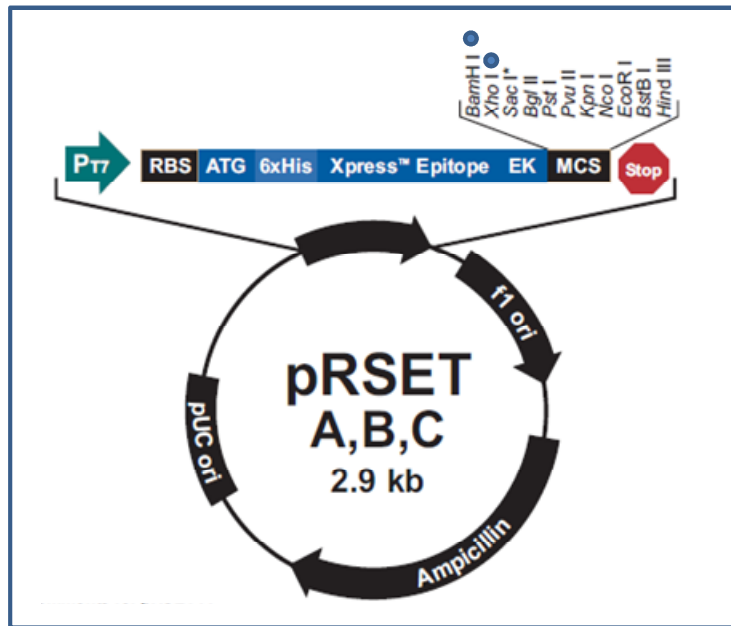


Figure 6: *Invitrogen's pRSET diagram. P7: T7 promoter. RBS: ribosomal binding site. ATG: codon encoding for methionine. 6xHis: histidine tag. Xpress Eptope: synthetic epitope tag. EK: enterokinase cleavage site. MCS: multiple cloning sites. Stop: stop codon. Blue circles denote location of restriction sites for BamHI and XhoI.*

5.3 Cloning of the NSP 3 gene into the pRSETA vector

The nsp-3 gene was cloned into pRSETA vector using T4 DNA ligase (New England Biolabs). Both vector and NSP 3 gene were digested with *BamHI* and *XhoI* restriction endonucleases (New England Biolabs) in a 20 μl reaction. The reaction

was set as follows: 10 μ l of pRSET A (390 ng/ μ l) or 10 μ l of NSP 3 gene (100 ng/ μ l), 2 μ l of 10X NEB buffer 2 (New England Biolabs), 0.5 μ l of 100X BSA, 1 μ l of *Bam*HI enzyme (20,000U/ml) (New England Biolabs), 1 μ l of *Xho*I enzyme (20,000U/mL) (New England Biolabs), and 5.5 μ l ddH₂O. The reaction was then incubated at 37°C in a water bath for 2 hr. After digestion, linearized pRSET A and NSP 3 DNAs were resolved in a 1% agarose-TAE-ethidium bromide gel, and a bands of appropriate sizes were excised and purified from the gel using QIAquick Gel Extraction Kit (Qiagen) as described above (Figure 7).

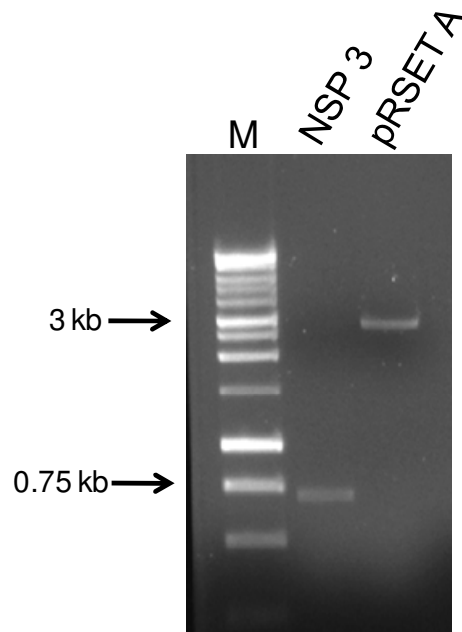


Figure 7: PRRSV NSP 3 and pRSET A were digested with *Bam*HI and *Xho*I and resolved by agarose gel electrophoresis in a 1% 1X-TAE buffer (Tris-acetate-EDTA) stained with ethidium bromide. M: 1Kb ladder molecular weight marker (Invitrogen), N: non-template control, NSP 3 gene, and linearized pRSET A (2.9 kb).

5.4 Ligation reaction

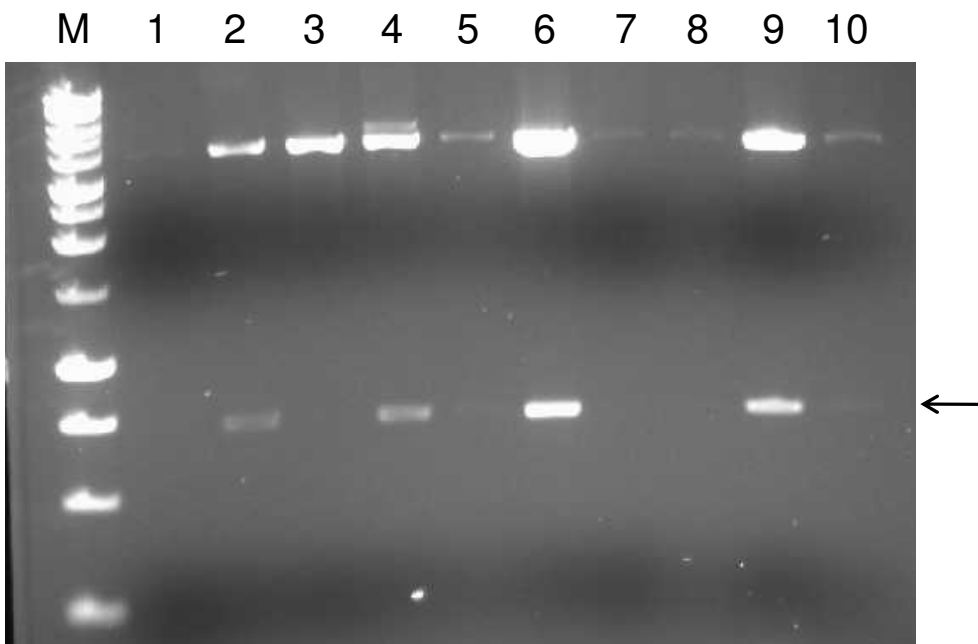
The linearized pRSETA vector and digested nsp-3 gene insert were combined in a 10 µl ligation reaction. The reaction was set up as follows: 1 µl of vector, 1 µl of insert, 1 µl of 10X T4 DNA ligase buffer (New England Biolab), and 400 units of T4 DNA ligase (New England Biolab). The reaction was incubated overnight at 14°C. After incubation the ligated DNA was used to transform *E. coli* TOP 10 competent cells (Invitrogen).

5.5 Purification of Prseta-nsp3 vector

After transformation, *E. coli* TOP 10 competent cells were placed on Terrific Broth (Invitrogen) agar plates containing 50 µg/ml of ampicillin and incubated overnight at 37°C. Ampicillin resistant colonies were picked from the plate and grown overnight at 37°C in Terrific both containing 50 µg/ml of ampicillin. Bacterial cultures were spun down in bench-top centrifuge at 4000 rpm 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°C 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 μ L Buffer EB (elution buffer) to the center of the column, and spinning 1 min at maximum speed.

Recovered plasmids were digested with *Bam*HI and *Xho*I as described above



and resolved in a 1% agarose TAE gel stained with ethidium bromide (Figure 8).

Figure 8. Recovered plasmids were digested with *Bam*HI and *Xho*I as described above and resolved in a 1% agarose TAE gel. Arrow indicates the presence of an insert of appropriate site in clones 2, 4, 6, and 9.

Clones carrying inserts of the correct size (Figure 8) were sequenced (Genewiz, South Plainfield, NJ) to further assessing the fidelity of cloned NSP 3 gene. Sequences were analyzed using Bioedit software.

5.6 Maxiprep of selected pRSETA-nsp3

Once fidelity of the NSP 3 gene in pRSETA was determined, the concentration of plasmid DNA was increased by using the Hi-speed Plasmid Maxi Kit (Qiagen). The importance of this step is to increase the amount of nsp-3 (as well as the plasmid in which it lies) for further research uses. An *E.coli* stock harboring pRSETAnsp3 was cultured overnight at 37°C with shaking in 150 ml of Terrific Broth (Invitrogen) containing 50 µg/ml of ampicillin. 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 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). This maxiprep was successful and an increased concentration of plasmid DNA and nsp-3 were produced.

5.7 Expression of PRRSV NSP 3 in *E.coli* BL21 cells

E. coli BL21 (DE3)pLysS chemically competent cells (Invitrogen) were transformed with pRSETAnsp3 by heat shock. Briefly, BL21 cells (50 μ l) were thawed on ice and mixed with 100 ng of pRSETAnsp3. The mixture was incubated for 30 min on ice. After incubation, the tube containing cells and plasmid DNA was incubated for 40 sec at 42^oC in a water bath and place immediately on ice for additional 2 min. After incubation on ice, 250 μ l of SOC media (Invitrogen) were added to the mixture and incubated at 37^oC for 1 hr in an orbital shaker at 250 rpm. Transformed cells were selected by plating in Terrific Broth agar plates containing 50 μ g/ml of ampicillin and 35 μ g/ml of chloramphenicol, followed by incubation overnight at 37^oC.

Two BL21 cell bacterial colonies were picked from the plate containing the selective media and transferred to 5 mL of Terrific Broth containing 50 μ g/ml of ampicillin and 35 μ g/ml of chloramphenicol and grown with shaking overnight at 37^oC. Chloramphenicol selects for maintenance of the pLysS plasmid required for T7 lysozyme expression and ampicillin selects for the pRSETAnsp3 plasmid. After incubation, 150 μ L of the culture were then added to 150 mL of Invitrogen's Magic Media broth. The culture was incubated for 18 hr at 37^oC, after which it was centrifuged at 4,000rpm for 30 min and the supernatant was discarded. The BL21 cell pellet expected to contain expressed NSP 3 kept at -20^oC prior to lysis.

To extract NSP 3, BL21 cells pellets were thawed on ice and 20 mL of CellLytic B buffer (Sigma, St Louis, MO) containing 100 μ L of protease inhibitor (Sigma) was added per 1 g of cells. The pellet was resuspended by pipetting and vortexing, and further incubated at 37°C 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°C. To determine nsp-3 expression, an SDS-PAGE NuPAGE® Novex 12% Bis-Tris Gel (Invitrogen) was run. Samples were run under “reducing” conditions by mixing 2.5 μ L of NuPAGE® LDS Sample Buffer (4X), 1 μ L of NuPAGE® Reducing Agent (10X), and 6.5 μ L 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 transferred to a clean tray and stained with Coomassie blue (SimplyBlue Self Stain, Invitrogen) (Figure 9). The result was a dark stain in the 25-31 kDa region indicating the presence of nsp-3 in high concentration.

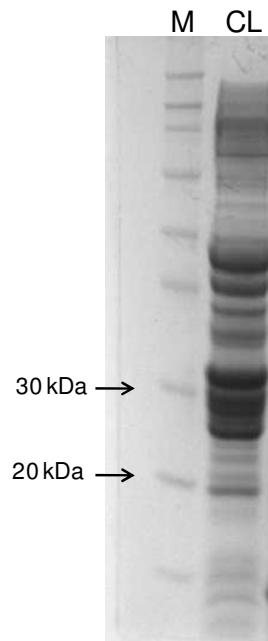


Figure 9. BL21 cell protein lysates (CL) were separated by SDS-PAGE using a NuPAGE® Novex 12% Bis-Tris Gel (Invitrogen) and stained with SimplyBlue Self Stain (Invitrogen). M: molecular size marker expressed in kDa.

The fusion protein expressed from pRSETA-nsp3 in BL21 cells (Figure 10) is expected to have a molecular size of approximately 29 kDa.

```

MRGSHHHHHHGMASMTGGQQMGRDLYDDDDKDRWGSGGPHLIAALHV
ACSMALHMLAGVYVTAVGSCGTGTNDPWCTNPFVPGYGPGLCTSRLCI
SQHGLTLPLTALVAGFGLQEIALVVLIFVSIGGMAHRLSCKADMLCVLLAIAS
YVWVPLTWLLCVFPCWLRWFSLHPLTLWLVFLLMSVNMPGILTVVLLVAL
WLLGRYTNVVGLVTPYDIHHYTNGPRGVAALATAPDGTYLAAVRRRAALTGR
TVLFTPSQLGSLLLE

```

Deleted:

Figure 10: Amino acid sequence of PRRSV NSP3 fusion protein expressed in this work. In red is the fused sequence that includes 6x-His, Express® epitope, and enterokinase cleavage site sequences.

5.8 Purification of NSP3

This time, purification was carried out of the nsp-3 grown in Magic Media. Chemically competent One Shot BL21 (DE3) pLysS *E. coli* (Invitrogen) cells were transformed with 10 ng of pRSETAnsp3 plasmid encoding PRRSV nsp-3, cultured in Magic Media (Invitrogen) for 21 hours at 37°C 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, the columns were inverted a few times and the Equilibration/Wash Buffer was drained from the columns for 4 min. To purify nsp-3, 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 with 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 it was labeled Wash 1. The process was repeated two more times and Wash 2 and Wash 3 were collected. His-tagged nsp-3 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 so that Elutions 2 and 3 could be collected. All fractions were stored at -20°C . Collected fractions were then analyzed on a 10% NuPage Novex Bis-Tris gel (Invitrogen) using a discontinuous SDS-PAGE system as described above. The gel was stained using Simply Blue (Invitrogen) (Figure 11). Though the band is very faint, the gel picture still shows the presence of nsp-3, although the protein-yield was low.

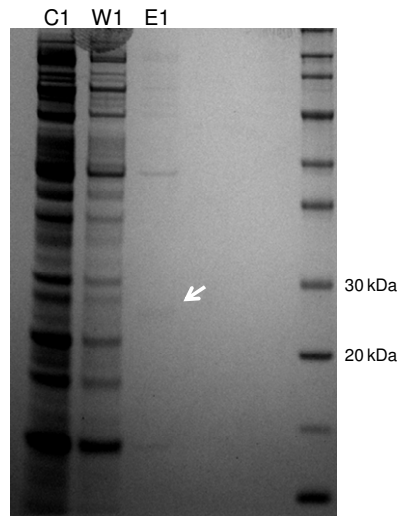


Figure 11. Purification of His-tagged PRRSV NSP 3. SDS-PAGE electrophoresis stained with Simple Blue (Invitrogen). M: molecular size markers expressed in kDa. C1: collection 1. W1: wash 1. E1: elution 1. Arrow indicates NSP 3 His-tagged fusion with a molecular size of 28 kDa.

5.9 Detection of NSP3 by Western Immunoblot

To specifically detect NSP 3, protein lysates from BL21 cells transformed with pRSETAnsp3 were run in a SDS-PAGE 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 transferred to 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 (Invitrogen) 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 ddH₂O, 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 μ L 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 (Figure 12). Using this approach a protein of

approximately 28 kDa was detected by western immunoblot using an anti His-tag monoclonal antibody.

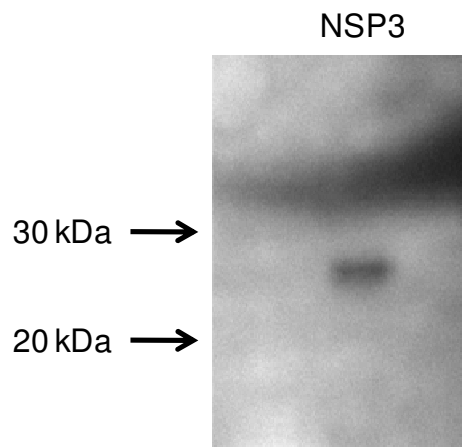


Figure 12. Western immunoblot showing His-tagged PRRSV NSP 3.

Summary

NSP 3 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 3 was purified by affinity chromatography and its purity verified by SDS-PAGE and Western immunoblot using a monoclonal antibody against the 6xHis tag. 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 3 will be used for production of antibodies. These antibodies against PRRSV NSP 3 are critical for studies such as protein-protein interactions between viral and host proteins.

References

- Barfoed, A.M., Blixenkroner-Møller, M., Jensen, M.H., Botner, A. and Kamstrup, S. (2004) DNA vaccination of pigs with open reading frame 1-7 of PRRS virus. *Vaccine* 22(27-28), 3628-41.
- Botner, A., Strandbygaard, B., Sørensen, K.J., Have, P., Madsen, K.G., Madsen, E.S. and Alexandersen, S. (1997) Appearance of acute PRRS-like symptoms in sow herds after vaccination with a modified live PRRS vaccine. *Vet Rec* 141(19), 497-9.
- Cafruny, W.A., Duman, R.G., Wong, G.H., Said, S., Ward-Demo, P., Rowland, R.R. and Nelson, E.A. (2006) Porcine reproductive and respiratory syndrome virus (PRRSV) infection spreads by cell-to-cell transfer in cultured MARC-145 cells, is dependent on an intact cytoskeleton, and is suppressed by drug-targeting of cell permissiveness to virus infection. *Virology* 3, 90.
- Calvert, J.G., Slade, D.E., Shields, S.L., Jolie, R., Mannan, R.M., Ankenbauer, R.G. and Welch, S.K. (2007) CD163 expression confers susceptibility to porcine reproductive and respiratory syndrome viruses. *J Virol* 81(14), 7371-9.
- Collins, J.E., Benfield, D.A., Christianson, W.T., Harris, L., Hennings, J.C., Shaw, D.P., Goyal, S.M., McCullough, S., Morrison, R.B., Joo, H.S. and et al. (1992) Isolation of swine infertility and respiratory syndrome virus (isolate ATCC VR-2332) in North America and experimental reproduction of the disease in gnotobiotic pigs. *J Vet Diagn Invest* 4(2), 117-26.
- Das, P.B., Dinh, P.X., Ansari, I.H., de Lima, M., Osorio, F.A. and Pattnaik, A.K. (2010) The minor envelope glycoproteins GP2a and GP4 of porcine

reproductive and respiratory syndrome virus interact with the receptor CD163. *J Virol* 84(4), 1731-40.

Das, P.B., Vu, H.L., Dinh, P.X., Cooney, J.L., Kwon, B., Osorio, F.A. and Pattnaik, A.K. (2011) Glycosylation of minor envelope glycoproteins of porcine reproductive and respiratory syndrome virus in infectious virus recovery, receptor interaction, and immune response. *Virology* 410(2), 385-94.

Done, S.H. and Paton, D.J. (1995) Porcine reproductive and respiratory syndrome: clinical disease, pathology and immunosuppression. *Vet Rec* 136(2), 32-5.

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

Halbur, P.G., Paul, P.S., Meng, X.J., Lum, M.A., Andrews, J.J. and Rathje, J.A. (1996) Comparative pathogenicity of nine US porcine reproductive and respiratory syndrome virus (PRRSV) isolates in a five-week-old cesarean-derived, colostrum-deprived pig model. *J Vet Diagn Invest* 8(1), 11-20.

Kim, H.S., Kwang, J., Yoon, I.J., Joo, H.S. and Frey, M.L. (1993) Enhanced replication of porcine reproductive and respiratory syndrome (PRRS) virus in a homogeneous subpopulation of MA-104 cell line. *Arch Virol* 133(3-4), 477-83.

Kimman, T.G., Cornelissen, L.A., Moormann, R.J., Rebel, J.M. and Stockhofe-Zurwieden, N. (2009) Challenges for porcine reproductive and respiratory syndrome virus (PRRSV) vaccinology. *Vaccine* 27(28), 3704-18.

Knox, Robert V. "PRRS Is Still A Mystery Disease" (Swine Reproduction) University of Illinois Extension. *Illinois Livestock Trail* (2011).

<http://www.livestocktrail.uiuc.edu/swinerepronet/paperDisplay.cfm?ContentID=7595>>.

- Linghua, Z., Xingshan, T. and Fengzhen, Z. (2007) Vaccination with porcine reproductive and respiratory syndrome killed virus vaccine and immunostimulatory oligodeoxynucleotides induces specific immunity in piglets. *Vaccine* 25(10), 1735-42.
- Mardassi, H., Massie, B. and Dea, S. (1996) Intracellular synthesis, processing, and transport of proteins encoded by ORFs 5 to 7 of porcine reproductive and respiratory syndrome virus. *Virology* 221(1), 98-112.
- Meng, X.J., Paul, P.S., Halbur, P.G. and Lum, M.A. (1995) Phylogenetic analyses of the putative M (ORF 6) and N (ORF 7) genes of porcine reproductive and respiratory syndrome virus (PRRSV): implication for the existence of two genotypes of PRRSV in the U.S.A. and Europe. *Arch Virol* 140(4), 745-55.
- Music, N. and Gagnon, C.A. (2010) The role of porcine reproductive and respiratory syndrome (PRRS) virus structural and non-structural proteins in virus pathogenesis. *Anim Health Res Rev* 11(2), 135-63.
- Neumann, E.J., Kliebenstein, J.B., Johnson, C.D., Mabry, J.W., Bush, E.J., Seitzinger, A.H., Green, A.L. and Zimmerman, J.J. (2005) Assessment of the economic impact of porcine reproductive and respiratory syndrome on swine production in the United States. *J Am Vet Med Assoc* 227(3), 385-92.
- Nielsen, H.S., Oleksiewicz, M.B., Forsberg, R., Stadejek, T., Botner, A. and Storgaard, T. (2001) Reversion of a live porcine reproductive and respiratory syndrome virus vaccine investigated by parallel mutations. *J Gen Virol* 82(Pt 6), 1263-72.

- Otake, S., Dee, S.A., Rossow, K.D., Moon, R.D., Trincado, C. and Pijoan, C. (2003) Transmission of porcine reproductive and respiratory syndrome virus by houseflies (*Musca domestica*). *Vet Rec* 152(3), 73-6.
- Patel, D., Opriessnig, T., Stein, D.A., Halbur, P.G., Meng, X.J., Iversen, P.L. and Zhang, Y.J. (2008) Peptide-conjugated morpholino oligomers inhibit porcine reproductive and respiratory syndrome virus replication. *Antiviral Res* 77(2), 95-107.
- Plana-Duran, J., Bastons, M., Urniza, A., Vayreda, M., Vila, X. and Mane, H. (1997) Efficacy of an inactivated vaccine for prevention of reproductive failure induced by porcine reproductive and respiratory syndrome virus. *Vet Microbiol* 55(1-4), 361-70.
- Rossow, K.D. (1998) Porcine reproductive and respiratory syndrome. *Vet Pathol* 35(1), 1-20.
- Terpstra, C., Wensvoort, G. and Pol, J.M. (1991) Experimental reproduction of porcine epidemic abortion and respiratory syndrome (mystery swine disease) by infection with Lelystad virus: Koch's postulates fulfilled. *Vet Q* 13(3), 131-6.
- Van Gorp, H., Van Breedam, W., Delputte, P.L. and Nauwynck, H.J. (2008) Sialoadhesin and CD163 join forces during entry of the porcine reproductive and respiratory syndrome virus. *J Gen Virol* 89(Pt 12), 2943-53.
- Vincent, A.L., Thacker, B.J., Halbur, P.G., Rothschild, M.F. and Thacker, E.L. (2006) An investigation of susceptibility to porcine reproductive and respiratory syndrome virus between two genetically diverse commercial lines of pigs. *J Anim Sci* 84(1), 49-57.

Wensvoort, G., Terpstra, C., Pol, J.M., ter Laak, E.A., Bloemraad, M., de Kluiver, E.P., Kragten, C., van Buiten, L., den Besten, A., Wagenaar, F. and et al. (1991) Mystery swine disease in The Netherlands: the isolation of Lelystad virus. *Vet Q* 13(3), 121-30.

Wissink, E.H., Kroese, M.V., van Wijk, H.A., Rijsewijk, F.A., Meulenber, J.J. and Rottier, P.J. (2005) Envelope protein requirements for the assembly of infectious virions of porcine reproductive and respiratory syndrome virus. *J Virol* 79(19), 12495-506.