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VIRUS-LIKE PARTICLES GENERATED BY EXPRESSING PROTEINS OF
PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (PRRSV)
USING THE RECOMBINANT BACULOVIRUS EXPRESSION SYSTEM

BY

APRIL ANNE NELSEN

A thesis submitted in partial fulfillment of the requirements of the

Master of Science

Major in Biological Sciences

Specialization in Microbiology

South Dakota State University

2015

VIRUS-LIKE PARTICLES GENERATED BY EXPRESSING PROTEINS OF
PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (PRRSV)
USING THE RECOMBINANT BACULOVIRUS EXPRESSION SYSTEM

This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science degree and is accepted for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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ABBREVIATIONS

APC: antigen presenting cells

BTV: bluetongue virus

CPV: canine parvovirus

DIVA: Differentiating Infected from Vaccinated Animals

E: envelope

EM: electromagnetic

ER: endoplasmic reticulum

GAG: glycosaminoglycan

GP2a: glycoprotein-2a

GP3: glycoprotein-3

GP4: glycoprotein-4

GP5: glycoprotein-5

IFA: immunofluorescence assay

IL-10: interleukin 10

INF- α : interferon α

INF- β : interferon β

INF- γ : interferon γ

M: membrane

MOI: multiplicity of infection

N: nucleocapsid

NA: neutralizing antibody

NE: neutralizing epitope

NLS: nuclear localization signal

NSP: non-structural protein

ORF: open reading frame

PAM: porcine alveolar macrophages

PCR: polymerase chain reaction

PCV: porcine circovirus

PBMC: peripheral blood mononuclear cells

PBS: phosphate buffered saline

PBST: phosphate buffered saline plus tween 20

pi: post infection

PI: persistent infection

PL2: papain-like protease domain

PRR: pattern recognition receptors

PRRS: porcine reproductive and respiratory syndrome

PRRSV: porcine reproductive and respiratory syndrome virus

pSn: porcine sialoadhesion

qRT-PCR: real time quantitative reverse transcription polymerase chain reaction

TEM: transmission electron microscope (microscopy)

TM: transmembrane

TNF- α : tissue necrosis factor α

TLR: toll-like receptors

VLP: virus-like particles

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ABSTRACT

VIRUS-LIKE PARTICLES GENERATED BY EXPRESSING PROTEINS OF
PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (PRRSV)
USING THE RECOMBINANT BACULOVIRUS EXPRESSION SYSTEM

APRIL ANNE NELSEN

2015

Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped, single stranded, positive sense RNA virus and a member of *Arteriviridae*. Its genome encodes 10 open reading frames for at least 7 structural proteins and 14 non-structural proteins. Membrane (M), Nucleocapsid (N), and Glycoprotein-5 (GP5) are the major structural proteins of PRRSV, while Envelope (E), Glycoprotein-2 (GP2), Glycoprotein-3 (GP3), and Glycoprotein-4 (GP4) are the minor structural proteins of PRRSV. GP5 induces neutralizing antibodies and forms heterodimers with M, while N is the most immunogenic protein of PRRSV. Previous studies have shown viral structural proteins are able to form virus-like particles (VLPs) that can induce an immune response in respective hosts by use of the recombinant baculovirus expression system in insect cells. We sought to find if structural proteins, M, N, GP5, GP4, and E, could be expressed using the recombinant baculovirus expression system and produce VLPs.

After purifying and amplifying PRRSV GP4, GP5, and E genes from PRRSV viral RNA, we inserted those genes into the pCAGEN plasmid, and expressed them in mammalian cells (cos-1). Protein expression of E and GP5 in both the supernatant and cell lysate was verified through western blot, while GP4 was not. We inserted the expressed genes into the pOET-1 plasmid for production of recombinant baculoviruses

using the flashBAC expression system in insect cells. Expression of GP5 and E proteins were verified with IFA. Viral titers were collected using bacuQUANT qRT-PCR, and used to co-infect TriEx SF9 cells with recombinant baculoviruses containing PRRSV M, N, GP5, and E at a multiplicity of infection (MOI) of 2 for N, GP5, and E proteins and a MOI of 3 for M protein for 72 hours. Protein expression was confirmed through western blot. The results of this experiment show that PRRSV M, N, GP5, and E proteins are expressed after co-infection of TriEx SF9 cells using the recombinant baculovirus system, and that VLPs of similar shape and size to the PRRSV virion are produced. These results will aid in further research of vaccine production using PRRSV VLPs.

CHAPTER 1: INTRODUCTION AND BACKGROUND

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS), first observed in the United States in the late 1980s and in Europe the early 1990s, and is characterized by reproductive losses in sows and pneumonia and reduced growth in piglets. (Shi, M., Lam, T.T., et al. 2010). PRRS has shown huge economic losses in the swine industry, with an estimated \$664 million loss annually (Holtkamp, D.J., Kliebenstein, J.B., et al. 2013). These losses are not only due to direct production losses, but also from animal health costs for pharmaceuticals, biological, diagnostics, as well as prevention and treatment of secondary infections (Holtkamp, D.J., Kliebenstein, J.B., et al. 2013).

The causative agent of PRRS is Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), which is an enveloped positive strand RNA virus belonging to the family *Arteriviridae*, order *Nidovirales*, and primarily infects macrophages, especially porcine alveolar macrophages (PAMs), and dendritic cells, which are antigen presenting cells that play an important role in immune activation (Gorbalenya, A.E., Enjuanes, L., et al. 2006, Suarez, P. 2000). PRRSV is easily transmitted through bodily fluids of pigs in close contact, and has been observed to be transmitted through the mosquito *Aedes vexans* and housefly *Musca domestica*, and therefore can move through a herd of pigs very easily (Otake, S., Dee, S.A., et al. 2003).

When a pig is infected, the initial antibodies produced do not confer protection to the pig. It is the neutralizing antibodies (NAs) to PRRSV structural protein, Glycoprotein-5 (GP5), that have been shown to provide protection and clear viremia.

Unfortunately, these NAs do not show up until 28 days post infection or later (Mateu, E., Diaz, I. 2008, Lopez, O.J., Osorio, F.A. 2004). This gives the virus the ability to replicate and mutate while evading the host immune system, which leads us to the crucial feature of PRRSV infection: its ability to remain a persistent infection (PI); where the infected animal continues to shed virus, thus increasing the duration of transmission (Lopez, O.J., Osorio, F.A. 2004). This feature of PRRSV is also of concern when using live attenuated vaccines, due to the fact that persistent infection can be re-activated, or they could become re-infected with the live attenuated vaccine (Murtaugh, M.P., Genzow, M. 2011, Nielsen, H.S., Oleksiewicz, M.B., et al. 2001).

Currently, live attenuated vaccines and inactivated virus vaccines are commercially available for PRRSV; none of which confer heterologous protection, safety, or the ability to distinguish vaccinated pigs from non-vaccinated pigs (Kimman, T.G., Cornelissen, L.A., et al. 2009). Attenuated vaccines have been shown to revert to virulence, spread from vaccinated to non-vaccinated individuals, produce infection, and recombine with field isolates (Murtaugh, M.P., Genzow, M. 2011, Nielsen, H.S., Oleksiewicz, M.B., et al. 2001). Killed vaccines have been considered ineffective or of limited efficiency (Kimman, T.G., Cornelissen, L.A., et al. 2009, Murtaugh, M.P., Genzow, M. 2011). Because of the possibility of severe reactions, resulting in an atypical PRRS complex of live attenuated vaccines, the ineffectiveness of the inactivated virus, and the overall lack of heterologous protection, a need for a cost-effective, heterologous protective vaccine is urgent.

Virus-like particles (VLPs) are multiprotein complexes that mimic the virion structure, but lack the genetic material for reproduction. Because VLPs lack genetic

material (non-structural proteins), PRRSV VLPs would be able to avoid the immune escape mechanism of non-structural proteins of PRRSV (Mateu, E., Diaz, I. 2008, Crisci, E., Barcena, J., et al. 2012, Miller, L.C., Laegreid, W.W. 2004). VLPs are also easily and quickly manufactured, giving them the advantage over other vaccine options for the highly mutable PRRSV (Crisci, E., Barcena, J., et al. 2012, Noad, R., Roy, P. 2003). VLP vaccines have been shown to be safe, effective, and offer the ability to differentiate non-vaccinated from vaccinated individuals for several viruses, including human papilloma virus (HPV), hepatitis B virus (HBV), and bluetongue virus (BTV) (Noad, R., Roy, P. 2003, Crisci, E., Barcena, J., et al. 2012).

The primary objectives of this study were to construct plasmids containing the GP4, GP5, and E genes of PRRSV that can be expressed in both mammalian and insect cells, to construct recombinant baculoviruses that express GP4, GP5, and E proteins of PRRSV for the purification of PRRSV VLPs and production VLPs similar in size and shape to PRRSV virions by expressing M, N, GP4, GP5, and E proteins simultaneously in insect cells.

LITERATURE REVIEW

1. CLASSIFICATION AND EVOLUTION

Porcine reproductive and respiratory syndrome virus (PRRSV) belongs to the family *Arteriviridae*, under the order *Nidovirales*. *Nidovirales* consists of three other families, which include *Coronaviridae*, *Roniviridae*, and *Toroviridae*. The viruses of *Nidovirales* are linear, positive sense, single-stranded RNA viruses that are enveloped and contain a 5' cap structure as well as a 3' poly (A) tail (Gorbalenya, A.E., Enjuanes, L., et al. 2006). PRRSV belongs in the family *Arteriviridae* along with equine arteritis virus (EAV) of horses, lactate dehydrogenase-elevating virus (LDV) of mice, and simian hemorrhagic fever virus (SHFV) of monkeys. Each of these viruses is species specific (Shi, M., Lam, T.T., et al. 2010).

PRRSV was first recognized to be the causative agent of porcine reproductive and respiratory syndrome (PRRS) in the late 1980s in the United States and in the early 1990s in Europe (Shi, M., Lam, T.T., et al. 2010). Although the emergence was almost concurrent, the types of PRRSV are genetically different. PRRSV Type 1 consists of viruses mainly from Europe, while PRRSV Type 2 consists of viruses mainly from Northern America and Asian countries. The two types only share 55-70% nucleotide and 50-80% amino acid similarity throughout the various PRRSV viral genes (Shi, M., Lam, T.T., et al. 2010).

There are two hypotheses as to the evolution of PRRSV pre-emergence. The first hypothesis is that Type 1 and Type 2 diverged not long before emergence followed by an extremely high substitution rate (Shi, M., Lam, T.T., et al. 2010). Most RNA viruses

evolve at a rate of 10^{-3} to 10^{-5} per site per year, where PRRSV seems to have evolved at a rate of 10^{-2} per site per year (Hanada, K., Suzuki, Y, et al. 2005). This hypothesis would put the estimated time of divergence for Type 1 and Type 2 PRRSV around 1972-1988, which is just before the virus seemed to have emerged (Hanada, K., Suzuki, Y, et al. 2005). According to this study, the evolutionary rate of PRRSV would be $4.71-9.8 \times 10^{-2}$, making it the highest among RNA viruses (Hanada, K., Suzuki, Y, et al. 2005). The problem with this study, pointed out by Forsberg 2005, was the problematic methodology and the unproductive data set.

This leads us to the second hypothesis that Type 1 and Type 2 diverged way ahead of the emergence and evolved independently on two continents for a long time (Shi, M., Lam, T.T., et al. 2010). Forsberg used an alternative methodology and reanalyzed the existing data on PRRSV to conclude that the most recent ancestor, or tMRCA, of all PRRSV to be around 1880 ± 15 years (Forsberg, R. 2005). The tMRCA of Type 1 is estimated to be around 1946-1967 and the tMRCA of Type 2 is estimated to be around 1977-1981.

Both studies indicate that PRRSV originated from another host species, possibly wild boar, and was transmitted to swine within 10 years of the “original” outbreak of PRRS.

The “original” outbreak of PRRS began in the late 1980s in the United States and in the early 1990s in Europe. The first PRRSV Type 1 was isolated in the Netherlands and was termed the Lelystad virus because it formed highly homologous phylogenetic (Lelystad-like) clusters with other European samples (Shi, M., Lam, T.T., et al. 2010). More samples were collected and formed well-supported clads that had diverged prior to

the epidemic clad, which would indicate that they were not progenies of the Lelystad-like PRRSV and gave scientists the indication that Type 1 PRRSV viruses had been present in the field long before the “original” epidemic (Shi, M., Lam, T.T., et al. 2010). There is an exceptionally large diversity and longer establishment of Type 1 PRRSV in Eastern Europe, but there seems to be a limited gene flow from Eastern Europe Type 1 PRRSV viruses to Western Europe Type 1 PRRSV (Shi, M., Lam, T.T., et al. 2010).

In the late summer of 1996, an outbreak of “acute PRRS” emerged in southeast Iowa and several other states (Shi, M., Lam, T.T., et al. 2010). This severe, acute PRRS resulted in elevated abortion rates and a slow mortality rate (Shi, M., Lam, T.T., et al. 2010). Of the herds affected by this PRRSV, many of them were vaccinated with Ingelvac PRRS MLV or both Ingelvac PRRS MLV and PrimePAC PRRSV, but the isolates of the acute PRRS were heterogenetic and very different from the isolates of the vaccines (Shi, M., Lam, T.T., et al. 2010).

The next outbreak occurred in late 2001 and is termed the MN184 outbreak, due to the fact that the virus had Restriction Fragmented Length Polymorphism (RFLP) 1-8-4 pattern and was identified as a highly virulent variant of PRRSV in the state of Minnesota (Shi, M., Lam, T.T., et al. 2010). The MN184 outbreak has been hypothesized to have originated from Canada (Shi, M., Lam, T.T., et al. 2010).

The most recent highly pathogenic outbreak of PRRSV occurred in China in the summer of 2006 causing prolonged high fever, red colorations on the body, and a high mortality rate in adult pigs (Shi, M., Lam, T.T., et al. 2010). This highly pathogenic PRRSV spread for 12 months over more than 20 provinces in China and was believed to have evolved from the local diversity of PRRSV in China (Shi, M., Lam, T.T., et al.

2010). It can be traced back to a single introduction from North America (Shi, M., Lam, T.T., et al. 2010).

2. TRANSMISSION AND PATHOGENESIS

Direct routes or indirect routes can transmit PRRSV. Direct transmission is accomplished through infected pigs via blood, semen, saliva, feces, aerosols, milk, colostrum, as well as vertical transmission of sow to piglet (Cho, J.G., Dee, S.A., 2006). One study in England showed PRRSV could be transmitted up to 3 kilometers by aerosol (Cho, J.G., Dee, S.A., 2006). Another study has shown pathogenicity of the isolate to be more of a factor for aerosol transmission. The PRRSV MN-184 isolate, a highly pathogenic isolate that produces a higher viral load in blood and tissues with clinical symptoms that are more severe and of longer duration, was observed to be transmitted via aerosol, while PRRSV MN-30100, an isolate with low pathogenicity, was unable to be transmitted via aerosol in this study (Cho, J., Deen, J., et al. 2007). Clinical symptoms of PRRSV MN-30100 are mild and the viral concentration in blood and tissue is lower than more pathogenic strains (Cho, J., Deen, J., et al. 2007).

Several indirect routes of PRRSV transmission, including fomites, transport vehicles, and insects, have been observed. Needle sharing, as well as, contaminated boots and coveralls can transmit PRRSV (Cho, J.G., Dee, S.A., 2006). According to Otake, S, Dee, S.A., et al 2002, when following the “biosecurity protocols”, transmission can be minimized, if not eliminated. “Biosecurity protocols” enforced during this study included combinations of hand washing, changing or discarding clothing and footwear, showering into and out of facility, and refraining from contact with swine for 12-72 hours

before entering the facility. PRRSV and PRRSV antibodies were not detected at any time during the study after the protocol (Otake, S, Dee, S.A., et al 2002).

Transmission via transport vehicles of pigs has also been observed. Because of the properties of PRRSV: its survivability outside the host is affected by temperature (viability decreases with increased temperatures), pH (infectivity is reduced when pH is less than 6.0 or greater than 7.65), and exposure to detergents (decreased infectivity with lipid solvents, chloroform, and ether); transmission via transport vehicles can be minimized (Cho, J.G., Dee, S.A., 2006). After drying and sanitizing transport vehicles, risk of infection was reduced (Cho, J.G., Dee, S.A., 2006).

Although insects, including mosquitoes (*Aedes vexans*) and houseflies (*Musca domestica*), are not biological vectors of PRRSV, transmission of PRRSV can be accomplished if viral load post-ingestion and environmental temperatures are optimal (Otake, S., Dee, S.A., et al. 2003). In one study, PRRSV isolated from infected recipient pigs was found to be 100% homologous with donor pig after nucleic-acid sequencing, indicating transmission of PRRSV from infected swine to non-infected swine via mosquito was successful (Otake, S., Dee, S.A., et al. 2002).

Avian and non-porcine mammalian species, including rodents, raccoons, dogs, cats, opossums, skunks, house sparrows, and starlings, have not been found capable of transmitting PRRSV (Cho, J.G., Dee, S.A., 2006).

Once infected with PRRSV, sows begin to show inappetence and anorexia, with reproductive symptoms progressing to abortion, premature birth, late term abortion (around 107-112 days), increased incidence of fresh or autolytic stillborn, fetal death with or without mummification, and weak born piglets that die shortly after birth (Nodelijk, G.

2011, Wensvoot, G. 1993, Rossow, K.D., Collins, J.E., et al 1995). Other clinical symptoms of sows can include transient discoloration (cyanosis) of ears, abdomen, and vulva, respiratory distress, and slightly elevated body temperatures (Nodelijk, G. 2011, Wensvoot, G. 1993)

Respiratory disorders manifest in piglets infected shortly after birth and include clinical symptoms of anorexia, transient fever, respiratory distress, roughened hair coats, slow growth, interstitial pneumonia, respiratory disease, and sometimes death (Wensvoot, G. 1993). Older piglets or fattened piglets seem less affected by PRRSV and show shorter periods of anorexia and mild respiratory distress (Wensvoot, G. 1993). Disease symptoms tend to be aggravated by secondary infections, especially in neonatal pigs with PRRSV pneumonia (Wensvoot, G. 1993, Nodelijk, G. 2011). As PRRSV becomes established in a herd, the number of abortions decrease, and infection manifests mostly in weaned and growing pigs (K.D., Collins, J.E., et al 1995).

Gross and/ or microscopic lesions can be seen in lymph nodes, lungs, heart and blood vessels; therefore PRRS could be considered a multisystem disease (K.D., Collins, J.E., et al).

Boars shed PRRSV in semen and can therefore be a cause of infection during coitus (K.D., Collins, J.E., et al 1995).

Clinical symptoms in sows and piglets led to the multitude of names including, Mystery Swine Disease, Swine Infertility and Respiratory Syndrome (SIRS), Porcine Epidemic Abortion and Respiratory Syndrome (PEARS), Seuchenhafter Spatabort Schweine, Abortus Blauw, and Blue-eared Pig Disease, which ultimately led to the

current name Porcine Reproductive and Respiratory Syndrome (PRRS) (Nodelijk, G. 2011).

The crucial feature of PRRSV infection is its ability to remain a persistent infection (PI), where the infected animal continues to shed virus, thus increasing the duration of transmission (Lopez, O.J., Osorio, F.A., 2004). Early, or acute, PI follows initial exposure to PRRSV, which is characterized by mentioned clinical symptoms, as well as abundant replication in alveolar macrophages and other tissue macrophages (Lopez, O.J., Osorio, F.A., 2004, Allende, R., Laegreid, W.W., et al 2000). Acute PI may last up to one month and is characterized by high viral loads in tissues and presence of cell-free viremia (Lopez, O.J., Osorio, F.A., 2004). Low levels of viral replication and low PRRSV load levels in lymph nodes and tonsils, sites of replication, are easily recovered during the late infectious phase of PI (Lopez, O.J., Osorio, F.A., 2004, Rowland, R.R.R., Lawson, S., et al. 2003). One study showed no evidence of virus in lung or other non-lymphoid organs during PI (Rowland, R.R.R., Lawson, S., et al. 2003). Viremia peaks between 0-1 month and can last up to 2 months, which is followed by a total antibody response that can last up to a year or longer (Lopez, O.J., Osorio, F.A., 2004). Viral load in tissues begins to increase around 7-9 days post infection and begins a steady decrease around 5 months post infection (Lopez, O.J., Osorio, F.A., 2004, Allende, R., Laegreid, W.W., et al 2000). This corresponds with the appearance of Neutralizing Antibodies (NAs) and Interferon λ (INF- λ) producing cells which exponentially increases (Lopez, O.J., Osorio, F.A., 2004). PRRSV is eventually cleared from the body at around 150 days post infection (Lopez, O.J., Osorio, F.A., 2004, Allende, R., Laegreid, W.W., et al 2000).

PRRSV has been shown to cause apoptosis in infected and non-infected macrophages, monocytes, germ cells, and cells of endometrium/ fetal placentas, which peaks at 14 days post infection, and is preceded by a peak in interleukin-10 (IL-10) and interleukin-1 (IL-1) production (Labarque, G., Van Gucht, S., et al 2003, Sur, J.H., Doster, A.R., et al. 1998 Karniychuk, U.U., Saha, D., et al. 2011). There is also evidence PRRSV interferes with cellular innate immune responses, which are closely tied with apoptosis (Miller, L.C., Fox, J.M. 2004). The interference of host immune responses contributes to the risk of secondary infections to PRRSV. There is an increased severity of clinical disease in animals co-infected with PRRSV and different bacteria, including *Streptococcus suis*, *Haemophilus parasuis*, *Salmonella choleraesuis*, *Mycoplasma hyopneumoniae*. (Thanawongnuwech, R., Brown, G.B., et al 2000). One explanation of PRRSV infected animals increased susceptibility to *S. suis* could be PRRSV induces suppression of pulmonary intravascular macrophage function (Thanawongnuwech, R., Brown, G.B., et al 2000).

3. GENOME ORGANIZATION

The PRRSV 15kb long RNA genome is capped at the 5' end and polyadenylated at the 3' end (Kim, W., Yoon, K. 2008). The genome encodes at least nine open reading frames (ORFs), each of which is expressed via the generation of a 3' - co-terminal nested set of subgenomic mRNA (Kim, W., Yoon, K. 2008). ORF 1a and 1b make up about 80% of the genome and encode the RNA-dependent RNA polymerase, aka RNA replicase, and non-structural proteins (nsps) (Meulenber, J.J.M. 2008). ORF1a is translated directly from genomic RNA, while the ribosomal frameshifting 5' end of the

genome expresses ORF1b (Den Boon, J.A., Faaberg, K.S., et al. 1995, Dokland, T. 2010). The two large ORFs, ORF1a and ORF1b, encode two multidomain replicase polyproteins, pp1a and pp1ab (Sun, Y., Xue, F., et al 2009, Dokland, T. 2010). These polyproteins are processed by viral proteases to yield at least 14 non-structural proteins (Dokland, T. 2010, Sun, Y., Xue, F., et al 2009). First, nsp1 α , nsp1 β , and nsp2 (proteases) release themselves from pp1a, and then nsp4 (the fourth protease) cleaves the remaining nsps from the polyproteins (Dokland, T. 2010, Sun, Y., Xue, F., et al 2009). Remaining nsps include the RNA-dependent RNA polymerase (nsp9), a helicase (Hel/ nsp 10), and a *Xenopus Laevis* homolog poly (u), which is an endonuclease (nsp11) (Dokland, T. 2010, Sun, Y., Xue, F., et al 2009). These nsps form a replication/transcription complex essential for viral RNA synthesis (Sun, Y., Xue, F., et al 2009).

ORFs 2-7 are located at the 3' end of the genome and encode the structural proteins that are associated with the PRRSV virion (Meulenberg, J.J.M. 2008). These ORFs are expressed from the 3' nested set of subgenomic mRNAs synthesized during PRRSV replication, and are composed of a leader sequence that is derived from the 5' end of the viral genome (Meulenberg, J.J.M. 2008). ORF2b is completely embedded within ORF2a and encodes a non-glycosylated, minor structural protein, Envelope (E) (Meulenberg, J.J.M. 2008, Kim, W., Yoon, K. 2008). ORF2a, ORF3, and ORF4, encode N-glycosylated minor envelope proteins Glycoprotein-2a (GP2a), Glycoprotein-3 (GP3), and Glycoprotein-4 (GP4), respectively (Kim, W., Yoon, K. 2008, Meulenberg, J.J.M. 2008). GP2a, GP3, and GP4 form heterotrimers by disulfide linkages (Kim, W., Yoon, K. 2008). Major envelope protein, Glycoprotein-5 (GP5), which was previously known as Envelope, is encoded by ORF5 and forms heterodimers with the major protein

Membrane (M) that is encoded by ORF6 (Kim, W., Yoon, K. 2008). This GP5/ M heterodimer dominates the virion surface (Kim, W., Yoon, K. 2008). Nucleocapsid (N) protein, another major structural proteins expressed from ORF7, is the first antigen that produces a non-neutralizing antibody response in pigs during early infection (Kim, W., Yoon, K. 2008).

4. VIRION STRUCTURE

PRRSV virion structure is spherical or oval in shape and ranges from 50-74 nm in diameter, with 54nm being the median (Dokland, T. 2010). Using electron microscopy (EM), as well as X-ray crystallography, a smooth outline with a few protruding features can be observed (Dokland, T. 2010). The protruding features are thought to be small ectodomains, about 2nm, of the major envelope proteins M, 18-19kDa, and GP5, 25kDa heterodimer with approximately 16 and 30 residues, respectively (Dokland, T. 2010, Dea, S., Gagnon, C.A., et al. 2000). This interaction between GP5-M is required for proper post-translational processing of proteins (Das, P.B., Dinh, P.X., et al. 2009). The lipid bilayer envelope has a thickness of approximately 4.5nm and some cross-striations can be observed in the membrane, which are assumed to correspond to envelope (E) protein transmembrane (TM) domains (Dokland, T. 2010). E protein possesses ion-channel-like properties that may function as a viroporon on the envelope as well, which may account for the striations observed (Das, P.B., Dinh, P.X., et al. 2009). There are also a few protruding features from the membrane surface, about 10-15nm, which are most likely the less abundant, more bulky membrane proteins, GP2 for example (Dokland, T. 2010). The GP2-GP3-GP4 heterotrimer, 29kDa, 31kDa, and 42kDa, respectively, has 436

residues on the outside membrane, which are a 5-10nm structure (Dokland, T. 2010, Gagnon, C.A., et al. 2000). There has also been a strong interaction between GP4 and GP5 observed, as well as in interaction of GP2a and GP4 with CD163 (Das, P.B., Dinh, P.X., et al. 2009). GP4 is critical for mediating interglycoprotein interactions, and serves as a viral attachment protein that is responsible for mediating interactions with CD163 for virus entry into susceptible cells, along with GP2a (Das, P.B., Dinh, P.X., et al. 2009).

The internal core, whose size and shape correspond with its envelope, has an average diameter of 39 nm and is separated from the envelope by a 2-3nm gap (Dokland, T. 2010). The gap is transversed by only a few strands of density, which means that the interactions between the internal core and the envelope are weak and flexible (Spilman, M.S., Welbon, C., et 2009). The core is a 12nm thick double layer that surrounds a less dense area, indicating there is a hollow core (Dokland, T. 2010). N protein, 15kDa, which is the most abundant protein expressed in infected cells with 1415 copies, comprises the Nucleocapsid, or internal core (Spilman, M.S., Welbon, C., et 2009, Dokland, T. 2010, Gagnon, C.A., et al. 2000). This 123-128 amino acid protein, the smallest subgenomic mRNA, interacts with viral RNA to form the viral nucleocapsid (Dokland, T. 2010). It is the most immunogenic protein, but produces antibodies that are non-neutralizing and non-protective (Dokland, T. 2010). Rather than having an icosahedral shell, the nucleocapsid has been thought to be helical and filamentous (Spilman, M.S., Welbon, C., et 2009). One possible model for the nucleocapsid structure is that the N protein forms dimers that form two layers to make a linked, twisted chain with the viral RNA in the middle (Spilman, M.S., Welbon, C., et 2009). The chain then

interacts with the N-terminal RNA-binding domain of N and is bundled into a roughly spherical shape with a hollow interior (Spilman, M.S., Welbon, C., et 2009).

5. PRRSV LIFE CYCLE

PRRSV prefers to infect porcine alveolar macrophages (PAMs) that have been recently activated, but has also been known to infect monocytes, glial cells, porcine pulmonary cells, and macrophages of other tissues, including intravascular cells, endothelial cells, smooth muscle cells and fibroblasts (Suarez, P. 2000).

The virus must enter the cell for the cell to become infected. PRRSV particles have been seen at the cell surface with virus particles then observed within small vesicles circumscribed by a clathrin-like zone, but no virus particles have been detected fusing at the plasma membrane; therefore, receptor mediated endocytosis must be involved (Kreutz, L.C., Ackermann, M.R. 1996).

The first step for PRRSV entry is attachment. PRRSV GP5-M heterodimer on the viral envelope attaches to heparin sulfate glycosaminoglycan (GAGs) present on macrophage surface (Delputte, P.L., Costers, S., et al. 2005). Heparin sulfate GAGs are found in different tissues, as well as macrophages, therefore binding to non-permissive cells occurs (Van Breedam, W., Van Gorp, H., et al 2010). One study showed that washing cells, that had PRRSV attached, with heparin could remove viral attachment early, but not at a later time (Delputte, P.L., Costers, S., et al. 2005). This showed that there is another interaction with a receptor that takes place.

An interaction with a porcine sialoadhesion receptor (pSn) and GP5-M follows the attachment of PRRSV to heparin sulfate GAGs (Van Breedam, W., Van Gorp, H., et

al 2010, Delputte, P.L., Costers, S., et al. 2005, Van Breedam, W., Van Gorp, H., et al. 2010). pSn binds to the sialic acid of GP5, and the pSn-GP5-M interaction is critically dependent on the sialic-acid binding capacity of pSn as well as on sialic acids on viral GP5 glycoprotein (Van Gorp, H., et al. 2010). The pSn is macrophage restricted (Delputte, P.L., Costers, S., et al. 2005). Upon binding of the virus to the pSn receptor, the virus is internalized via the mechanism of clathrin-mediated endocytosis and enters the endocytic pathway (Van Breedam, W., Van Gorp, H., et al 2010, Delputte, P.L., Vanderheijden, N., et al. 2002, Delputte, P.L., Costers, S., et al. 2005). Fusion does not occur between the endosomal membrane and viral envelope (Delputte, P.L., Costers, S., et al. 2005). For proper virus replication and for genome release into the cytoplasm of target cells, a drop in pH in the endosome is required (Delputte, P.L., Vanderheijden, N., et al. 2002, Van Breedam, W., Van Gorp, H., et al 2010).

Once the virus has been internalized, the viral genome is released from the early endosome and into the cytoplasm of host cells (Van Breedam, W., Van Gorp, H., et al 2010). PRRSV virions do not continue to late endosomes (pH 5.0-6.0) or lysosomes (pH 4.6-5.0) (Van Breedam, W., Van Gorp, H., et al 2010). CD163, a putative PRRSV scavenger receptor, is essential for the genome release, and may do so by interactions with GP2a and GP4 (Van Breedam, W., Van Gorp, H., et al 2010, Calvert, J.G., Slade, D.E., et al. 2007, Van Gorp, H., Van Breedam, W., et al 2008). PRRSV is uncoated through this process, as well as by different cellular proteases, membrane protein E, and serine proteases (Van Breedam, W., Van Gorp, H., et al 2010). More research is needed in this area to determine the exact functions of these proteins.

Initiation of transcription and translation is next required for the formation of new virions. PRRSV genome is expressed through subgenomic transcripts (mRNA1-mRNA7), each of which encodes PRRSV ORFs (Conzelmann, K., Visser, N., et al. 1992). The translation of ORF1a and expression through a ribosomal frame shift of ORF1ab yield the pp1a and pp1ab polyproteins, respectively, that are then processed extensively by ORF1a encoded viral proteases, yielding at least 14 PRRSV NSPs (Sun, Y., Xue, F., et al. 2009, Dokland, T., 2010). Four proteases (NSP1 α , NSP1 β , NSP2, and NSP4), the RNA-dependent RNA polymerase (NSP9), a helicase (NSP10), and an endonuclease (NSP11) are included in the expressed NSPs (Dokland, T., 2010). NSP1 α and NSP1 β are at the N-terminus of pp1a, each including a papain-like cysteine protease domain called PCP α and PCP β , respectively, and cleave themselves from the polyprotein in their C-terminal junctions (Dokland, T., 2010). NSP1 β is cleaved from NSP2 autocatalytically by PCP β , followed by the papain-like protease (PL2) of NSP2 cleaving at the NSP2-3 junction (Dokland, T., 2010). NSP4, the “main” viral protease, carries out the remaining cleavages in both pp1a and pp1ab, which is activated upon the cleavage by the NSP2 protease at the NSP2-3 junction (Dokland, T., 2010). The cleavage products of pp1a, NSP5-8, are not well described, while the cleavage products contained within pp1ab, NSP9-11, are the most conserved with the nidoviruses (Dokland, T., 2010).

ORFs 2-5 encode glycosylated membrane proteins GP2-GP5, ORF6 encodes non-glycosylated M protein, and ORF7 encodes the N protein (Dokland, T., 2010). ORF2b is fully enclosed with ORF2, and encodes the E protein, or GP2b, which is non-glycosylated (Dokland, T., 2010).

N protein forms homodimers through covalent and non-covalent interactions, and is distributed between cytoplasmic and nuclear compartments (Rowland, R.R.R., Yoo, D. 2003). Covalent interactions are formed through disulfide linkages, while the non-covalent region holds two basic regions, nuclear localization signal 1 (NLS-1), and nuclear localization signal 2 (NLS-2) (Rowland, R.R.R., Yoo, D. 2003). NLS-1 and NLS-2 are important for the packaging of viral RNA genome within the nucleocapsid of the virus (Rowland, R.R., Kervin, R., et al. 1999). Vesicles containing enveloped nucleocapsids are derived from the endoplasmic reticulum, and then transported to the premedial Golgi apparatus, where viral budding takes place and virus particles are released (Suarez, P. 2000). This budding and accumulation of enveloped particles can only be observed between the smooth ER and the medial Golgi, where the virions seem to mature (Suarez, P. 2000). The virus assembly is completed when the enveloped particles interact with the N protein in the cytosol and are transported to the plasma membrane (Suarez, P. 2000, Wissink, E.H.J., Krose, M.V., et al. 2005).

6. PRRSV IMMUNOLOGY

PRRSV immunology has been a challenge and is not fully understood. Infected pigs develop a strong humoral response 5-7 days post infection (pi), with IgM antibodies reaching a peak at day 14 pi and declining to undetectable levels by day 42 pi, and IgG antibodies reaching a maximum at 21-49 days pi (Mateu, E., Diaz, I. 2008, Lopez-Fuertes, L., Campos, E., et al 2000). Although the response is strong, these antibodies are non-neutralizing and do not confer any sort of protection against PRRSV (Mateu, E., Diaz, I. 2008). These early antibodies may aid in the development of PRRS

by enhancing internalization and viral replication in alveolar macrophages (Mateu, E., Diaz, I. 2008). The earliest antibodies are to the N protein, followed by the M protein, and finally to the GP5 protein (Mateu, E., Diaz, I. 2008). Neutralizing antibodies (NAs), which appear 28 days pi or later, are mainly directed against the neutralization epitope (NE) in the ectodomain of GP5, which correlates with the clearance of viremia (Mateu, E., Diaz, I. 2008, Lopez-Fuertes, L., Campos, E., et al 2000). Other proteins that may contain a neutralizing epitope include GP4 and M (Mateu, E., Diaz, I. 2008). One of the challenges of immunology with PRRSV is its ability to mutate; with the NE being a typical candidate for mutation, although NEs can be located within a region of pathogenic function, in which case, the virus would need to find other means to evade the immune system (Lopez-Fuertes, L., Campos, E., et al 2000). This mutation is a typical mechanism of RNA viruses is called antigenetic drift. PRRSV can use a decoy epitope, as well as sugars surrounding the NE, to hinder the response to NE, which could account for the late appearance of NAs (Lopez-Fuertes, L., Campos, E., et al 2000). NAs alone have been found to fully prevent transplacental infection, as well as infection in pregnant sows (Lopez-Fuertes, L., Campos, E., et al 2000).

Interferons and cytokines are also important for protection from and removal of PRRSV. A strong lymphocyte proliferative response can be seen around four weeks pi, which would parallel with appearance of NAs (Mateu, E., Diaz, I. 2008). One study showed that interferon γ (INF- γ) starts no sooner than four weeks pi and builds until the virus is eliminated, which coincides with PRRSV specific INF γ and NA appearance (Lopez-Fuertes, L., Campos, E., et al 2000). According to Miller, L.C., Laegreid, W.W., 2004, PRRSV infection directly interferes with type 1 interferon transcriptional activation

at the level of interferon β (INF- β), which is early in its pathway. Type 1 interferons are synthesized and secreted by eukaryotic cells in response to viral infections, and PRRSV is highly susceptible to type 1 interferons. They found low levels, if any, interferon α (INF- α), an interferon that is synthesized following INF- β synthesis and secretion, in the lungs or in-vitro infected alveolar macrophages of PRRSV-infected pigs (Miller, L.C., Laegreid, W.W., 2004). Type 1 interferons are inhibited by another cytokine, interleukin 10 (IL-10) (Mateu, E., Diaz, I. 2008). IL-10 is a powerful cytokine that downregulates host immune responses and several proinflammatory cytokines, including tissue necrosis factor α (TNF- α) and INF- α (Suradhat, S., Thanawongnuwech, R., et al. 2003). Levels of IL-10 increase in pig peripheral blood macrophage cells (PBMCs) and pig lungs after PRRSV infection while levels of INF- γ expression decrease, which seems consistent among intracellular pathogens that target macrophage (Mateu, E., Diaz, I. 2008, Suradhat, S., Thanawongnuwech, R., et al. 2003). IL-10 may also have an inhibitory effect on the function of antigen presenting cells (APCs), which could contribute to the delayed induction of protective immune response to PRRSV (Suradhat, S., Thanawongnuwech, R., et al. 2003). IL-10 inhibits the production of cytokines and proliferation of CD4⁺ cells, which results in the inhibition and activation of T cells, and in turn interferes with cell-mediated immune responses and correct antigen presentation (Suradhat, S., Thanawongnuwech, R., et al. 2003, Mateu, E., Diaz, I. 2008). PRRSV also down-regulates the expression of MHC-I and MHC-II in monocyte-derived dendritic cells (Mateu, E., Diaz, I. 2008). The effect of this under-expressed, down-regulated immune system is a delayed response to the virus, which results in clinical manifestation

and shedding of virus for longer periods of time, and therefore more spreading of PRRSV and deaths.

7. CURRENT VACCINES

There are currently several types of vaccines available for PRRSV; they include, live attenuated, killed, and subunit vaccines, none of which function ideally for PRRSV. They are unable to completely prevent respiratory infection, transplacental transmission, or pig-to-pig transmission (Kimman, T.G., Cornelissen, L.A., et al. 2009). Challenges faced when producing a vaccine include effectiveness, heterogeneity, safety, cost, and differentiation of vaccinated versus naturally infected animals. The North American and European strains of PRRSV are clinically, antigenetically, and genetically heterogeneous, and isolates within each genotype differ by approximately 20%, with GP5 being the most variable, and most immunogenic (Kimman, T.G., Cornelissen, L.A., et al. 2009). The evolution of PRRSV by random mutation and intragenic recombination events also adds to the difficulty of producing an effective vaccine (Kimman, T.G., Cornelissen, L.A., et al. 2009).

Attenuated vaccines are live viruses derived by cell culture attenuation of virulent isolates (Murtaugh, M.P., Genzow, M. 2011). PRRSV live attenuated vaccines have been shown to revert to virulence under field conditions, spread from vaccinated to non-vaccinated, produce lung and lymphoid tissue infections, and maintain infection for periods of time (Murtaugh, M.P., Genzow, M. 2011, Nielsen, H.S., Oleksiewicz, M.B., et al. 2001). Natural recombination between attenuated vaccine strains and field strains has also been found to occur, which could affect PRRSV evolution (Li, B., Fang, L., et al.

2009). There has been evidence of some immune protection with attenuated vaccines, but this has been in homologous situations, and the risks outweigh any immune advantages (Kimman, T.G., Cornelissen, L.A., et al. 2009).

Killed vaccines are inactivated preparations of virulent isolates that may reduce levels of viremia in some pigs, but have ultimately been considered ineffective or of limited efficiency at best (Kimman, T.G., Cornelissen, L.A., et al. 2009, Murtaugh, M.P., Genzow, M. 2011). In trials, there has been no consistent benefit against infection or respiratory disease (Murtaugh, M.P., Genzow, M. 2011).

Subunit vaccines express only selected proteins and they allow the immune system to be focused on protective B cell and/ or T cell epitopes of a particular viral protein (Hu, J., Ni., Y., et al. 2012, Murtaugh, M.P., Genzow, M. 2011). They eliminate any safety concern of virulent reversion of modified live vaccines, but being based on recombinant proteins can suffer poor immunogenicity due to incorrect folding of target protein or poor presentation to the host immune system (Noad, R., Roy, P. 2003 Hu, J., Ni., Y., et al. 2012). Subunit vaccines are more costly due to the fact that more doses of increases concentration of antigen are needed to offer protection (Hu, J., Ni., Y., et al. 2012).

A vaccine that can provide protection against PRRSV by neutralizing virus entry, can offer heterologous protection without reversion or recombination, and can be differentiated from field strains, would be the ideal candidate for vaccine production.

8. VIRUS LIKE PARTICLES AS VACCINE CANDIDATES

Virus-like particles (VLPs), for use as vaccines, have been produced for over 30 different viruses that infect humans and animals (Noad, R., Roy, P. 2003). VLPs are a specific class of subunit vaccines that do not contain infectious genetic material while mimicking the structure of authentic virus particles (Noad, R., Roy, P. 2003, Crisci, E., Barcena, J., et al. 2012). They are easily recognized by host immune system and are able to present viral antigen with a more authentic conformation than other subunit vaccines (Noad, R., Roy, P. 2003). VLPs are based on an expression system of viral capsid proteins that lead to spontaneous assembly of virus particles that are structurally similar to authentic viruses (Noad, R., Roy, P. 2003). There are several advantages of VLPs for vaccine use over current vaccines available.

A well-defined geometry and uniformity with repetitive and ordered surface structures that mimic the overall structure of authentic virions is one major advantage of VLPs (Crisci, E., Barcena, J., et al. 2012). Using a recombinant expression system, multiple copies of one or more major viral capsid or envelope proteins are overexpressed to self-assemble into VLPs (Noad, R., Roy, P. 2003, Crisci, E., Barcena, J., et al. 2012). These particles lack DNA or RNA, but have the authentic viral capsid or envelope proteins that are seen in attenuated or inactivated vaccines, and therefore lack the drawbacks of recombination, virus replication, insertion, re-assortment or reversion, and are much safer than attenuated or inactivated vaccines (Crisci, E., Barcena, J., et al. 2012, Noad, R., Roy, P. 2003). Because VLPs are only structural proteins that do not contain DNA, RNA, or NSPs, they are able to be differentiated from a field infection and have

the possibility to follow Differentiating Infected from Vaccinated Animals (DIVA) compliance (Crisci, E., Barcena, J., et al. 2012).

VLPs have a particulate and multivalent nature while being able to preserve the native antigenic conformation of virions, and can trigger the innate immune system by being recognized by toll-like receptors (TLRs) and pattern-recognition receptors (PRRs), as well as stimulate a strong B-cell, CD4⁺ T cell proliferative responses and cytotoxic T cell responses (Crisci, E., Barcena, J., et al. 2012, Noad, R., Roy, P. 2003). The antigens are highly stable when compared to soluble antigens in extreme environmental conditions (Crisci, E., Barcena, J., et al. 2012).

There are several different expression systems that can be used to produce VLPs. The most popular is recombinant baculovirus expression in insect cells, but bacteria, yeast, mammalian, and plant cells have also been used (Crisci, E., Barcena, J., et al. 2012). The major advantages of using the recombinant baculovirus expression system are that large amounts of correctly folded recombinant proteins are produced in eukaryotic-like post-translational modifications with a high recovery of correctly folded antigens, the complexity of VLPs produced is higher than in yeast or bacterial cells, there is a limited host range (insects) and is therefore safe for vertebrates, the method is simple, fast for large grade vaccine production, and provides high versatility, which is very important because of the highly mutable surface proteins of viruses. (Crisci, E., Barcena, J., et al. 2012, Noad, R., Roy, P. 2003). A disadvantage to the recombinant baculovirus expression system is the possibility of contaminated virions, but the use of chemical treatment or the use of a recombinant baculovirus lacking the vp80 gene, the gene that is

essential for virus formation, can easily eliminate that problem and will not affect foreign gene expression (Crisci, E., Barcena, J., et al. 2012, Noad, R., Roy, P. 2003).

Several VLPs have been produced for various viruses, and some are even used in practice today. The hepatitis B virus and human papilloma virus are both human viruses that have VLP vaccines produced from the expression of the S gene in yeast and the expression of the L1 protein in both insect or yeast cells, respectively (Harro, C.D., Pang, Y.S., et al. 2001 Noad, R., Roy, P. 2003). Ebola VLPs have also been produced by expressing VP40 or GP proteins using the recombinant baculovirus expression system in insect cells (Ye, L., Lin, J., et al. 2006). Animal VLP vaccines have also been produced, and include porcine circovirus type 2 (PCV2), canine parvovirus (CPV) and bluetongue virus (BTV). CPV overexpresses its VP2 gene and assembles in insect cells, where BTV overexpresses four major structural proteins, VP2, PV2, PV5, and VP9 using the recombinant baculovirus expression system (Stewart, M., Dovas, C.I., et al. 2012, Noad, R., Roy, P. 2003). The four proteins are co-expressed in the same cell and form VLPs structurally indistinguishable from virus particle VLPs, only lacking the genome (Noad, R., Roy, P. 2003).

Influenza is another candidate for VLP vaccine production. Its major structural protein, M1, has been shown to be sufficient to produce VLPs, but M1-HA and M1-HA-NA VLPs have been shown to confer protection from lethal challenge of the same type of Influenza virus (Roy, P., Noad, R. 2008).

Not only are VLPs used for vaccines, but they can be used in research as well. Norwalk virus is unable to be grown in cell culture, so scientists use VLPs to monitor the disease (Noad, R., Roy, P. 2003). Norwalk virus VLPs have also been shown to be

effective at stimulating IgG, IgA, and humoral responses in mice, and could therefore be a potential vaccine.

With the nature of PRRSV and the previous research done on VLPs, PRRSV seems to be an ideal candidate for VLP vaccine production.

CHAPTER 2: VIRUS-LIKE PARTICLES GENERATED BY EXPRESSING
PROTEINS OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME
VIRUS (PRRSV) USING THE RECOMBINANT BACULOVIRUS EXPRESSION
SYSTEM

INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) belongs to the family *Arteriviridae*, and is a linear, positive sense, single-stranded, enveloped RNA virus (Gorbalenya, A.E., Enjuanes, L., et al 2005). PRRSV genome encodes at least nine open reading frames (ORF), which express at least 14 non-structural proteins and seven structural proteins (Meulenberg, J.J.M. 2000, Dokland, T. 2010). The seven structural proteins form the spherical or oval virion, with the nucleocapsid (N) protein, membrane (M) protein, and glycoprotein 5 (GP5) being the major structural proteins (Dokland, T. 2010). The M and GP5 proteins are essential for viral attachment and entry into susceptible porcine cells (Delputte, P.L., Costers, S., et al. 2005). N protein is the most abundant protein expressed in infected cells and is the most immunogenic (Spilman, M.S., Welbon, C., et 2009, Dokland, T. 2010, Gagnon, C.A., et al. 2000). GP4 is critical for mediating interglycoprotein interactions, and serves as a viral attachment protein that is responsible for mediating interactions with CD163 for virus entry into susceptible cells, along with GP2a (Das, P.B., Dinh, P.X., et al. 2009). E protein possesses ion-channel-like properties that may function as a viroporin on the envelope (Das, P.B.,

Dinh, P.X., et al. 2009). Virion assembly and budding then take place in the endoplasmic reticulum and Golgi apparatus of host cells (Suarez, P. 2000).

Clinical symptoms of PRRSV infected pigs range from respiratory distress of piglets to reproductive failure in sows, and may reduce the annual production of a herd by 15% (Wensvoot, G. 1993, Holtkamp, D.J., Kliebenstein, J.B., et al 2013). The crucial feature of PRRSV infection is its ability to remain persistent. Animals continue to shed virus during persistent infection (PI), thus continuing to transmit virus, and increase the likelihood of contracting a secondary infection (Lopez, O.J., Osorio, F.A. 2004). PRRSV is eventually cleared from the body around 150 days post infection (pi), with the help of neutralizing antibodies (NAs) to the neutralizing epitope (NE) of GP5 (Lopez, O.J., Osorio, F.A. 2004, Mateu, E., Diaz, I. 2008).

There are currently several types of vaccines commercially available for PRRSV; none of which confer heterologous protection, safety, or the ability to distinguish vaccinated pigs from infected pigs (Kimman, T.G., Cornelissen, L.A., et al. 2009). Attenuated vaccines have been shown to revert to virulence, spread from vaccinated to non-vaccinated individuals, produce infection, and recombine with field isolates (Murtaugh, M.P., Genzow, M. 2011, Nielsen, H.S., Oleksiewicz, M.B., et al. 2001). Killed vaccines have been considered ineffective or of limited efficiency (Kimman, T.G., Cornelissen, L.A., et al. 2009, Murtaugh, M.P., Genzow, M. 2011). Due to economic losses associated with PRRSV, there is a dire need for a safe, effective vaccine to PRRSV. Virus-like particle (VLP) vaccines have been shown to be safe, effective, and offer the ability to differentiate non-vaccinated from vaccinated individuals for several viruses, including Ebola virus, canine parvovirus (CPV), porcine circovirus type 2

(CPV2) and bluetongue virus (BTV) (Noad, R., Roy, P. 2003, Crisci, E., Barcena, J., et al 2012, Ye, L., Lin, Y., et al. 2006).

In this study, we show how PRRSV VLPs, derived from PRRSV M, N, GP5, and E proteins by using the recombinant baculovirus expression system in insect cells, are expressed and produced.

MATERIALS AND METHODS

CELLS AND VIRUSES

CV-1 (simian) in Origin carrying the SV40 genetic material cells (Cos-1) derived from monkey kidney tissue and show high efficiency transfection, were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies Grand Island, NY), and maintained at 37°C in a 5% CO₂ atmosphere. Experiments performed with Cos-1 cells were in a 6-well plate with a concentration of 4x10⁵ cells/ well. Sf9 insect cells, derived from *Spodoptera frugiperda*, were cultured in HyClone™ TNM-FH Insect Cell Culture Media (GE Healthcare Life Sciences, Logan, Utah) with 1X penicillin. TriEx Sf9 cells, serum-free adapted Sf9 derived cells, were cultured in filtered Novagen TriEx™ Insect Cell Media (Novagen, San Diego, CA). Sf9 cells are adherent cells and are used only in the transfection of recombinant baculovirus, while the TriEx Sf9 cells are free floating cells that yield high levels of protein expression. Incomplete media, used for recombinant baculovirus transfection of Sf9 cells, was filtered Grace's Insect Media (Sigma, St. Louis, MO). Sf9 cells and the initial TriEx Sf9 cell culture were maintained in a T-75 flask at 28°C in a foil covered Tupperware box. Once 80-90% confluent, TriEx Sf9 cells were transferred to a shake culture and were maintained at 150rpm and 28°C. Sf9 cell concentration for T-75 flasks was 4x10⁶ cells/ flask and 2x10⁵ cells/ well for 24-well plates. TriEx Sf9 shaker cultured cells maintained a cell concentration between 2x10⁵ and 5x10⁵ cells/ ml in 50 mL, and 2x10⁶ cells/ mL in 20 mL for amplification of recombinant baculovirus. cDNA was produced from previously extracted RNA of PRRSV-23983.

POLYMERASE CHAIN REACTION (PCR)

Previously extracted PRRSV-23983 RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) and the cDNA produced was then subjected to first round PCR for amplification of PRRSV GP5, GP4, and E genes using the New England Biolabs Taq PCR kit (New England BioLabs Inc., Ipswich, MA). Primers used for GP5 were forward primer (FP): 5'-GAT GAT CTC GAG GCC ACC ATG TTG GAG AAA TGC TTG ACC-3' and reverse primer (RP): 5'-CGT ATG GGT AAG GAC GAC CCC ATT GTT CCG C-3' (IDT Ref #'s FP: 79600681; RP: 79600682), primers used for E were FP: 5'- GAT GAT CTC GAG GCC ACC ATG GGG TCC ATG CAA AGC CTT-3' and RP: 5' - CGT ATG GGT ATA AGA TCT TCT GTA ATT GCT C-3' (IDT Ref #'s FP: 7900683; RP: 79600684), and primers used for GP4 were FP: 5'- GAT GAT CTC GAG GCC ACC ATG GCT TCG TCC CTT CTT TTC-3' and RP: 5'- CGT ATG GGT AAA TTG CCA ACA GAA TGG C-3' (IDT Ref #'s FP: 52083932; RP: 52083933), which were synthesized by Integrated DNA Technologies (IDT, Coralville, IA). The GeneAmp PCR system 2400 thermocycler (Applied Biosystems, Carlsbad, CA) was used to complete 35 cycles of step 2 PCR according to the programming below:

Step 1: Hold 94°C for 5 minutes

Step 2: 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 30 seconds

Step 3: Hold 72°C for 10 minutes

Step 4: Hold 4°C for ∞

After the PCR reaction was completed, loading dye was mixed with each PCR product, then was loaded and separated in an ethidium bromide-stained 1% agarose gel (w/v) and visualized by the UV Transilluminator (UVP, Upland, CA). Concentration of PCR product was determined through NanoDrop ND-1000 Spectrophotometer.

HA Stop tag, which will be used to identify proteins in future experiments, was incorporated into the first round PCR product via second round PCR using the respective first round PCR product as a template with the HA Stop RP (5'-ACT ACT GAA TTC GAT ATC GCG GCC GCT TAC GCG TAG TCC GGG ACG TCG TAT GGG TA-3' (IDT Ref # 52385787)) and respective FP. PCR was completed for GP4 and E proteins for 35 cycles of step 2 using the following programming:

Step 1: Hold 94°C for 5 minutes

Step 2: 94°C for 30 seconds, 47°C for 30 seconds, 72°C for 30 seconds

Step 3: Hold 72°C for 10 minutes

Step 4: Hold 4°C for ∞

PCR was completed for GP5 protein for 35 cycles of step 2 using the following programming:

Step 1: Hold 94°C for 5 minutes

Step 2: 94°C for 30 seconds, 40°C for 30 seconds, 72°C for 30 seconds

Step 3: Hold 72°C for 10 minutes

Step 4: Hold 4°C for ∞

Concentrations of PCR product were determined through the NanoDrop ND-1000 Spectrophotometer.

RESTRICTION ENZYME DOUBLE DIGESTION, GEL EXTRACTION, AND LIGATION WITH pCAGEN PLASMID

PCR products, as well as pCAGEN plasmid (acquired from Dr. Li's Lab) were subject to restriction enzyme double digestion with Xho1, Not1, and Cutsmart Buffer (New England BioLabs, Ipswich, MA) in a 37° water bath for 3 hours and 1 hour, respectively. pCAGEN plasmid is used to contain our genes of interest after restriction enzyme double digestion and ligation, and will be replicated in competent cells. The replication will include both pCAGEN as well as our genes of interest. After restriction enzyme double digestion was complete, loading dye was mixed with each sample, then was loaded and separated in an ethidium bromide-stained 1% agarose gel (w/v) and visualized by the UV Transilluminator (UVP, Upland, CA) and cut from the gel. PCR products or pCAGEN was then extracted from the gel via the QIAquick Gel Extraction Kit (QIAGEN Sciences, Maryland). Each PCR product was then mixed with pCAGEN, T4 DNA Ligase, and T4 DNA Ligase buffer (10X) (New England BioLabs, Ipswich, MA) in a 15°C water bath for ligation of plasmid with gene of interest.

Each ligation reaction was added to competent cells (*Escherichia coli* DH-5 α) (New England BioLabs, Ipswich, MA), incubated on ice for 30 minutes, heat shocked for 45 seconds in a 42°C waterbath, placed back on ice for 5 minutes, and incubated on the rocker for 1 hour at 37°C and 250rpm after adding Lennox L broth (LB) (Research

Products International Corp Mount Prospect, IL) for transformation. The mixture was plated on LB plates with Ampicilin and allowed to incubate overnight at 37°C. An isolated colony was selected from each LB plate, added to LB medium plus ampicillin, and incubated overnight on the rocker at 37°C and 250rpm. pCAGEN-GP4, pCAGEN-GP5, and pCAGEN-E was then extracted from respective competent cells following the Wizard Plus SV Minipreps DNA Purification System Protocol (Promega, Madison, WI). Restriction enzyme double digestion and gel electrophoresis were again performed to be certain the colony selected contained the insertion. We discontinued our work with GP4 at this point because this was the second unsuccessful experiment with GP4 and we needed to move forward with the project.

Restriction enzyme double digestion was performed on pCAGEN-GP5 and pCAGEN-E, as well as transfer plasmid pOET1 (Oxford Expression Technologies, Oxford, UK) with NOT1, XHO1, and Cutsmart Buffer in 37°C waterbath for 1 hour. Gel extraction, ligation, transformation and screening of plasmid, and plasmid DNA extraction were performed on pOET1-GP5 and pOET1-E following protocols previously stated. pOET1 transfer plasmid was used due to its ability to express high levels of foreign genes and its compatibility with recombinant baculovirus expression systems, which we will be using in further experiments.

TRANSFECTION OF COS-1 CELLS

Extracted pCAGEN-GP5, pCAGEN-GP4, and pCAGEN-E was calculated at 4 µg DNA/ 6-well plate and added to raw DMEM media each in separate tubes, while Lipofectamine 3000 (Invitrogen, Carlsband, CA) was added to raw media in separate

tubes, and allowed to incubate at room temperature for 5 minutes. Each DNA tube was combined with each Lipofectamine 3000 tube and allowed to incubate at room temperature for 20 minutes, up to 6 hours. Each DNA/ Lipofectamine 3000 mixture was added to Cos-1 cells in separate wells of a 6-well plate, and allowed to incubate at 37°C with 5% CO₂ for 4-6 hours. Media was then removed and complete DMEM media was added to each well and allowed to incubate for 48 hours.

WESTERN BLOT

Harvested supernatant from pCAGEN-GP5, pCAGEN-GP4, and pCAGEN-E transfection of Cos-1 cells were added to TSE Buffer containing 10mM TrisHCl, 1M EDTA, 100mM NaCl with 20% sucrose, and ultracentrifuged for 1.5 hours at 45000rpm and 4°C. pCAGEN-GP5, pCAGEN-GP4, and pCAGEN-E transfected Cos-1 cells were harvested and lysed with a lysis buffer containing 0.01M Tris-HCl, 0.14M NaCl, 0.025% NaN₃, 1% Triton X-100, and with a protease inhibitor for 2 hours in 4°C and vortexed every 20 minutes. Proteins were then separated in a SDS-PAGE gel (Novex by Life Technologies, Carlsband, CA) and transferred to a nitrocellulose membrane (Life Technologies, Gaithersburg, MD). The membrane was then blocked for 1h by rocking slowly at room temperature with 5% (w/v) milk powder in PBS plus 0.05% Tween 20 (PBST). Next, primary antibody, monoclonal mouse anti-HA antibody (Sigma-Aldrich, St. Louis, MO) diluted 1:5,000 in blocking buffer (5% (w/v) milk powder in PBST) was added to the membrane and incubated overnight at 4°C on the rocker. In a dark room, the secondary antibody, goat anti-mouse IRDye (LI-COR, Lincoln, NE) diluted 1:10,000 in PBST was added to the membrane and incubated for 1 hour rotating at room temperature. Bands were visualized using the ODESSY Infrared Imaging System (LI-COR, Lincoln,

NE).

The same procedure was performed on harvested supernatant and cells from TriEx Sf9 cells co-infected with recombinant baculovirus containing PRRSV proteins M, N, E, and GP5.

TRANSFECTION OF SF9 CELLS

Sf9 cells were incubated in a 24-well plate for 1 hour at room temperature for the transfection of recombinant baculovirus. The transfection mixture of flashBAC (Oxford Expression Technologies, Oxford, UK), Baculofectin (Oxford Expression Technologies, Oxford, UK), raw media, and LacZ (Oxford Expression Technologies, Oxford, UK) pOET1-E, or pOET1-GP5 at 500ng/ well was prepared and incubated for 15 minutes at room temperature. With pOET1-E or pOET1-GP5 and flashBAC, a homologous recombination occurs which restores the function of a gene in the flashBAC DNA that is essential for replication of the recombinant baculovirus simultaneously inserting the gene of interest for the overexpression of that gene. Next, each transfection mixture was added to the center of its respective well of Sf9 cells and incubated for 5 hours at 28°C when complete media was then added. After complete media was added, the transfection was allowed to incubate for 5 days at 28°C. Supernatants were harvested and stored at 4°C. The supernatants contained the generated recombinant baculoviruses that encompassed PRRSV GP5 or E genes.

IMMUNOFLUORESCENCE ASSAY (IFA) OF GP5 AND E INFECTED SF9 CELLS

Formaldehyde in PBS (1:10) was added to pOET1-GP5 and pOET1-E infected

Sf9 cells for 10 minutes, followed by 0.2% Triton X-100 for 10 minutes. Next a blocking buffer (10% fetal bovine serum (FBS) in PBS 1X) was added for 1 hour. Primary antibody, mouse anti-HA at a 1:5,000 dilution in blocking buffer, was added and allowed to incubate at room temperature for 1.5 hours. The mouse anti-HA is a monoclonal antibody for the HA stop tag that was added during second round PCR. Secondary antibody, 5 μ L goat anti-mouse Alexa-546 (Life Sciences Advanced Technologies, St. Petersburg, FL) diluted in 1mL PBS, was incubated for 1 hour in the dark at room temperature. Fluorescing cells were visualized with Olympus 1X70 Inverted Microscope (Olympus, Center Valley, PA).

AMPLIFICATION AND TITRATION USING qRT-PCR OF RECOMBINANT BACULOVIRUS CONTAINING PRRSV GP5 AND E GENES

Using a suspension culture of TriEx Sf9 cells passed 3 hours prior containing 4×10^7 cells/ 20 mL, 0.5mL recombinant baculovirus containing PRRSV GP5 or E from the collected supernatant after Sf9 transfection in previous experiments was added to each respective flask and incubated for 5 days on the shaker at 28°C and 150rpm. Recombinant baculovirus was harvested at day 5 by pouring cells into 50mL centrifuge tube and spun down at 2000rpm for 10 minutes. The supernatant was collected and filtered through a 0.45 μ m syringe filter. The same procedure was repeated to continue to amplify the recombinant baculovirus until desired amount of recombinant baculovirus is obtained.

Virus titration was performed using the baculoQUANT ALL-IN-ONE™ Baculovirus DNA Extraction and Quantification Kit (Oxford Expression Technologies,

Oxford, UK). To begin virus titration, a 10 fold serial dilution of virus internal standard was prepared by adding 10 μ L of the virus internal standard (10^8 pfu/ mL) to 90 μ L of RNase free water and diluted to 10^3 pfu/ mL, making a total of 6 internal standard dilutions. 80 μ L of each recombinant baculovirus stock, as well as the 6 internal standard dilutions, were centrifuged at 16,000x g/ 13,000rpm for 5 minutes in a microfuge. The supernatant was removed and the virus pellet was resuspended in 20 μ L of lysis buffer by briefly vortexing. All tubes were placed into the GeneAmp PCR system 2400 thermocycler and ran according to the lysis program as follows:

Step 1: 65°C for 15 minutes

Step 2: 96°C for 2 minutes

Step 3: 65°C for 4 minutes

Step 4: 96°C for 1 minute

Step 5: 65°C for 1 minute

Step 6: 96°C for 30 seconds

Step 7: 20°C hold

Next, a master mix was prepared using reagents from the baculoQUANT ALL-IN-ONE™ Baculovirus DNA Extraction and Quantification Kit and Brilliant III Ultra-Fast QPCR Master Mix (Agilent Technologies, Santa Clara, CA), and run in duplicate for each standard and sample. Reference dye was diluted 50X (1 μ L reference dye in 49 μ L RNase free water). Master mix was prepared by adding 0.3 μ L diluted reference dye per sample, 3 μ L primer/ probe mix per sample, 10 μ L PCR mix (Brilliant II Ultra-Fast QPCR Master Mix) per sample, and 4.7 μ L water per sample. 18 μ L of master mix was added to each qRT-PCR tube, followed by viral DNA or diluted virus internal standard in their respective tubes. Tubes were capped and centrifuged for 3 minutes at 12000rpm.

qRT-PCR was run on the ABI7900HT Real-Time Thermocycler (Applied Biosystems, Grand Island, NY) according to the following conditions: Reporter FAM, Quencher TAMRA, Stage 1: 95°C for 3 minutes, and Stage 2: 40 repeats of 95°C for 5 seconds and 60°C for 15 seconds.

CO-INFECTION OF TRIEX SF9 CELLS TO DETERMINE OPTIMAL MULTIPLICITY OF INFECTION (MOI) AND INCUBATION TIMES

TriEx SF9 cells were co-infected with recombinant baculoviruses containing PRRSV N, M, GP5, and E proteins at MOI of 2, 3, 5, or 10 with cell concentrations of 1.5×10^7 cells/ 10mL. Recombinant baculoviruses containing PRRSV N and M were produced through previous experiments in our lab that has not been published. Cells and supernatant were harvested at 24, 48, 72, and 96 hours by centrifuging at 2000rpm for 10 minutes, and were subjected to western blot for protein expression analysis.

CO-INFECTION OF TRIEX SF9 CELLS WITH OPTIMAL MOI AND INCUBATION TIMES FOR VLP PRODUCTION

TriEx SF9 cells were co-infected with recombinant baculoviruses containing PRRSV N, M, GP5, and E proteins at MOI of 2 for N, GP5, and E proteins, and MOI of 3 for M protein with cell concentrations of 75×10^6 cells/ 50mL. Supernatants were harvested at 72 hours by centrifuging at 2000rpm for 10 minutes and filtering through a .45µm filter.

TRANSMISSION ELECTRON MICROSCOPY (TEM)

Harvested supernatant from TriEx SF9 cells co-infected with recombinant

baculovirus containing PRRSV M, N, GP5, and E genes or uninfected TriEx cells were added to TSE Buffer containing 10mM TrisHCl, 1M EDTA, 100mM NaCl with 20% sucrose, and ultracentrifuged for 1.5 hours at 45000rpm and 4°C. Supernatant was removed after untracentrifugation and the pellet was resuspended in 100µL 1X PBS. Samples were sent to Karen Bently, MS and the EM core facility of the University of Rochester Medical Center in Rochester, NY to be analyzed.

RESULTS

AMPLIFICATION AND ISOLATION OF PRRSV GP4, GP5, ANE E PROTEINS

For insertion of PRRSV GP4, GP5, and E proteins into the pCAGEN plasmid, amplification through PCR of genes of interest and isolation through gel extraction followed by restriction enzyme double digestion of PCR products was performed. Amplification and isolation of proteins were determined through gel electrophoresis. Bands were seen for GP4 at approximately 540bp, 600bp for GP5, and 200bp for E. Concentrations of PCR product after second round PCR were then determined to be 730 ng/µL GP4, 530 ng/µL GP5, and 650 ng/µL E. Concentrations of PCR product and pCAGEN after restriction enzyme double digestion and gel extractions were determined to be 4.9 ng/µL GP4, 7.2 ng/µL GP5, 6.9 ng/µL E.

INSERTION AND SCREENING OF PRRSV GP4, GP5 AND E GENES INTO pCAGEN PLASMID

Genes were inserted into pCAGEN plasmid through ligation, followed by transformation of plasmids containing respective PRRSV genes into competent cells,

which were then plated on LB plus amp media and allowed to grow. Colonies seen on plates had the complete pCAGEN plasmid that contained the cloned PRRSV GP4, GP5, or E genes. The cut pCAGEN plasmid was used as the negative control and showed no growth. The pCAGEN plasmid contains an ampicillin resistant gene when complete. If it is cut, the plasmid will be susceptible to ampicillin. After allowing each of the transformed bacteria to grow in LB broth for 24 hours at 37° and 250rpm, plasmids were extracted and once again double digested for verification that the correct gene was inserted into the plasmid. Bands were seen at approximately 540bp for GP4, 600bp for GP5, and 200bp for E after gel electrophoresis. Concentrations of extracted plasmid were determined to be 252 ng/μL for GP4, 132 ng/μL for GP5, and 25.3 ng/μL for E.

PRRSV GP5 AND E PROTEINS ARE EXPRESSED IN MAMMALIAN COS-1 CELLS

Each purified plasmid DNA, pCAGEN-GP4, pCAGEN-GP5, and pCAGEN-E, was transfected into cos-1 mammalian cells at 4μg plasmid DNA per well of a 6 well plate, and allowed to incubate for 48 hours before harvesting the supernatant and cells. Protein expression of PRRSV GP4, GP5, and E proteins in mammalian cells was then verified through western blot (**Figure 2.1**). We saw that both PRRSV GP5 and E were expressed in both cell lysate and supernatant of mammalian cells, with bands at 25kDa and 10kDa, respectively. PRRSV GP4, 31kDa, was not seen in either cell lysate or supernatant, and therefore was eliminated from future studies. Bands or slurs seen above protein bands can be explained by aggregates of structural proteins.

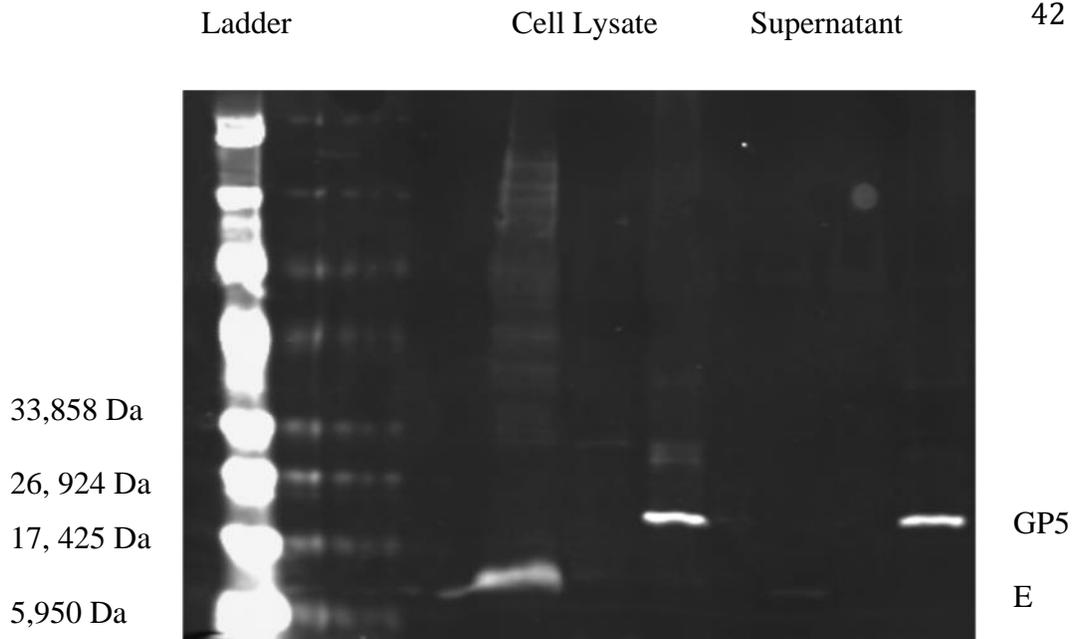


Figure 2.1: Western Blot analysis of PRRSV GP4, GP5, and E protein expression in transfected cos-1 cells. After transfecting mammalian cos-1 cells with pCAGEN-GP4, pCAGEN-GP5, or pCAGEN-E for 48 hours, western blot analysis was performed on cell lysate and supernatant for protein expression. Bands are seen for E protein (10 kDa) and GP5 (25 kDa) proteins in both the supernatant and cell lysate, but not for GP4.

INSERTION AND SCREENING OF PRRSV GP5 AND E PROTEINS INTO pOET-1 PLASMID

Plasmids, pCAGEN-GP5, pCAGEN-E, and pOET-1, were subjected to restriction enzyme double digestion and gel extraction with the resulting concentrations of 11.3ng/ μ L for GP5, 8.2ng/ μ L E, and 36.5ng/ μ L pOET-1. PRRSV genes were inserted into pOET-1 plasmid through ligation, followed by transformation of plasmids containing respective PRRSV genes into competent cells, which were then plated and allowed to grow. Colonies seen on plates contained successfully inserted PRRSV GP5 and E genes into the pOET-1 plasmid. The cut pOET-1 plasmid was used as the negative control and

showed no growth, due to its susceptibility to ampicillin when not complete. The complete plasmid will show resistance to ampicillin. After allowing each of the transformed bacteria to grow in LB broth for 24 hours at 37° and 250rpm, plasmids were extracted and once again double digested for verification that the correct gene was inserted into the plasmid. Bands were seen at approximately 600bp for GP5 and 200bp for E after gel electrophoresis. Concentrations of extracted plasmid were determined to be 97.2 ng/μL for GP5, and 92.8 ng/μL for E.

PRODUCTION OF PRRSV RECOMBINANT BACULOVIRUS GP5 AND E

SF9 cells were transfected using the flashBAC transfection mixture, pOET1-GP5, and pOET1-E for the production of recombinant baculoviruses containing PRRSV GP5 and E. According to Oxford Expression Technologies, the flashBAC system works by co-transfecting insect cells, SF9 cells, with the flashBAC virus DNA and pOET-1 containing the gene of interest to produce recombinant baculoviruses. The flashBAC DNA contains a gene that is partially deleted. The homologous recombination of the flashBAC DNA with pOET-1 containing GP5 or E restores function of that partially deleted gene and thus allows for replication of the recombinant baculovirus in insect cells, while inserting the GP5 or E gene into the flashBAC DNA (**Figure 2.2-** retrieved from oetltd.com). The produced recombinant baculovirus will replicate in the inset cells allowing for overexpression of GP5 and E.

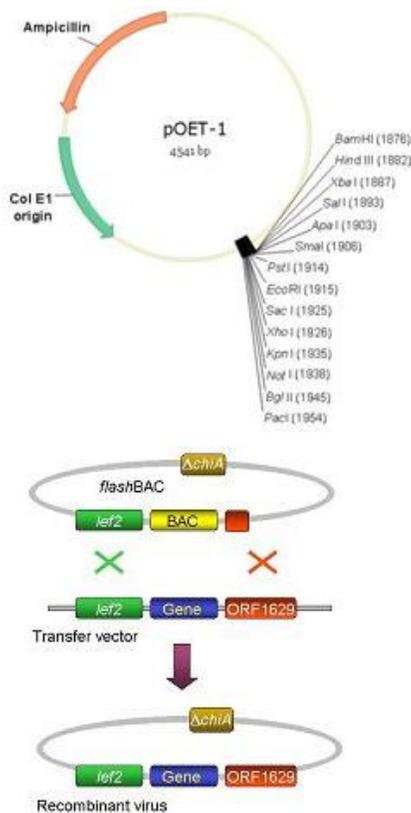


Figure 2.2 FlashBAC Recombinant Baculovirus Expression System. (Retrieved from oetltd.com) The FlashBAC Recombinant Baculovirus Expression System creates a Recombinant Baculovirus that overexpresses our proteins of interest (GP5 and E). It works by co-transfecting insect cells (SF9) with flashBAC with pOET1- GP5 or pOET1- E. Homologous recombination of the virus with the insect cells restores function to the virus' deleted gene, simultaneously inserting the gene of interest. The restored recombinant baculovirus genomes, and thus the inserted genes (GP5 and E), replicate to be harvested as the recombinant virus seed stock that is used to produce GP5 and E proteins.

Transfection was proven to be effective by adding 2% x-gal to the control transfection well of LacZ and observing change in media color to blue. An immunofluorescence assay (IFA) was also performed to confirm transfection of SF9 cells

with PRRSV recombinant baculovirus GP5 or E. Results of the IFA are shown in **Figure 2.3**. PRRSV recombinant baculovirus GP5 and E were both observed under the fluorescent microscope and phase contrast microscope, while mock cells could only be visualized by the phase contrast microscope. This shows that the transfection was effective for PRRSV GP5 and E.

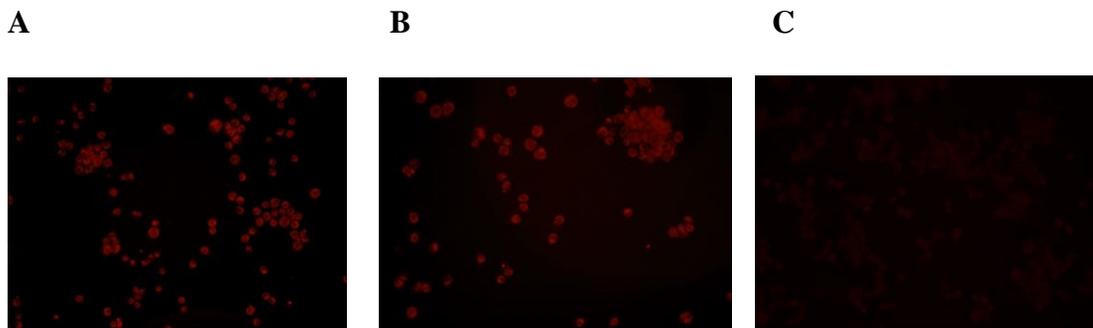


Figure 2.3: IFA of transfected SF9 cells with PRRSV recombinant baculovirus GP5 or E. SF9 cells were incubated for 5 days after being transfected with PRRSV recombinant baculovirus GP5 or E, followed by an IFA. Figure 2.2A shows PRRSV recombinant baculovirus GP5 is present in the transfected cells. Figure 2.2B shows that PRRSV recombinant baculovirus E is also present in the transfected cells. Figure 2.2C shows a negative control of mock cells with no staining. This indicated that the transfection was effective.

After infection of TriEx Sf9 cells with PRRSV recombinant baculovirus containing GP5 or E for amplification, viral titers were shown to be 6.94×10^8 pfu/mL for GP5 and 6.19×10^8 pfu/mL for E.

CO-INFECTION OF TRIEX SF9 CELLS WITH PRRSV RECOMBINANT BACULOVIRUS N, M, GP5, AND E FOR PROTEIN EXPRESSION

To determine the optimal MOI and incubation time for protein expression co-infection of TriEx SF9 cells with MOI of either 2, 5, or 10 of PRRSV recombinant baculoviruses N and M (from previous studies not published), and E at time points of 24, 48, 72, and 96 hours was performed. Results from the Western Blot show that MOI of 2 at the 72-hour time point was the most ideal for protein expression for N and E, while M was only faintly visible at a MOI of 5 and time points of 72 and 96 hours (**Figure 2.4**).

To further explore M and GP5, co-infection of M and GP5 in TriEx SF9 cells with MOI of 3 for M and MOI of 1 or 3 for GP5 was performed. The results showed that the MOI of 3 worked for M, and MOI of either 1 or 3 worked for GP5 (**Figure 2.5**). There are bands seen above and below bands for proteins that can be explained by aggregates of proteins or break down of proteins. Structural proteins have been known to form heterodimers or homodimers. For further experiments, MOI of 2 was used for GP5.

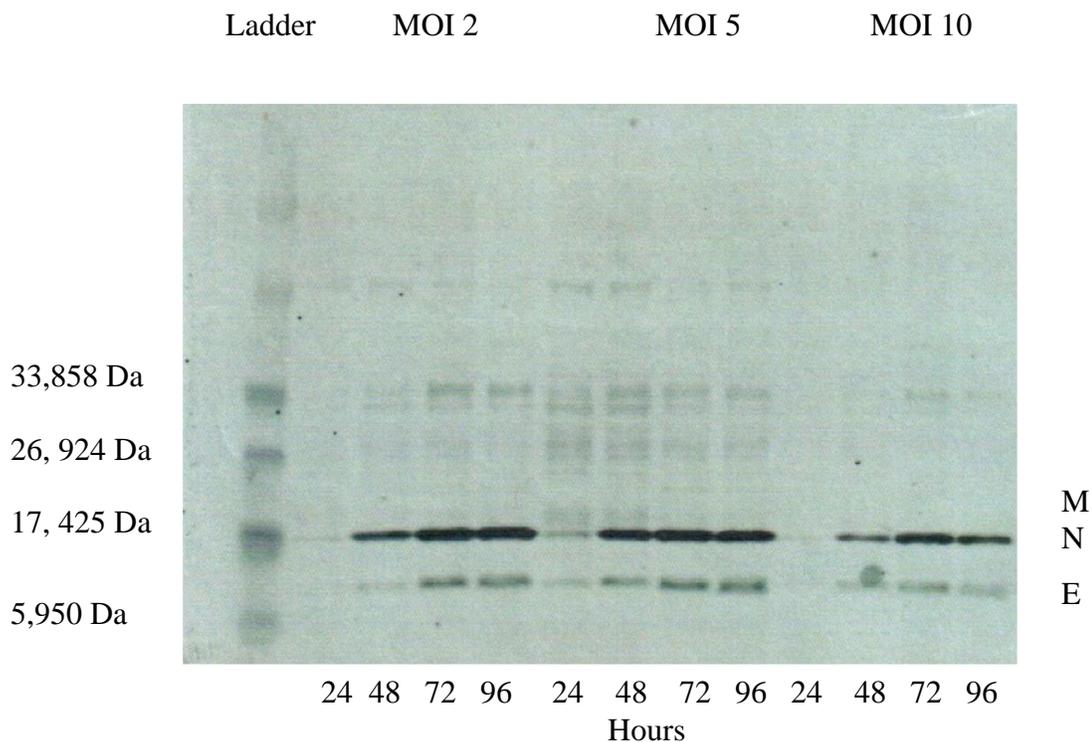


Figure 2.4: Protein Expression of PRRSV proteins M, N, and E post co-infection of TriEx SF9 cells. TriEx SF9 cells were co-infected with M, N, and E at MOI of 2, 5, or 10, and collected at 24, 48, 72, or 96 hours post infection for all samples. The band for the M protein was only slightly visible at MOI of 5 at 72 and 96 hours post infection. The N protein was visible at all time points, as well as MOI, and showed the strongest band at 72 and 96 hours of 2 MOI. The E protein was visible at all time points and MOI except for the 24 hour of 2 and 10 MOI.

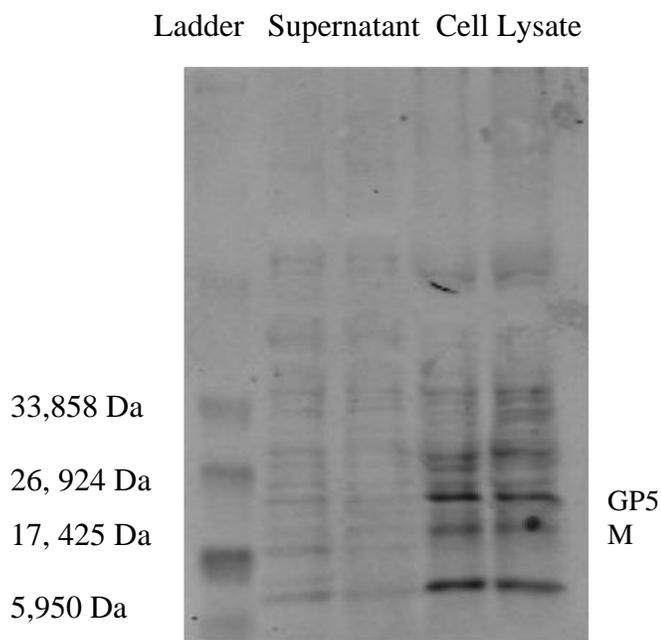


Figure 2.5: Protein Expression of PRRSV GP5 and M 72 hours post co-infection of TriEx cells. Lanes 1 and 3 show bands for both GP5 and M with an MOI of 1 for GP5 and 3 for M. Lanes 2 and 4 show bands again for both GP5 and M with an MOI of 3 for both GP5 and M.

PRODUCTION OF VLPs

TriEx SF9 cells (75×10^6 cells/ 50 mL) were co-infected with recombinant baculovirus containing PRRSV M at an MOI of 3 and recombinant baculovirus containing PRRSV N, GP5, and E at an MOI of 2 for a large-scale production of VLPs. Western blot analysis was performed to confirm the expression of PRRSV proteins (**Figure 2.6**), and transmission electron microscopy (TEM) was performed to confirm the production of VLPs (**Figure 2.7**). The negative control for TEM was TriEx Sf9 cells that had been grown at same conditions, but were not co-infected. All desired proteins are expressed; GP5 is seen around 25 kDa, M at 18-19 kDa, N at 15 kDa, and E is seen around 10 kDa. Again, bands can be seen above the protein bands, which could possibly be aggregates of heterodimers or homodimers of structural proteins. VLPs that are similar in shape (spherical or oval) and size (approximately 50nm in diameter) were seen on TEM.

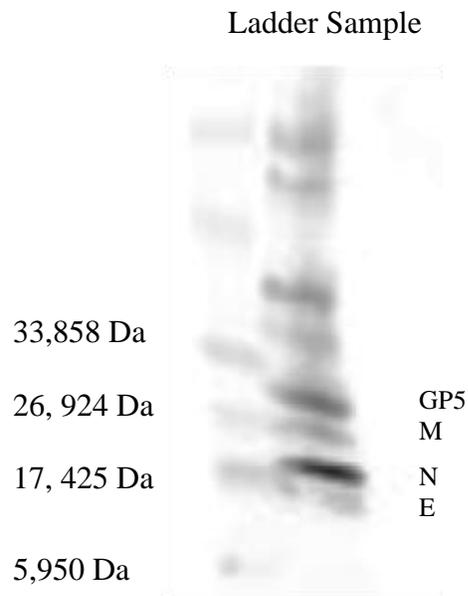


Figure 2.6. Expression of four PRRSV proteins after co-infection. Under optimal conditions, all four proteins of interest, PRRSV M, N, GP5, and E are expressed.

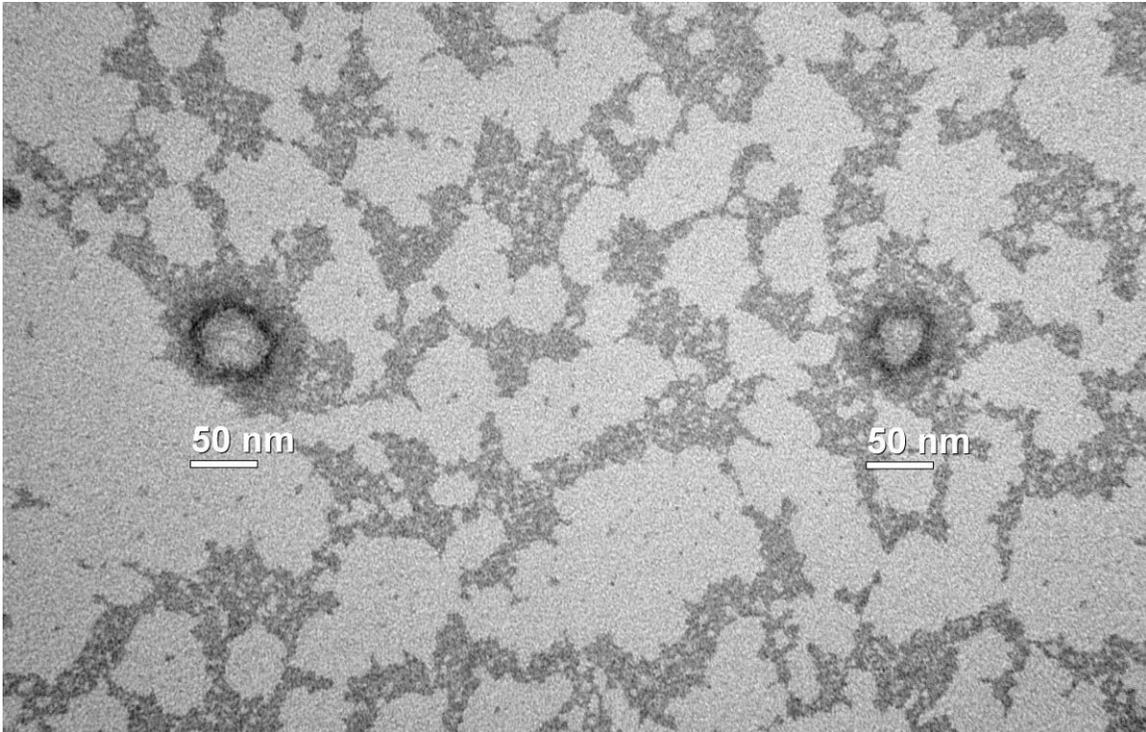


Figure 2.7 TEM of VLPs. VLPs were seen to be produced in a similar shape (spherical or oval) and size (approximately 50 nm in diameter) after co-infection of TriEx Sf9 cells with recombinant baculoviruses containing PRRSV structural proteins M, N, GP5, and E.

DISCUSSION

The use of virus-like particles (VLP) in vaccines and research has been well established in both humans and animal viruses (Noad, R., Roy, P. 2003). VLPs are generated by the use of expression systems, including recombinant baculovirus expression in insect cells, bacteria, yeast, mammalian, or plant cells, that overexpress one or several structural proteins (non-infectious proteins) of the target virus, and spontaneously self-assemble into a structure similar to the desired virion (Noad, R., Roy, P. 2003, Crisci, E., Barcena, J., et al. 2012). Bluetongue virus (BTV), canine parvovirus (CPV), Ebola virus, and porcine circovirus type 2 (PCV2) have utilized recombinant

baculovirus expression systems in the production of VLPs, and have significant potential for the use in vaccines, with Porcilis PCV® for PCV2 currently licensed and commercially available as a VLP-based vaccine (Harro, C.D., Pang, Y.S., et al. 2001 Noad, R., Roy, P. 2003, Ye, L., Lin, J., et al. 2006, Crisci, E., Barcena, J., et al. 2012). The four major structural proteins of BTV, VP2, VP3, VP4, and VP5, are overexpressed using the recombinant baculovirus expression system for the formation of VLPs, while the VLPs produced for CPV are assembled from just one structural protein, VP2, in insect cells (Harro, C.D., Pang, Y.S., et al. 2001 Noad, R., Roy, P. 2003). Being able to understand virus particle assembly is also an important aspect of VLPs, with an example of Norwalk virus that cannot be grown in cell culture (Roy, P., Noad, R. 2008). Our previous unpublished studies have shown that the Membrane (M) and Nucleocapsid (N) proteins of PRRSV are able to assemble into VLPs when expressed both separately or in combination using the recombinant baculovirus expression system. The exploration of other structural proteins of PRRSV, including Glycoprotein 4 (GP4), Envelope (E), and Glycoprotein 5 (GP5), which contains the major neutralizing epitope for PRRSV, for the production of VLPs has yet to be examined. The objectives of this study were to construct plasmids containing the GP4, GP5, and E genes of PRRSV that can be expressed in both mammalian and insect cells, to construct recombinant baculoviruses that express GP4, GP5, and E proteins of PRRSV for the purification of PRRSV VLPs, and produce VLPs similar in size and shape to PRRSV virions by expressing M, N, GP4, GP5, and E proteins simultaneously in insect cells.

We were able to isolate PRRSV GP4, GP5, and E genes through PCR by using constructed primers specific for each gene. During second round PCR, the HA stop tag

was added to the C-terminus of each gene of interest for future identification of protein expression. There are currently no monoclonal antibodies for these PRRSV specific proteins, so the HA stop tag was incorporated. Once we confirmed isolation through gel electrophoresis, we inserted the genes of choice into the pCAGEN plasmid, which is ideal for transfection of mammalian cells. The pCAGEN-GP4, pCAGEN-GP5, and pCAGEN-E were transfected into competent *E. coli* cells and insertion was confirmed by the presence of colony growth on LB plates with ampicillin. Bacteria that grew contained the complete plasmid with the gene insertion making them resistant to ampicillin. No growth on the plate containing the cut pCAGEN transfected cells was our negative control showing susceptibility to ampicillin.

PRRSV GP5 and E proteins were expressed in cos-1 mammalian cells, as shown in Figure 2.1. We first needed to show the expression of PRRSV proteins in mammalian cells due to PRRSV being a pig (mammal) virus and cos-1 cells have been shown to have a high efficiency for transfection. We did not observe the expression of PRRSV GP4. This could be due to the possible interaction of the added HA stop tag at the C-terminal of GP4 (Du, Y., Pattnaik, A.K., et al., 2012). The C-terminal of GP4 contains a hydrophobic domain that acts as the anchor of the GP4 protein to the ER membrane, where protein synthesis takes place (Du, Y., Pattnaik, A.K., et al., 2012).

For the production of VLPs, we choose the recombinant baculovirus expression system, which has been proven effective at over expressing multiple virus proteins, and was effective in previous studies with PRRSV M and N proteins (Stewart, M., Dovas, C.I., et al. 2012, Noad, R., Roy, P. 2003). We showed the insertion of the genes of choice into the pOET1 plasmid by growth of colonies on LB plates with ampicillin. They

showed resistance to ampicillin, while an unsuccessful transfection of cut pOET1 would have showed no growth or susceptibility to ampicillin. The pOET1 plasmid was a suitable transfer vector for the flashBAC system. Recombinant baculoviruses containing PRRSV GP5 and E were both successfully produced and confirmed through IFA (**Figure 2.2**).

PRRSV N and E proteins showed the highest levels of expression at a MOI of 2 and 72 hours post co-infection with recombinant baculoviruses N, E, and M (**Figure 2.4**). PRRSV M showed the highest level of expression at a MOI of 3 and 72 hours post co-infection with recombinant baculovirus GP5 (**Figure 2.5**). PRRSV GP5 showed the highest level of expression at MOI of 1-3 at 2 hours post co-infection with recombinant baculovirus M (**Figure 2.5**), and therefore we used an MOI of 2 throughout the experiments. It is known that PRRSV N protein is the most abundant protein expressed in PRRSV infected cells, while M, E, and GP5 proteins have known interactions with other structural proteins, M and GP5 have been established to form heterodimers that are important for viral attachment (Spilman, M.S., Welbon, C., et 2009, Dokland, T. 2010, Gagnon, C.A., et al. 2000, Das, P.B., Dinh, P.X., et al. 2009). This information gives us the insight to why the N protein band is consistently stronger than the bands for M, GP5 or E proteins during co-infection, as well as to the unknown bands seen in Figure 2.4 and 2.5. These results showed the optimal MOI and incubation period for expression of each of our proteins of interest to up-scale our VLP production.

Our experiments showed that all four PRRSV proteins of interest are expressed 72 hours after co-infection with recombinant baculoviruses containing PRRSV N, M, GP5, and E (**Figure 2.6**). The heterodimer formed between M and GP5 is important in viral

attachment and entry into host cells, while the E protein has been shown in the uncoating of PRRSV once internalized in host cells (Van Breedam, W., Van Gorp, H., et al 2010). N protein is the most immunogenic protein of PRRSV, and GP5 is important in the host's production of neutralizing antibodies (Mateu, E., Diaz, I. 2008). Based on this information and previous studies with PRRSV M and N proteins, a stable VLP similar in size and shape of PRRSV; PRRSV virion structure is spherical or oval in shape and approximately 50nm in diameter, should be produced by the overexpression of these four proteins and become an ideal vaccine candidate for PRRSV (Dokland, T. 2010). We were able to confirm this with the help of Karen Bently, MS and the EM core facility of the University of Rochester Medical Center in Rochester, NY (**Figure 2.7**).

In conclusion, PRRSV GP5 and E are readily expressed in cos-1 mammalian cells while PRRSV GP4 was not. PRRSV GP5 and E are also expressed in SF9 insect cells as part of recombinant baculovirus DNA through the flashBAC system. Combined with previous experiments, PRRSV M, N, GP5, and E can be overexpressed in TriEx SF9 insect cells when co-infected. We can also conclude that VLPs are produced in a similar size and shape to the PRRSV virion by TEM. Future studies include purification of produced VLPs to test the immunogenicity and protection efficacy of the PRRSV VLPs. More experiments are also needed to examine the nature of PRRSV GP4 and its expression in mammalian cells. Studies to look at the production of VLPs with GP5 or E alone and different combinations of structural proteins would also be useful for understanding which combination of structural proteins produce the most VLPs similar in shape and size to the PRRSV virion.

CHAPTER 3: SUMMARY AND CONCLUSIONS

Virus-like particles (VLPs) have been produced for several viruses, including Ebola virus, porcine circovirus type 2 (PCV2), bluetongue virus (BTV), and canine parvovirus (CPV), using the recombinant baculovirus expression system in insect cells (Stewart, M., Dovas, C.I., et al. 2012, Noad, R., Roy, P. 2003, Ye, L., Lin, J., et al. 2006, Crisci, E., Barcena, J., et al. 2012). VLPs have been shown to be ideal candidates for vaccines due to their lack of viral DNA or RNA and similarities in viral capsid shape and size, resulting in a safe, effective, and differentiable immunogens (Crisci, E., Barcena, J., et al 2012). Licensed and commercially available is the VLP-based vaccine Porcilis PCV® for PCV2, which is produced using the recombinant baculovirus expression system and has been proven safe, highly immunogenic, and effective against different PCV genotypes (Crisci, E., Barcena, J., et al. 2012). Currently, the vaccines available for PRRSV have proven ineffective, unsafe, and costly (Kimman, T.G., Cornelissen, L.A., et al. 2009). Previous studies our lab conducted showed PRRSV membrane (M) and nucleocapsid (N) proteins are able to produce VLPs alone or in combination with each other using the recombinant baculovirus expression system.

In this study, we demonstrated that PRRSV Glycoprotein-5 (GP5), Envelope (E), M and N proteins are expressed in TriEx SF9 cells when co-infected with recombinant baculoviruses containing PRRSV GP5, E, M, and N genes. We found the highest level of protein expression to be at 72 hours and 96 hours post co-infection, with a MOI of 2 for GP5, E, and N proteins, and a MOI of 3 for M protein.

We were unable to incorporate the GP4 protein in our studies, which has known interactions with GP5 and aids in viral attachment leading to viral entry. It seems that the

GP4 protein needs its intact C-terminal to anchor to the host's ER membrane for protein synthesis (Du, Y., Pattnaik, A.K., et al. 2012). The added HA stop tag, which is needed to identify desired proteins, may have been involved in the elimination of GP4 from our experiments. At this time, there are not commercially available monoclonal antibodies to each of these proteins, so the HA stop tag is essential for identification. Therefore further experiments that include moving the position of the added HA stop tag, as well as the removal of the HA stop tag, would be necessary to explore the reasoning for GP4 to not be expressed in our experiments with mammalian cells.

Based on previous studies of PRRSV M and N proteins, we would expect to see VLPs produced from the co-infection of PRRSV recombinant baculovirus GP5, E, M, and N proteins that are similar in shape and size to PRRSV virions; figure 2.7 shows this. More experiments would be needed to determine if different combinations of PRRSV structural proteins or GP5 or E alone would be the optimal combination for VLP production. With the immunogenic nature of the N and GP5 proteins, along with the interactions of E, GP5, and M and their attachment and entry properties, we would expect to produce highly immunogenic VLPs that would be safe for the use in a vaccine (Mateu, E., Diaz, I. 2008).

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