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Assessment of the Interactions of Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) Non Structural Protein 3 (NSp3) with Cellular Proteins from Virus Permissive Cells

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**Assessment of the interactions of Porcine Reproductive and
Respiratory Syndrome Virus (PRRSV) Non Structural Protein 3
(NSp3) with cellular proteins from virus permissive cells**

Rene M. MacKinnon

A Thesis

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ABSTRACT

The purpose of this research is to develop a method for assessing *in vivo* protein-protein interactions in cultured cells that are permissive to Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) infection. The Non Structural Protein 3 (NSp3) expressed by PRRSV was used as a prey to capture interacting cellular proteins in protein cell lysates using a Pull-Down assay. Interacting proteins were resolved by SDS-PAGE, extracted and identified by means of mass spectrometry. Different alternatives have been tested to create the optimal conditions for performing the technique that will allow further confirmation of virus-host cells protein-protein interaction in PRRSV permissive cells.

REVIEW OF LITERATURE: PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS

Etiology:

The Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) is an enveloped, spherical, single-stranded positive-sense RNA virus 40-60 nm in diameter. PRRSV is in the order of Nidovirales, family Arteriviridae, genus *Arterivirus* (Botner, 1994). There are two types of PRRSV viruses, one is the European isolate and the other is the North American isolate. Sequence analysis showed that the virus isolates are evolving by random mutation and intragenic recombination (Cho, 2006). Other arteriviruses include equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV), and simian hemorrhagic fever virus (SHFV). EAV, LDV and SHFV were isolated and characterized in 1960s and 1970s; however, PRRSV was not isolated or characterized until the 1990s (Snijder, 1998).

The polycistronic 3'-polyadenylated and 5'-capped arterivirus genome is 12-16 kb long. The arterivirus genomes have a 5' nontranslated region (NTR) of 156-224 nucleotides, and a 3' NTR of 59-117 nucleotides. Arteriviruses contain 10-15 known open reading frames (ORFs). Specifically, PRRSV contains eight ORFs (Xiang-Jin, 1995). Arteriviruses also contain two large ORFs named 1a and 1b, which take up three-quarters of the genome at the 5' proximal end (Snijder, 2013).

With very strict host cell specificity, LDV has been found to grow but not replicate in primary cultures of mouse macrophages, and SHFV and PRRSV have been found to replicate in primary macrophage cultures of simian and porcine origin as well as in African green monkey kidney cells (MA-104) and their derivatives (CL2621 and MARC-

145) (Snijder, 1998). Arteriviruses assemble by budding of nucleocapsids into the lumen of the endoplasmic reticulum and/or Golgi apparatus, and are released from cells via exocytosis. Although little is known about the replication cycle of arteriviruses, the replication cycle begins with the expression of non-structural protein genes encoded by ORF1a and ORF1b. Both arteriviruses and coronaviruses show discontinuous subgenomic mRNA transcription of their genomes, which is one reason why they are grouped together in the order of *Nidovirales* (Snijder, 1998).

Arteriviruses are spherical, enveloped virions with a diameter of 40-60 nm, containing an isometric core particle of 25-35 nm in diameter. Furthermore, arteriviruses contain a nucleocapsid protein, the N protein, with a molecular mass between 12-15 kDa, which represents 20-40% of the protein content of the virion. Immunoprecipitation of N from purified PRRSV showed that the protein is present as disulfide-linked homodimer. The non-glycosylated M protein of arteriviruses is the most conserved structural protein, playing a role in the formation of disulfide bridges in receptor binding (Snijder, 1998).

The severity of diseases resulting from arteriviruses varies on the specific virus and strain as well as the condition and age of the animal infected. However, a variety of symptoms from arteriviruses infections generally include respiratory distress, hemorrhagic fever, and necrosis of small muscular arteries. Additionally, abortion in pregnant animals, and a persistent presence of the virus in the semen of infected male animals can be seen depending on the particular arterivirus (Snijder 1998).

History of PRRSV

Porcine Reproductive and Respiratory Syndrome (PRRS), while the etiology was unknown at the time, emerged in 1987 in the United States and Canada (Mystery Disease); and, immediately impacted economics in the swine industry at a cost of approximately \$560 million US dollars per year in the 1990's (Cho, 2006). By 1991, PRRS had become widespread in Europe after similar clinical outbreaks were reported in Germany in 1991. PRRSV as the viral agent for PRRS was identified by researchers in The Netherlands and the United States, determining that there are two strains of PRRSV: North American and European strains (Forsberg, 2002).

Clinical Aspects of PRRSV

Clinical signs of PRRSV in swine include severe reproductive failure, post-weaning pneumonia, growth reduction, decreased performance, and increased mortality. Specific reproductive failures include third-trimester abortions, premature parturition, and higher levels of fetal losses. The degree of clinical PRRS varies depending on the virulence of the PRRSV isolate as well as the animal age and presence of bacterial co-infections (Cho, 2006).

Abortion rates from PRRSV infection vary greatly, and were reported as 3% in The Netherlands, and over 80% in Spain. Other symptoms include periocular edema, conjunctivitis, and eyelid edema, which were reported in Spain, Germany, and England. In Europe and Canada, but not the United States, a blue discoloration of ears, teats, snout, and ventral cervical skin, vulva, and abdomen was reported. Early signs of a PRRSV infection include influenza-like symptoms such as conjunctivitis, depression, lethargy, and anorexia lasting up to 2 weeks. Although death in older pigs is not

common with a PRRSV infection, the virus can persist in infected herds and lead to difficulty in breeding and growing pigs. In adult pigs, the signs tend to be milder and consist of anorexia lasting 5-7 days, with an increased respiratory rate, listlessness, and hyperexcitability. Additionally, adult pigs may show changes in body temperatures ranging between 104°-106°F. Chronic pneumonia is also a complication of PRRSV and contributes to an increase in mortality (Meulenberg, 2000).

Clinical manifestations are more severe in newborn and nursing pigs than in adult pigs especially in the form of respiratory distress. A respiratory disorder called “thumping” can occur with PRRSV infection in nursing pigs, which is caused by a virus induced interstitial pneumonia. Newborn and nursing pigs also experience mouth breathing, listlessness, lateral recumbency, paddling, sneezing, and vomiting. The mortality rate of preweaning pigs could be up to 50-60% due to starvation or diarrhea.

The clinical signs in breeding pigs include late term abortions, stillbirths (50-70%), and premature farrowing. Recovery can start up to 2-3 weeks after infection and can result in a poor conception rate and a slower return to heat. Some herds have been found to recover back to normal breeding routines after an outbreak, whereas other herds show persistent effects including smaller litter sizes, and piglets born with a risk of developing other diseases including atrophic rhinitis, enzootic pneumonia, and more severe forms of *Salmonella* and other bacterial infections (Rossow, 1998)

Diagnosis of PRRSV

Diagnosis of PRRSV can be determined by clinical signs or laboratory based testing including histopathology, serology, virus isolation and RT-PCR in clinical samples obtained from sows and/or fetuses. However, diagnosis based on clinical signs

alone is not ideal because of the great variety of clinical manifestations of PRRS from pig to pig and from herd to herd. Additionally, secondary bacterial infections as well as other diseases confound the clinical diagnosis of PRRS. Overall, if swine herds are showing more than a 20% rate of stillbirths, late-term abortions, pre-mature farrowing higher than 8%, or an increase in mortality rates of pigs within the first week of life (>25%), then PRRS should be suspected. Histopathologically, a characteristic sign of PRRSV infection of lungs includes the manifestation of interstitial pneumonia as thickened alveolar septa with macrophages present (Figure 1). However, infection of lungs with H1N1 and H3N2 strains of Influenza virus also result in thickened alveolar septa with macrophage infiltration.

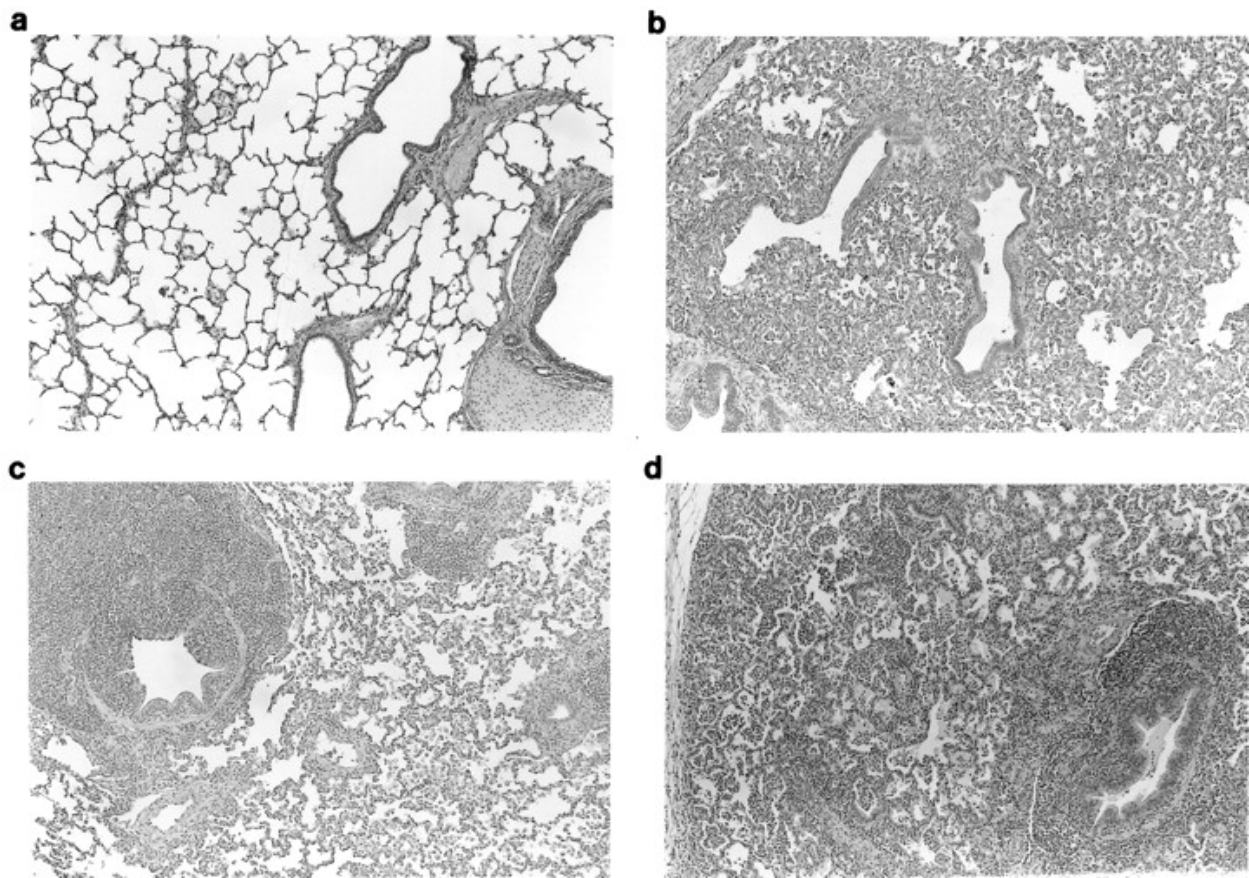


Figure 1. (a) Microscopic section of a normal pig lung. (b). Microscopic section of a lung from a pig infected with PRRSV after 10 days. Necrotic debris and inflammatory cells are present in the

alveolar spaces. (c). Microscopic section of a lung from a pig infected with PRRSV after 28 days. (d). Microscopic section of a lung from a pig infected with PRRSV after 28 days with both M. hyopneumonia and PRRSV-induced pneumonia (Thacker, 1999).

Antibody detection in fetal fluids or pre-colostral blood of stillborn and newborns as well as an increase in antibody titers in serum samples collected 3 weeks apart are indicative of PRRSV infections. For PRRSV surveillance purposes, a minimum of 30% of the herd should be serologically tested in order for a 95% confidence in the detection of PRRSV infections. Virus isolation is ideal for samples collected from acutely infected pigs presenting respiratory signs. PRRSV can be detected in lungs, spleen, lymph nodes and serum. Infectious virus can also be obtained from the lung, spleen, heart, blood, and thoracic fluids samples obtained from stillborn and aborted fetuses for up to 6-8 weeks after infection (Rossow, 1998).

PRRSV Transmission

Both infected pigs and contaminated semen contribute as direct routes for PRRSV transmission. PRRSV has been found in blood, semen, saliva, feces, milk, and colostrum of infected pigs. Vertical and horizontal transmissions of PRRSV have been reported. Indirect routes of PRRSV transmission include fomites such as boots and coveralls; transport vehicles; insects including mosquitoes and houseflies; avian and non-porcine mammalian species and possibly waterfowl; and aerosols (Cho, 2006). Aerosols are of high importance in the transmission of PRRSV, which is affected by high humidity, low temperatures, and low wind speed (Rossow, 1998).

Although exact information on the incubation period of PRRS is unknown, a 37 day lag time has been reported between the virus entry and manifestation of signs of the disease. Herds can remain infected for at least 2 months after signs of PRRS start

to fade away. The presence of the virus for a long period of time in infected herds is a source for new infections (Rossow, 1998).

Treatment and control

Avoiding the introduction of PRRSV to a herd by means of quarantine is one method of controlling PRRS. This also includes testing incoming pigs, restricting visitors, changing boots and clothes after dealing with the pigs, and keeping stray animals and rodents away. However, PRRSV can be transmitted as an aerosol, so control through quarantine may not be entirely effective. Other means of control include disposing of placentas, fetuses, and dead piglets immediately; cleaning farrowing areas thoroughly after abortions or pre-mature farrowing; and disinfecting all entrances/exits of breeding houses and transport vehicles (Rossow, 1998)

Vaccination against PRRSV also prevents the spread of disease in pigs. Modified-live virus (MLV) commercial PRRSV vaccines have been available since 1994, such as Ingelvac PRRS MLV (Boehringer Ingelheim Vetmedica, Inc) (Opriessnig, 2002). Experiments have shown that vaccination with MLV vaccines in pigs reduces clinical signs following a PRRSV challenge. Additionally, herd closure, sow acclimation, and mass exposure can help to reduce the risk of PRRSV shedding and spreading (Cano, 2007).

PRRS virus

Mature viral particles produced from PRRSV infected cells are composed of an envelope of 40-60 nm in diameter, surrounding a 20-30 nm isometric capsid that includes an approximately 15 kb linear positive-stranded RNA genome. Mature virions are released by exocytosis after being formed by the budding of preformed

nucleocapsids into the lumen of the smooth endoplasmic reticulum and/or Golgi apparatus (Dea, 2000).

PRRSV, as an enveloped virus, has a survivability outside the host that can be affected by temperature, pH, and exposure to detergents. PRRSV can survive for over four months at temperatures between -70 to -20 °C, and remain stable at pH ranges between 6.5 and 7.5. Detergents such as lipid solvents like chloroform and ether effectively disrupt PRRSV's viral envelope (Cho, 2006).

The PRRSV genome has nine open reading frames, which are transcribed in cells as subgenomic mRNAs. ORF1a and ORF1b are at the 5' end of the genome and represent about 75% of the viral genome encoding non-structural proteins that are involved in replication of the virus (Dea, 2000).

PRRSV has a restricted cell tropism *in vivo* and *in vitro*. The virus can infect well-differentiated cells of the monocyte-macrophage lineage *in vivo* including particular porcine alveolar macrophages (PAM). PRRSV also targets interstitial macrophages in the heart, thymus, spleen, Peyer's patches, hepatic sinusoids, renal medullary interstitium and the adrenal gland. PRRSV RNA and nucleocapsid protein N were also found by *in situ* hybridization (ISH) and immunohistochemistry in testicular germ cells, endothelial cells in the heart, interdigitating cells in the thymus, dendritic cells in the spleen and Peyer's patches (Music, 2010).

PAM cells and blood-derived monocytes are the only porcine cells that can be used to propagate PRRSV *in vitro*. However, MARC-145 and CL2621, which are subclones of the MA104 monkey kidney line, are of non-porcine origin permissive cell lines to PRRSV infection (Snijder, 1998).

The virus enters cells via receptor-mediated endocytosis, this entry mechanism was confirmed by Duan *et al* in 1998. In the same study, it was shown that PRRSV interacts with heparin sulphate during binding to the cell surface, to sialoadhesins for binding and internalization, and to vimentin. Higher expression of CD163, which is most likely used for viral uncoating, also interacts with PRRSV on macrophages. PRRSV infections induce apoptosis in *cells in vivo* as well as *in vitro*, which ultimately manifests as DNA fragmentation and activation of caspases (Music, 2010).

PRRSV Protein Synthesis

The PRRSV genome encodes structural and non-structural (NS) proteins. The major structural protein of PRRSV is encoded by ORF5, a 25 kDa envelope glycoprotein known as GP5. The 18-19 kDa unglycosylated membrane protein M is encoded by ORF6, and the 15 kDa nucleocapsid protein N is encoded by ORF7. The N protein is highly antigenic, making it a prime target for detection of PRRSV infections (Dea, 2000).

The life cycle of the virus begins with the expression of the large replicase gene complex encoded by ORF1a and ORF1b, which translate polyproteins pp1a and pp1ab, respectively (Figure 2). The pp1a polyprotein is cleaved at eight sites by viral and cellular proteases, yielding nine non-structural proteins (NSps) including NSp1 α , NSp1 β , and NSp2-NSp8. Cleavage of pp1ab polyprotein yields NSp9-NSp12. Although little is known about the function of NSps of PRRSV, besides that together they show replicase, protease, and polymerase functions, it is known that NSps generated from the pp1a polyprotein express proteolytic activities; and that NSps generated from pp1ab polyprotein are involved with transcription and replication functions (Music, 2010).

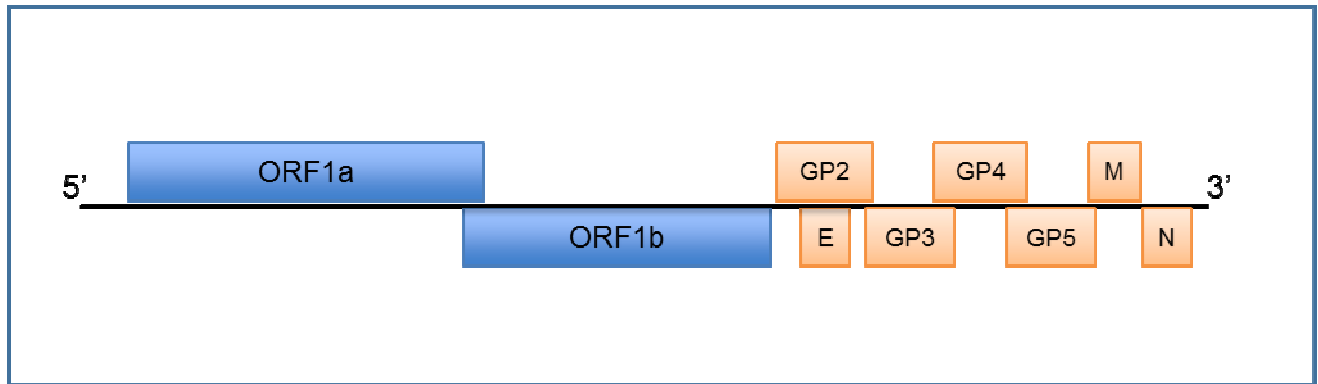


Figure 2. Genome organization of PRRSV containing the 5' proximal replicase ORFs 1a and 1b with downstream ORFs that encode glycoproteins GP2-5, the envelope (E), membrane (M), and nucleocapsid (N) towards the 3' end. Adapted from Fang, 2010.

Impact of the disease caused by PRRSV

Since the emergence of PRRSV in the 1980's-1990's in the United States, PRRS has been recognized as a disease with major economic implications to the swine industry (Albina, 1997). According to an economic assessment by the American Association of Swine Veterinarians in 2012, the total cost of productivity losses due to PRRSV in the United States was estimated at \$664 million yearly. This figure shows a significant increase from the estimated \$560 million annual cost in the 1990's to 2005. Therefore, PRRSV continues to be a critical virus to study as eradication efforts have failed to lower the economic burden of the disease (Neumann, 1995).

OBJECTIVE OF RESEARCH

The objective of this research is to develop a method to identify PRRSV-host protein-protein interactions in cells that are permissive to the virus infection. The method is based on a Pull-Down Assay that is designed to “capture” cellular proteins that bind to PRRSV NSp3. Virus-host protein-protein interactions are vital to understanding mechanisms underlying infection that result in manifestation of disease. Here, we are studying the interactions of PRRSV NSp3, a viral protein involved in replication, with cellular protein and its potential role in regulation of virus induced apoptosis. In addition, establishing a Pull-Down technique to study protein-protein interactions in PRRSV in general is of utmost importance for future research.

APPROACH

NSp3, a known protein expressed by PRRSV during infection, was used as a “prey” protein in a Pull-Down Assay to “capture” interacting cellular proteins in cell lysates obtained from PRRSV-permissive cells. The Pull-Down Assay protocol used here (Pierce® GST Protein Interaction Pull-Down Kit) uses a Glutathione S-Transferase (GST) based technique (Figure 3), where agarose gel beads bind to GST-tagged proteins. The GST-tagged protein in this study was PRRSV NSp3 whereas GST protein was used as control. GST-NSp3 was expressed in *E.coli* as a fusion protein and tested for the ability to bind to glutathione coated beads. Cell lysates were prepared under different conditions from MARC-145 cells that are permissive to PRRSV infection. After testing the ability of GST-NSp3 to bind cellular proteins by incubating coated beads with cellular lysates, beads were recovered and bound material separated by SDS-PAGE. Identification of bound cellular proteins was performed by tryptic in-gel digestion of

these proteins that interacted with the bait protein was conducted and identified by means of mass spectrometry.

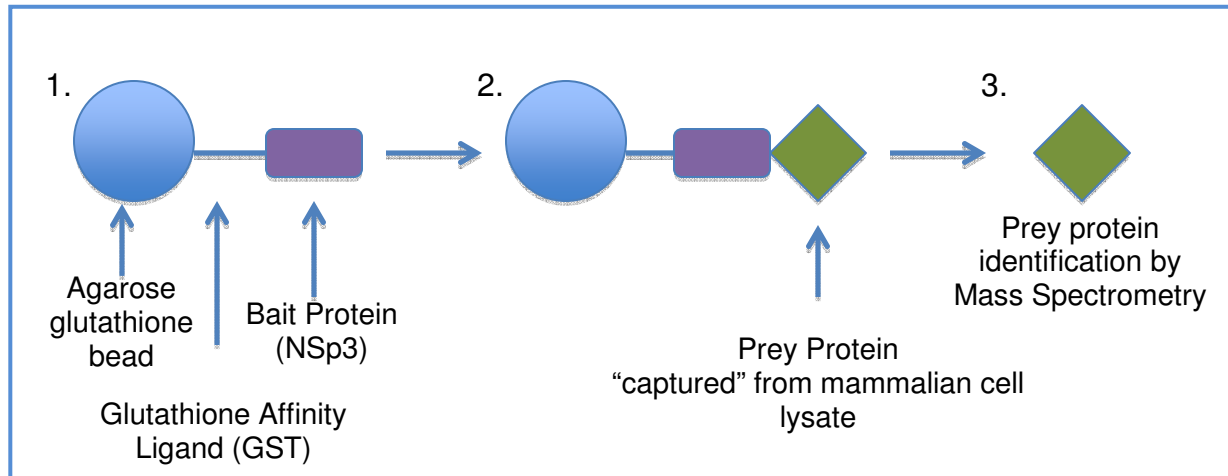


Figure 3. This schematic representation of a Pull-Down Assay describes the protocol in a simplified three-step process. First, the agarose glutathione bead bound to a glutathione affinity ligand (i.e.: GST) is prepared with the “bait” protein (i.e.: NSp3). Next, that “bait” protein, in this case NSp3, is used to “fish” for “prey” proteins in a mammalian cell lysate, in this case from PRRSV permissive MARC-145 cells. Finally, these “captured” “prey” proteins are eluted and proteins identified by Mass Spectrometry. Adapted from Pierce® GST Protein Interaction Pull-Down Kit Instructions.

PROCEDURES AND RESULTS

PRRSV NSp3 Synthesis using an *E. coli* Expression System: Cloning of GST-NSp3 fusion protein

The gene encoding for PRRSV NSp3 was amplified by means of the polymerase chain reaction (PCR) using primers NSp3_pENTR_For 5'caccatgggaggcccgcacctc 3' and NSp3_pENTR_Rev 5'gaattcttagtgatggtgatggtgatgctcaagaagggaccc 3' targeting plasmid pFL12 that contains a full-length DNA copy of PRRSV. The PCR conditions were as follows: incubation at 95 °C for 2 minutes, 35 cycles of 95 °C for 30 s, 58 °C for 30 s, and 68 °C for 2 minutes, and final extension step of 68 °C for 3 minutes. The amplification was undertaken in a thermal cycler (Mastercycler, Eppendorf, Westbury, NY). The resulting PCR fragment was eluted from a 1% agarose gel using the QIAEX II Gel Extraction Kit (Qiagen, Valencia, CA), and directionally cloned into pENTR/D-TOPO vector (Invitrogen, Carlsbad, CA) following instructions of the manufacturer. After cloning, recovered plasmids were sequenced to assess fidelity of the cloned NSp3 gene. The NSp3 gene was transferred to vector pDEST15 (Invitrogen) that contains a 5' end GST fusion tag, using homologous recombination. The reaction containing 7 µL of pENTR/D- TOPO-NSp3, 1 µL of pDEST15, and 2 µL of LR clonase was incubated for 1 hour at 25 °C. After incubation, 1 µL of proteinase K was added to the reaction that was further incubated for 10 minutes at 37 °C. The reaction mix was used to transform TOP-10 chemically competent *E. coli* cells (Invitrogen) that were plated in Terrific Broth agar plates in the presence carbenicillin for selection of bacteria containing pDEST15-NSp3. Carbenicillin resistant bacteria were selected from the plates and grown overnight at 37 °C in Terrific Broth-carbenicillin for isolation of plasmid DNA. After purification plasmid

DNA was sequenced to assess the fidelity of GST-NSp3 fusion. pDEST15-NSp3 was used for expressing the fusion protein.

Expression of GST-NSp3 fusion protein

BL21 (DE3) *E. coli* cells (Invitrogen) were transformed with plasmids pDEST15 and pDEST15-NSp3 harboring GST or GST-NSp3 fusion genes, respectively. Briefly; 2μL of Beta-mercaptoethanol (βME) were added to 100μL of BL21 cells containing microcentrifuge tubes labeled GST (control) or GST-NSp3. Cells were incubated on ice for 10 minutes, and 1μL of pDEST15 or pDEST15-NSp3 was added to each tube incubating on ice for 30 minutes. Next, BL21 cells were subject to a heat-shock at 42 °C for 45 seconds and placed back on ice for additional 2 minutes. Super Optimal Broth medium (SOC), 250μL, was added to the transformed cells and incubated while shaking for 1 hour at 37 °C. Cells, 150μL were plated in Terrific Broth-agar plates containing 100 ug/ml of carbecillin, and incubated overnight at 37 °C. Four colonies were selected from the plates and grown for 21 hours at 37 °C with shaking in MagicMedia (Invitrogen) to induce expression and harvested by centrifugation at 3,200 x g for 15 min. The cell pellets were treated with 1ml of CellLytic buffer (Sigma Aldrich, St. Louis, MO) in the presence of 20μL of lysozyme and 5mL of 10x Tris Buffered Saline (TBS) according to manufacturer's large scale extraction protocol and saved at -20⁰C. Cell lysates were analyzed on a NuPAGE® Novex 12% Bis-Tris Gel (Invitrogen) by a discontinuous SDS-PAGE system. MagicMark XP Protein Standard (Invitrogen) was used as a molecular weight standard. For SDS-PAGE, 13μL of cell lysates were mixed with 5μL of NuPage LDS Sample Buffer, 2μL of NuPage Reducing Agent, making a total volume of 20μL. Samples were heated at 70 °C for 10 minutes and ran for 50 minutes at 200V, 100mA.

Gels were stained with Coomassie blue stain (Colloidal Coomassie Blue). For Western blots, separated proteins were transferred to polyvinylidene fluoride (PVDF) membrane (Invitrogen). Briefly, blotting pads were soaked for 5 minutes in a transfer buffer containing 12.5mL of NuPAGE® Transfer Buffer (Invitrogen), 250µL of antioxidant (Invitrogen), 25mL of methanol and deionized water up to 250mL. PVDF membranes were soaked in methanol for 30 seconds, rinsed with water 3 times, and soaked in transfer buffer before transferring. The set up for the Western blot consisted of 2 blotting pads, filter paper, gel, PVDF activated membrane, and two additional blotting pads (Figure 4). The assembled unit was then placed in XCell II™ Blot module (Invitrogen) and the transfer was completed by applying 30 volts for 1 hour.

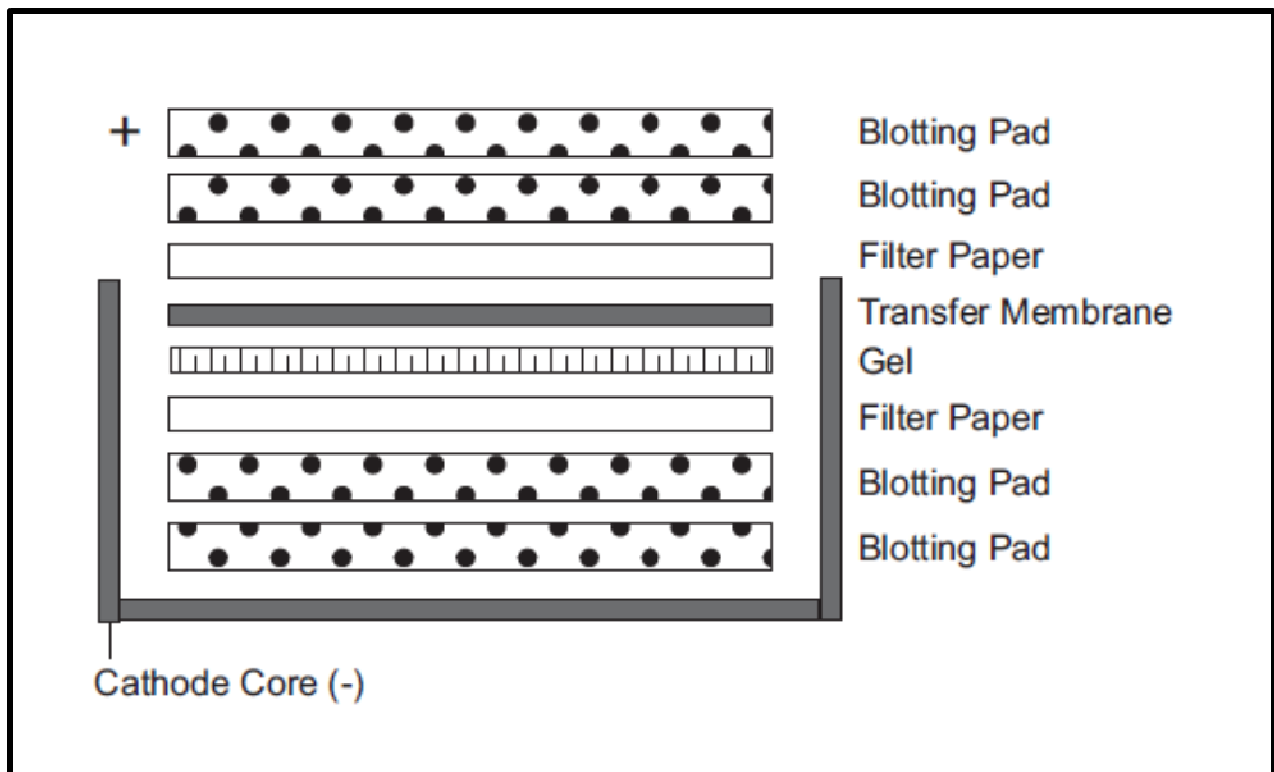


Figure 4. Assembly of SDS-PAGE gels for transferring of proteins to PVDF membranes using the XCell II™ Blot module (Invitrogen).

Western blots were developed using a Western Breeze Chemiluminescent Detection Kit (Invitrogen) according with instructions provided by the manufacturer. Briefly, after transfer, PVDF membranes were blocked for 4 hours at room-temperature, with shaking, in blocking buffer containing 5mL of ultrapure water, 2mL of Blocker/Diluent A, and 3mL of Blocker/Diluent B. After blocking, PVDF membranes were rinsed 3 times with ultrapure water for 5 minutes each time and incubated overnight with a mouse anti-GST antibody (Invitrogen) in a buffer containing 7mL of ultrapure water, 2mL of Blocker/Diluent A, 1mL of Blocker/Diluent B and 4 μ L (1:2500 dilution) of the anti-GST antibody. After exposure to the primary antibody, the membrane was washed 3 times for 5 minutes each wash with a washing buffer containing 150mL of ultrapure water and 10mL of the Antibody Wash. After washes, the membrane was then incubated in 10mL of anti-mouse secondary antibody solution for 30 minutes at room temperature, with shaking. After washing the excess of secondary antibody as described above, protein bands were detected using CDP-star chemiluminescent substrate provided with the kit and subsequently exposed to an X-ray film (Kodak X-OMAT LS film, Kodak, Rochester, NY).

The results from the Western immunoblot are shown in Figure 5. Lanes 1 and 2 show the expression of GST-NSp3 fusion proteins and lanes 3 and 4 show the expression of GST proteins.

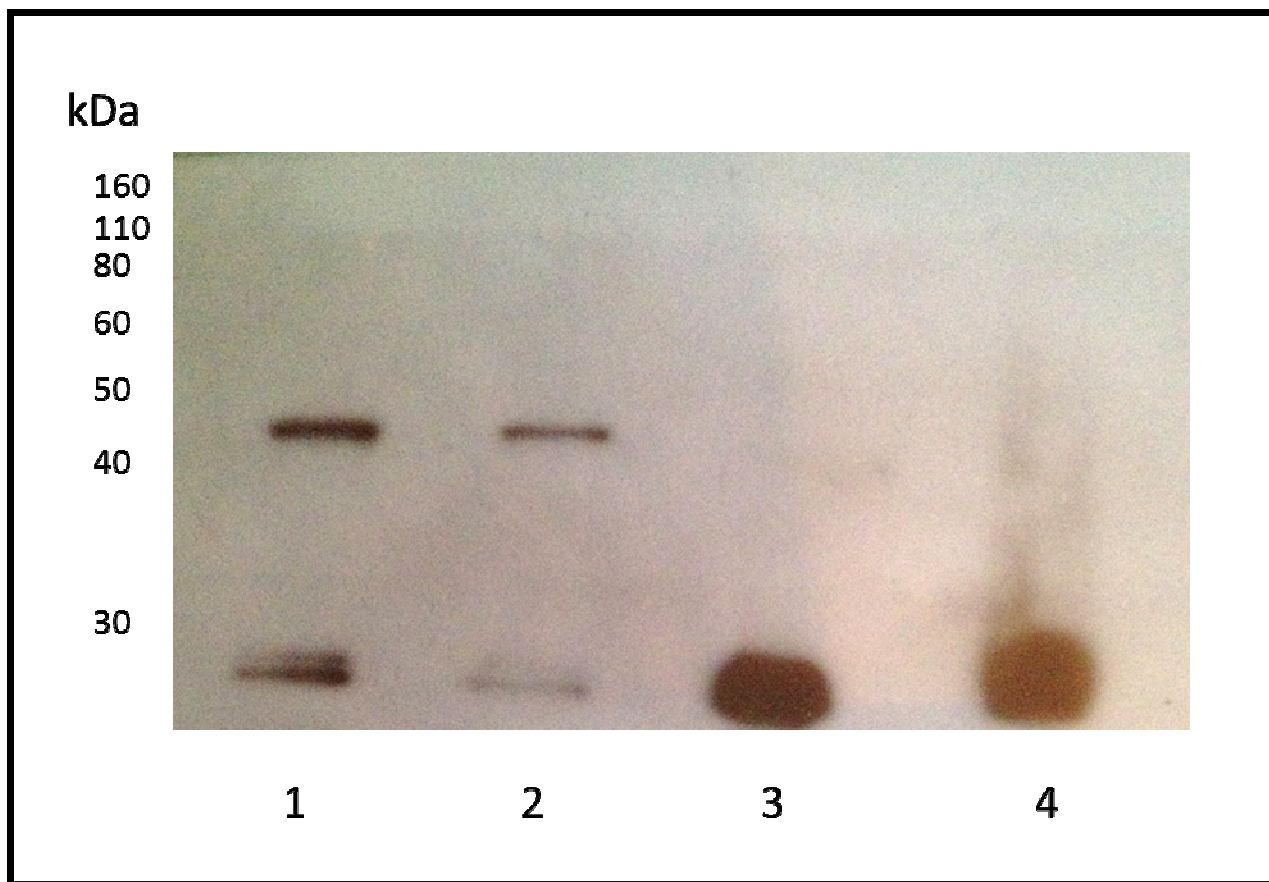


Figure 5. Detection of GST-NSp3 and GST proteins expressed in *E. coli*. Proteins were detected in a Western blot using Western Breeze Chemiluminescent Immunodetection Kit (Invitrogen). Lanes 1 and 2: GST-NSp3 fusion protein; lanes 3 and 4: GST protein.

Production of Glutathione-GST-NSp3 beads and detection of NSp3 binding proteins

After confirmation of expression of GST-NSp3 and GST proteins obtained with the *E.coli* based expression system, these “bait proteins” were immobilized on the surface of glutathione agarose beads. Here we used a Pierce™ GST Protein Interaction Pull-Down Kit (Pierce-Thermo Fisher Scientific). Glutathione Agarose was used for binding GST-fused NSp3 to the beads. Glutathione is linked by its central sulfhydryl to agarose beads and using affinity chromatography, glutathione-agarose permits rapid and highly selective binding and eventually purification of glutathione binding enzymes such as glutathione S-transferase (GST). The overall scheme used for the production of glutathione-GST-NSp3 beads and identification of NSp3 binding proteins is summarized in the following figure.

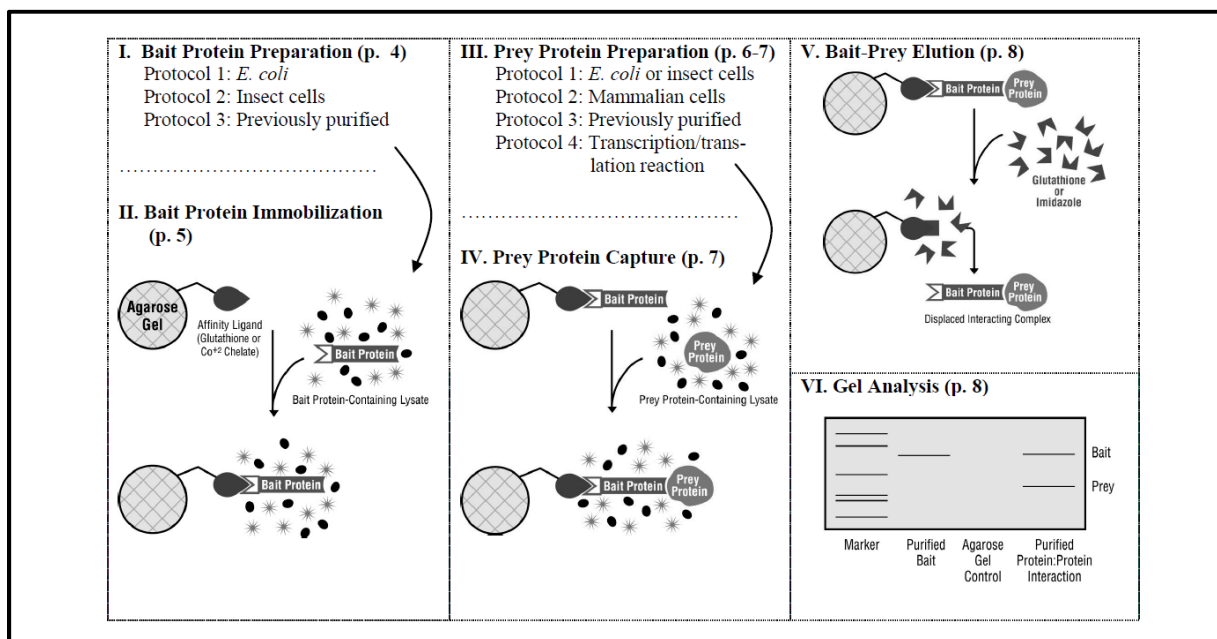


Figure 6. Summary of the procedure used to detect and identify cellular proteins that bind to NSp3. GST-NSp3 fusion was obtained from *E. coli* (step 1, bait preparation).

Immobilization of GST-NSp3

A- Equilibrate Glutathione Agarose

Glutathione agarose resin were thoroughly resuspended by vortexing and 50 μ L of the slurry was transferred to 1.5mL microcentrifuge tubes (**Note:** glutathione agarose is supplied as a 50% slurry. Settled resin volume per assay is 25 μ L). Then, 400 μ L of the wash solution buffer (1:1 solution containing TBS [25 mM Tris-HCL, 0.15 M NaCl, pH 7.2]-Pull-Down Lysis Buffer) were added to the tubes. Caps were closed and tubes inverted several times to equilibrate the glutathione agarose resin. Tubes were then spun at 1250 \times g for 30 seconds to 1 minute. Wash solution was discarded. The process was repeated for 5 times.

B- Immobilize Bait Protein

After equilibration, GST-NSp3 fusion proteins were added to the glutathione resin. Five different binding conditions were tested as described in Table 1.

Combination	Volume of GST-NSp3 prep. (μ L)	Volume of TBS (μ L)
1	50	450
2	150	350
3	200	300
4	300	200
5	400	100

Table 1. Binding of GST-NSp3 and GST proteins to GST beads. Combination of proteins and binding buffer tested.

GST-NSp3 and TBS were mixed and tubes were incubated at 4°C for at least 30 minutes with gentle rocking motion on a rotating platform (Note: Maximal binding may require a longer incubation time, which should be determined for each new GST-tagged fusion protein). After incubation, tubes were spun at 1250 \times g for 30 seconds to 1

minute. Supernatant was transferred to a fresh tube labeled “bait flow-through” and placed on ice. After binding the resin was washed five times using 400µL of the wash solution buffer followed by centrifugation $1250 \times g$ for 30 seconds to 1 minute each time. Western immunoblots were used to assess binding of GST-NSp3 and GST to the glutathione beads using an anti-GST antibody. Briefly, beads were mixed with 6µL of SDS-loading buffer and 1µL of reducing agent (Life Technologies), boiled for 10 minutes, and spun at $1250 \times g$ for 30 seconds. Protein containing supernatants were then resolved by Western blots following procedures explained above (Table 1). Obtained data showed that lysate-TBS combination 4 (Table 2), containing 300µL of *E. coli* derived lysate expressing GST-NSp3 protein, showed the highest binding as determined by the highest intensity of GST-NSp3 signal relative to the signals observed with the other combinations.

Lane	1	2	3	4	5	6	7	8	9	10	11	12
Sample	MW	PC	S-1	S-2	S-3	S-4	S-5	B-1	B-2	B-3	B-4	B-5

Table 2. After binding, supernatants (S) and beads (B) from the 5 combinations described in table 1 were resolved by SDS-PAGE followed by a Western blot to detect GST-NSp3 proteins. The highest band intensities were detected in lanes 6 and 11 that represent combination 4 of lysate-TBS.

The immobilization of bait protein experiment was repeated again for the purpose of testing additional combinations of *E.coli* lysate:TBS. Here, 200, 300, or 400µL of GST or GST-NSp3 were mixed with TBS up to a total volume of 500µL followed by the steps described above. Binding to glutathione beads was then assessed by Western immunoblots as described above (Figure 4).

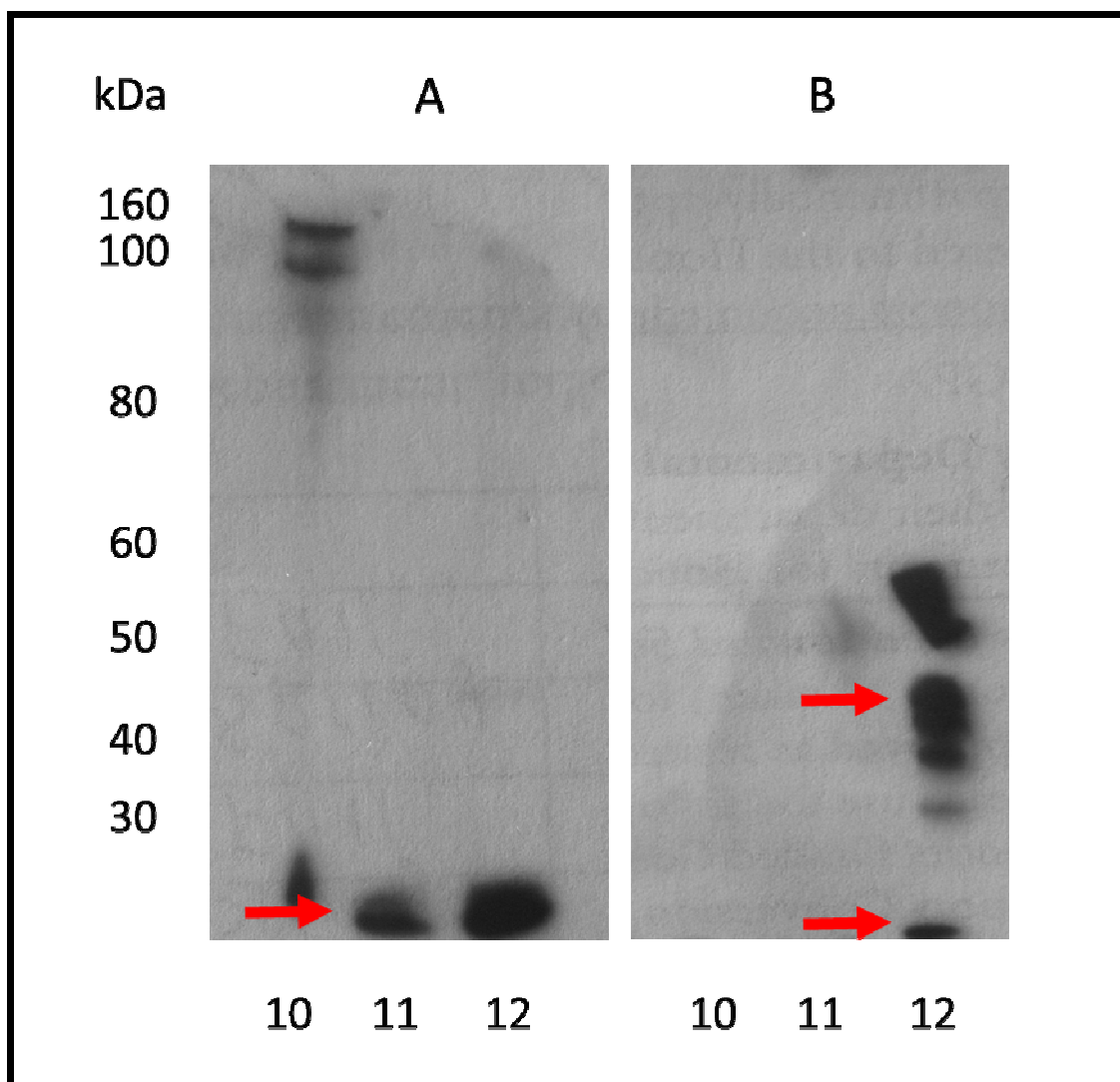


Figure 7. Detection of immobilized GST fusion proteins. X-ray film from a Western blot using anti-GST primary antibody (1:2500). (A) Lanes 11 and 12 show the presence of GST protein at ~27 kDa, red arrow (B) lane show the presence of GST at ~27 kDa and GST-NSp3 at ~48 kDa, red arrows.

These results show that the mixture containing 400 μ L of protein lysate (lanes 12 in figure 7) provided the highest binding to the glutathione beads.

Purification of the GST-NSp3 proteins

In an attempt to improve binding of the fusion protein to the glutathione agarose, GST-NSp3 was purified by affinity chromatography using the Pierce® GST Spin Purification Kit (Pierce-Thermo Fisher Scientific) according to instructions provided by the manufacturer. Purification is based on the affinity of Glutathione-S-transferase

(GST) for glutathione. Briefly, glutathione spin columns were centrifuged at 700 x g for 2 minutes in order to remove the storage buffer. Next, for equilibration, two resin-bed volumes relative to resin of kit supplied Equilibration/Wash Buffer (containing Tris-HCL, sodium chloride, pH8) was added to the columns, mixing by repeated inversions followed by centrifugation at 700 x g for 2 minutes. GST-NSp3 lysate (1 mL) obtained from *E.coli* was mixed in a 1:1 volume ratio with kit provided equilibration/ wash buffer. For binding, the lysate:buffer mixture was then applied to a equilibrated glutathione purification column followed by incubation at room temperature for 30 minutes with shaking in an orbital shaker. For elution purposes, the column was centrifuged at 700 x g for 2 minutes and flow through buffer collected in a centrifuge tube. The column was then washed with two resin-bed volumes of Equilibration/Wash Buffer, centrifuging at 700 x g for 2 minutes and collecting fraction in a centrifuge tube. This washing step was repeated two more times. GST-NSp3 protein was eluted from the resin by adding one resin-bed volume of Elution Buffer (10X Glutathione stock solution provided with the kit 10-fold with Equilibration/Wash Buffer). Centrifuge at 700 x g for 2 minutes and collecting the flow-through. This step was repeated two more times, collecting each fraction in a separate tubes.

For assessing purity of GST-NSp3 collected fractions were resolved by SDS-PAGE. Fractions (13µL) were combined with 5µL of SDS sample buffer, and 2µL of reducing agent (Life Technologies). The gel was run at 200V for 50 minutes and proteins transferred to PVDF membranes as described above. The presence of GST-NSp3 was determined by using an anti-GST antibody. No GST-NSp3 proteins were

detected in the blots. Thus, our experiments continued using crude extracts from *E.coli* expressing GST-NSp3.

Preparation of “prey” proteins from mammalian cells

The purpose of this experiment was to detect cellular proteins from mammalian MARC-145 cells that bind to PRRSV NSp3.

First, two different lysis buffers were tested for preparing lysates of cultured MARC-145 cells.

Cell monolayers containing approximately 10^7 cells were washed once with 5mL of 1X PBS (phosphate buffered saline pH 7.2). Cells were then detached from the flask using 2ml of 1X Trypsin-EDTA incubating at 37°C (~ 2 minutes). Four milliliters of complete tissue culture media (DMEM-10% fetal calf serum + antibiotics) were added to the flask and the entire volume was transferred to a 15mL centrifuge tube. Cells were centrifuged at 500 x g for 5 minutes, and supernatants discarded. Cell pellets were washed by adding 5mL of 1X PBS followed by centrifugation at 500 x g for 5 minutes. This step was repeated once.

After washing cells were resuspended in 3mL of RIPA buffer (Sigma-Aldrich) or 3mL of Cell Lytic B Buffer (Sigma-Aldrich) containing 20µL of lysozyme. Cells were then incubated on ice for 30 minutes with shaking in an orbital shaker. After incubation, tubes were centrifuged for 5 minutes at 12,000 x g to remove cellular debris. Supernatants were transferred to clean tubes and stored at -20°C until use. Aliquots of these lysates were resolved by SDS-PAGE and gels stained with Simply Blue (Invitrogen) to visualize cellular proteins. Higher protein yields were obtained when Cell Lytic B Buffer was used to prepare the lysates.

Capture of cellular proteins with Glutathione-GST-NSp3 beads

To capture cellular proteins with Glutathione-GST-NSp3 beads, a 1:1 volume mixture containing 400µL of MARC-145 cell lysates: 400µL of TBS was added to the beads. The mixture was then incubated in ice for 1 hour with shaking using an orbital shaker. After incubation, mixtures were centrifuged for 1 minute at 1250 x g and the supernatants saved as “prey flow-through GST” and “prey flow-through GST-nsp3”. Next, 400µL of Wash Buffer was added to the tubes for washing the beads while gently mixing by repeated inversions. Tubes were centrifuged at 1250 x g for 30 seconds and supernatants were removed and discarded. This washing step was repeated 5 times.

After the last wash, the supernatant was discarded and agarose beads were incubated at 70°C for 10 minutes in the presence of 4µL of reducing agent (Life Technologies) and 10µL of SDS sample buffer to elute bound proteins. Eluted proteins were then resolved by SDS-PAGE followed by staining of the gels with Colloidal Coomassie Blue.

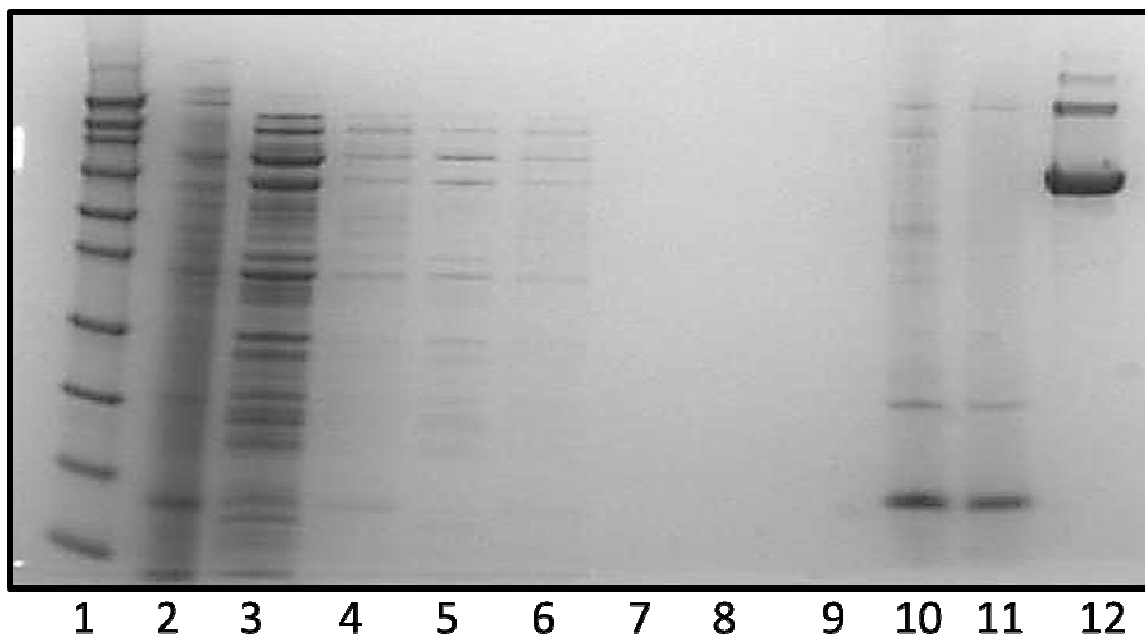


Figure 8. SDS PAGE Gel stained with Colloidal Coomassie Blue showing cellular proteins that interact with GST or GST-Nsp3. Lanes: 1- Molecular weight marker; 2- MARC-145 cell lysate; 3- "bait" GST protein; 4- "bait: GST-Nsp3 protein; 5- flow-through GST; 6- flow-through GST-Nsp3; 7- empty lane; 8- wash 1 GST; 9- wash 2 GST-Nsp3; 10- GST elute; 11- GST-Nsp3 elute; 12- BSA (bovine serum albumin).

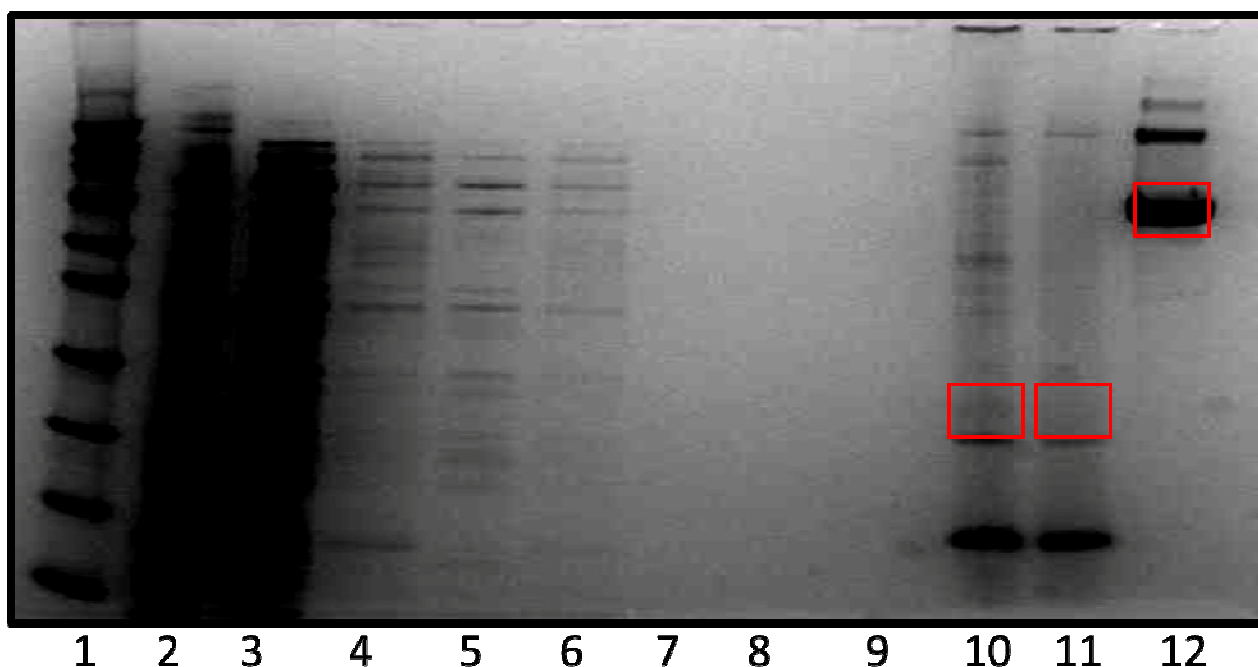


Figure 9. Same as in figure 8 with increased contrast. Squares indicates the portion of the gel that was excised for identification of cellular proteins using mass spectrometry (see below).

Identification of cellular proteins that bind to PRRSV-NSp3

Elucidating the function of viral proteins involves determining the role of these gene products during infection. In the cell, virus encoded proteins participate in an extensive and intricate network of protein:protein interactions. The interaction of viral proteins with cellular proteins most likely determines the fate of the infection. A preliminary step, as it has been taken in this work, is to identify cellular proteins that interact with PRRSV proteins, in this case PRRSV NSp3 protein. Here, we are attempting to identify these interacting cellular proteins by means of Mass Spectrometry. To accomplish that, “trapped” proteins by the glutathione-GST-NSp3 capture system that was established here, cellular proteins should be recovered and then subject to identification.

Recovery of cellular proteins that interact with GST-NSp3

In-Gel Digestion using proteases

After resolving “trapped” cellular proteins by SDS-PAGE (as described above), proteins were recovered from Colloidal Coomassie Blue (Life Technologies) stained gels by in-gel tryptic digestion using the In-Gel Tryptic Digestion Kit (Pierce-Thermo Fisher Scientific) according with instructions provided by the manufacturer.

Staining SDS-PAGE gels with Colloidal Coomassie Blue

To prepare SDS-PAGE gels for in-gel tryptic digestion, gels were stained using Colloidal Blue Staining Kit (Life Technologies) for high sensitive staining of proteins. After running, gels were washed 3 times for 5 minutes each time with ddH₂O. Next, 10 mL of fixing solution (4mL of ddH₂O, 5mL of methanol, and 1mL of acetic acid) was added to the gels incubating for 10 minutes at room temperature with shaking. The

fixing solution was then removed and a Staining Solution containing 5.5mL ddH₂O, 2mL of methanol, and 2mL of Stainer A Solution as provided with the kit, was added to gel incubating for 10 minutes at room temperature with shaking. After the 10 minutes of incubation, 0.5mL of Stainer B Solution was added to the gels that were further incubated overnight (~12 hours) at room temperature with shaking. After overnight incubation, the Staining Solution was removed and the gels were washed 3 times for 5 minutes each time with ddH₂O. Images of stained gels were captured for analysis purposes (Figures 8 and 9).

In-Gel Tryptic Digestion

Bands of interest observed in stained gels were recovered using an In-Gel Tryptic Digestion Kit (Pierce-Thermo Fisher Scientific). To digest the bands selected in the first pull-down experiment (Figure 9), stained gels were destained using a Destaining Buffer containing 80mg of ammonium bicarbonate, 20mL of acetonitrile, and 20mL of ultrapure water. Using a sterile scalpel, ~1cm² surrounding bands of interest in lanes 10, 11, and 12 (Figure 9) were excised from the gel and placed into tubes labeled GST, GST-NSp3, and BSA, respectively. Next, 200μL of the Destaining Buffer was added to the tubes incubating at 37 °C for 30 minutes with shaking. This step was repeated once.

After destaining reduction and alkylation of recovered proteins were performed. For reduction, 30μL of reducing buffer containing 3.3μL of TCEP (Tris [2-carboxyethyl] phosphine) and 30μL of the Digestion Buffer (10mg of ammonium bicarbonate in 5mL of ultrapure water) was added to the tubes incubating a 60⁰C for 10 minutes. After cooling, the buffer was discarded. For alkylation, 30μL of 1X Alkylating

Buffer (5X stock: 7mg of IAA [Iodoacetamide] in 70 μ L of ultrapure water) diluted in Digestion Buffer (25 mM ammonium bicarbonate) was added to the samples that were incubated in the dark at room temperature for 1 hour. Alkylation buffer was then discarded and samples were washed by adding 200 μ L of Destaining Buffer followed by incubation at 37 °C for 15 minutes with shaking. The washing step was repeated once.

For digestion purposes, excised gel pieces were shrunk by adding 50 μ L of acetonitrile incubating for 15 minutes at room temperature. Acetonitrile was discarded and gel pieces were air-dried for 5 to 10 minutes. For swelling the gel pieces, 10 μ L of Activated Trypsin solution (1 μ L of Trypsin Working Solution [5X trypsin stock diluted in ultrapure water] in 9 μ L of Digestion Buffer) was added to the tubes that were incubated for 15 minutes at room temperature. Then 25 μ L of Digestion Buffer was added to the samples that were incubated at 30 °C overnight with shaking. The digestion mixture, containing peptides, was transferred to clean tubes. At this step samples were ready for identification by means of Mass Spectrometry.

Mass Spectrometry of gel recovered proteins

Samples were submitted to the Laboratory of Mass Spectrometry and Omics Analysis at the Department of Chemistry, University of Connecticut for protein identification. Samples were processed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with a QSTAR Elite LC/MS/MS System (AB Sciex) and proteins were identified using Mascot software (Matrix Science). Mascot is a software search engine that uses mass spectrometry data to identify proteins from peptide sequence databases. By using this approach peptides were not detected or detected at low levels in samples obtained from the pull-down experiment (Figure 9 lanes 10 and

11), however bovine serum albumin was detected in the band excised from lane 12 (Figure 9) as expected. Most likely, the yield of cellular proteins obtained, as observed in Figures 8 and 9, in the pull-down assays is low and not sufficient for detection by LC-MS/MS. Although peptides that have no match with known proteins in the searched database were identified in protein samples “trapped” with glutathione-GST-NSp3 capture setting (Table 3). Further experiments should be conducted to improve the yield of NSp3 binding proteins.

Table 3. Peptide identified by LC-MS/MS data do not match to known proteins. Identified peptides were detected in samples “trapped” with glutathione-GST-NSp3 capture setting.

Peptide	Sequence
1	IATDPAIR
2	RTEVTEGCLGLMRK
3	VLSNQVMTCEVVPISMTK
4	KFSSKLTDSK
5	YQMNVANGFR
6	AVRFSFTMSISIK
7	VVIKARGK

DISCUSSION

The presence of GST-NSp3 observed by Western blots after expressing GST-NSp3 in the *E. coli* expression system confirmed that the transformation of BL21 cells was successful, and that GST-NSp3 can be efficiently expressed. This protein was used as “prey” to trap or capture cellular proteins in the pull-down assay developed here (Figure 3). Using a mouse anti-GST antibody, the Western blot showed bands of the expected molecular sizes. However, this GST-NSp3 proteins were only detected in samples that were treated with the Cell Lytic B Buffer. One possibility of the lack of GST-NSp3 could be from the Pull-Down Buffer’s properties not successfully working on a bacterial system, as it is a mammalian cell buffer. The Cell Lytic B Buffer was the lysis buffer of choice.

During the first round of GST-NSp3 and GST proteins binding and immobilization to glutathione agarose beads, different volumes of *E.coli* obtained lysates were combined with TBS and incubated with the glutathione beads to test for optimal binding conditions (Table 1). It was observed that combining at least 300μL of protein lysate with 200μL of TBS or less yielded the strongest signals in Western blots (Table 2 and Figure 7). With the purpose of improving binding of GST and GST-NSp3 to the glutathione beads, an attempt to purify proteins by affinity chromatography was made. However, the amount of protein obtained by using the Pierce® GST Spin Purification Kit was negligible. Further attempts should be made for improving purified protein yields. As a results, crude *E.coli* cell lysates were used in this work.

The pull-down assay using GST and GST-NSp3 and lysates from MARC-145 cells was successful although yielded low amounts of cellular proteins as observed in figures 8 and 9. Nonetheless, proteins were purified using an in-gel tryptic digestion and obtained peptides were submitted for Mass Spectrometry Analysis. Although the amount of peptides obtained in this pull-down was low and not readily detected by the LC-MS/MS analysis, BSA that was used as control was detected. Also, peptides with not matches in the protein database were detected in samples from the GST-NSp3 pull-down.

Overall, the data presented here suggest that the entire process established including cloning, expression, binding, pull-down, in-gel digestion and LC-MS/MS worked successfully and will require further adjustments.

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