Virus like particles as a vaccine against porcine reproductive and respiratory syndrome virus (PRRSV)

By

Ambika Mosale Venkatesh Murthy

Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Master of Science
In
Biological Systems Engineering

Chenming Zhang
Ryan S. Senger
Xiang-Jin Meng

April 26, 2013
Blacksburg, VA

Keywords: Porcine reproductive and respiratory syndrome virus, PRRSV, vaccine, VLP, inclusion bodies.

Virus like particles as a vaccine against porcine reproductive and respiratory syndrome virus (PRRSV)

Ambika Mosale Venkatesh Murthy

Abstract

Porcine reproductive and respiratory syndrome (PRRS) is the most significant infectious disease currently affecting the swine industry worldwide. Several inactivated and modified live vaccines (MLV) have been developed to curb PRRSV infections. The unsatisfactory efficacy and safety of these vaccines, drives for the development of new generation PRRS universal vaccines. Virus like particles (VLPs) based vaccines are gaining increasing acceptance compared to subunit vaccines, as they present the antigens in more veritable conformation and are even readily recognized by the immune system. Hepatitis B virus (HBV) core antigen (HBcAg) is very well studied and has been successfully used as a carrier for more than 100 other viral sequences. In this study, hybrid HBcAg VLPs are generated by fusion of the conserved protective epitopes of PRRSV and expressed in *E. coli*. An optimized purification protocol that overcomes issues from ultracentrifugation is developed to obtain hybrid HBcAg VLP protein from the inclusion bodies. This hybrid HBcAg VLP protein self assembled to 23nm VLPs that were shown to block virus infection of susceptible cells when tested on MARC 145 cells. Therefore, the safety of non-infectious and non-replicable VLPs and production through low-cost *E. coli* fermentation may make this vaccine competitive against current vaccines on both efficacy and cost.

Dedication

Dedicated to my parents, Rajalakshmi and Venkatesh Murthy

Acknowledgement

It is with immense gratitude that I acknowledge the support and help of my advisor, Dr Chenming Zhang. He continuously and convincingly conveyed a spirit of adventure in regard to research and scholarship. Without his guidance and constant help, this thesis would not have been possible. I would like to thank my committee members, Dr Ryan S Senger and Dr X.J.Meng for their encouragement and support.

In addition, I would like to thank Kathy Lowe for her help with electron microscopy. I thank Yanyan Ni for providing MARC 145 cells and Amy Egan for all her technical help. I am indebted to my colleagues Dr Jianzhong Hu, Dr Hong Zheng, Wei Huang, and Yun Hu for their constant support. Last but not the least, I would like to thank my parents and my family members for their constant support.

Attribution

Some colleagues helped with the research and writing behind part of one of my chapters of this thesis.

In Chapter 3, virus blocking assay experiment.

Yanyan Ni is currently a doctoral student in Biomedical and Veterinary Sciences at Virginia Tech, helped me with providing MARC 145 cell.

X.J.Meng is currently a professor in College of Veterinary Medicine, Virginia Tech, contributed editorial comments.

Contents

Figures

Chapter I: Introduction

Introduction

Porcine reproductive and respiratory syndrome (PRRS), also known as blue-ear pig disease, emerged as a disastrous disease in late 1980's. It was initially observed in the herds in North Carolina, which showed symptoms like severe reproductive losses, extensive post- weaning pneumonia, reduction of growth performance and increased mortality. As the causative agent was unknown, it was even referred as "Mystery Swine Disease" (MSD). Immense efforts were undertaken to identify MSD causing agent, as swine was the major source of animal protein in human diet (KK, 1989). Later in 1991, the cause of MSD was elucidated when Koch's postulates were fulfilled with a previously unrecognized, enveloped RNA virus (Terpstra et al., 1991). The first virus isolates were entitled in the Netherlands and U.S. as Lelystad virus and Swine Infertility and Respiratory Syndrome (SIRS) virus (BIAH-001), respectively. These isolates induced reproductive failure and respiratory signs under experimental conditions (Collins et al., 1992; Terpstra et al., 1991). This virus is now commonly addressed as porcine reproductive and respiratory syndrome virus (PRRSV) across the globe.

PRRSV is single stranded enveloped, RNA virus of order *Nidovirales* and family *Artiviridae.* PRRSV can be grouped into 2 genotypes, namely European (Type 1) and North American (Type 2)(Meng et al., 1996). At genomic sequence level they differ approximately by 40 % and they differ serologically. In the beginning, the isolates from both the genotypes were homogeneous. Over two decades later, genetically diverge, more virulent strains started appearing. The high rate of evolution $3.29x10^{3}$ substitutions per nucleotide site per year

causes new strains to frequently appear in the field. Genetically divergent strains are very difficult to control by the existing vaccines.

PRRSV has a genome size of about 15kb, where genomic RNA is capped and polyadenylated. It comprises of 10 Open Reading Frames (ORF1a, ORF1b, ORF2, ORF2a, ORF3, ORF4, ORF5a, and ORF5 - ORF7). ORF1a and 1b account to about 75% of viral genome and are translated into two large polyproteins by ribosomal frameshifting (Snijder and Meulenberg, 1998). These proteins are cleaved into 14 non-structural proteins (nsp), either co- or post translationally by four putative viral proteases. These non-structural proteins are involved in myriad functions like proteases (nsp1α, nsp1β, nsp2, nsp4), RNA dependent RNA polymerase (nsp9), helicase (nsp10), endoribonuclease (nsp11), subgenomic mRNA transcription (nsp1) and transmembrane proteins (nsp3 and nsp5) (Fang and Snijder, 2010). ORF2a, ORF3, ORF4 and ORF5 encode membrane associated N-glycosylated structural proteins GP2a, GP3, GP4 and GP5 respectively. Recently, ORF5a was discovered, which encodes ORF5a protein comprising 51 amino acids, of unknown function (Johnson et al., 2011). ORF2b and ORF6 encode E and M proteins respectively, which are non-glycosylated membrane protein. Nucleocapsid protein N is encoded by ORF7. GP5 and M proteins forms heterodimeric complexes via disulfide bonds in PRRSV infected cells and are indispensable for the formation of PRRSV particles. GP5 and M proteins interacts with cellular receptors, sialoadhesin and heparan sulfate respectively and are associated with virus attachment and internalization (Delputte et al., 2002; Dokland, 2010; Van Breedam et al., 2010a; Van Breedam et al., 2010b). GP4 along with GP2a interacts with CD163 and is involved in viral uncoating and release of genomic RNA (Das et al., 2010; Tian et al., 2009). These structural proteins are translated from six subgenomic mRNAs, which are capped and

polyadenylated and are synthesized as a 3'- coterminal nested set of mRNA's, which has a common leader sequence at the 5' end (Snijder and Meulenberg, 1998).

Several inactivated and modified live vaccines (MLV) have been developed to curb PRRSV infections. Inactivated PRRSV vaccines are considered ineffective, as they fail to prevent clinical signs and viremia caused by virus challenge even with homologous strains (Kimman et al., 2009; Zuckermann et al., 2007). Although MLV PRRSV vaccines are regarded more effective when compared to inactivated vaccines in reduction of clinical signs associated with PRRSV, it has several disadvantages. First, it confers partial protection against heterologous strains. Second, they were unstable, which resulted in intrinsic risk of reversion of the vaccine virus to virulence under farm conditions. Third, vaccinated pigs shed infective virus, which demands vaccination of all pigs in the pen at the same time (Botner et al., 1997; Madsen et al., 1998; Oleksiewicz et al., 1998). Therefore, development of new generation PRRSV vaccines vaccine that confers protection against PRRS field virus is more challenging.

Virus like particles (VLP s) are expedient candidate for vaccine design, as they mimic the 3D structure of native viruses and are devoid of viral genome. They are known to stimulate B cell mediated response, CD4 cell proliferating response and cytotoxic T Lymphocyte (CTL) responses. They cross-link the B-cell receptors and efficiently reach the MHC class I pathway (Bachmann and Jennings, 2010). They can even target dendritic cells (DC), which are essential to invoke both humoral and cell mediated immunity, making VLP based vaccine more attractive (Aguilar and Rodriguez, 2007).Hepatitis B virus (HBV) core antigen (HBcAg) VLP s were the first to be used as a epitope carrier, which displayed epitopes from

the foot-and-mouth disease virus (*Picornaviridae*) (Milich et al., 1987). HBcAg is highly immunogenic and flexible carrier of foreign epitopes.

The long term goal of this project is to develop a universal vaccine towards PRRSV. In order to accomplish this goal, hybrid HBcAg VLPs were generated by fusion of the conserved protective epitopes of PRRS and these VLPs were tested on MARC 145 to check its efficiency in blocking PRRSV.

References

1. Aguilar, J. C. and E. G. Rodriguez (2007). "Vaccine adjuvants revisited." Vaccine **25**(19): 3752-3762.
2. Bachmann, M. F. and G. T. Jennings (2010). "Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns." Nat Rev Immunol **10**(11): 787-796.
3. Botner, A., B. Strandbygaard, K. J. Sorensen, P. Have, K. G. Madsen, E. S. Madsen and S. Alexandersen (1997). "Appearance of acute PRRS-like symptoms in sow herds after vaccination with a modified live PRRS vaccine." Vet Rec **141**(19): 497-499.
4. Collins, J. E., D. A. Benfield, W. T. Christianson, L. Harris, J. C. Hennings, D. P. Shaw, S. M. Goyal, S. McCullough, R. B. Morrison, H. S. Joo and et al. (1992). "Isolation of swine infertility and respiratory syndrome virus (isolate ATCC VR-2332) in North America and experimental reproduction of the disease in gnotobiotic pigs." J Vet Diagn Invest **4**(2): 117-126.
5. Das, P. B., P. X. Dinh, I. H. Ansari, M. de Lima, F. A. Osorio and A. K. Pattnaik (2010). "The minor envelope glycoproteins GP2a and GP4 of porcine reproductive and respiratory syndrome virus interact with the receptor CD163." J Virol **84**(4): 1731-1740.
6. Delputte, P. L., N. Vanderheijden, H. J. Nauwynck and M. B. Pensaert (2002). "Involvement of the matrix protein in attachment of porcine reproductive and

respiratory syndrome virus to a heparinlike receptor on porcine alveolar macrophages." J Virol **76**(9): 4312-4320.

7. Dokland, T. (2010). "The structural biology of PRRSV." Virus Res **154**(1-2): 86-97.
8. Fang, Y. and E. J. Snijder (2010). "The PRRSV replicase: exploring the multifunctionality of an intriguing set of nonstructural proteins." Virus Res **154**(1-2): 61-76.
9. Johnson, C. R., T. F. Griggs, J. Gnanandarajah and M. P. Murtaugh (2011). "Novel structural protein in porcine reproductive and respiratory syndrome virus encoded by an alternative ORF5 present in all arteriviruses." J Gen Virol **92**(Pt 5): 1107-1116.
10. Kimman, T. G., L. A. Cornelissen, R. J. Moormann, J. M. Rebel and N. Stockhofe-Zurwieden (2009). "Challenges for porcine reproductive and respiratory syndrome virus (PRRSV) vaccinology." Vaccine **27**(28): 3704-3718.
11. KK, K. (1989). "Reproductive failure of unknown etiology." Swine Pract Newsl **1**: 1-9.
12. Madsen, K. G., C. M. Hansen, E. S. Madsen, B. Strandbygaard, A. Botner and K. J. Sorensen (1998). "Sequence analysis of porcine reproductive and respiratory syndrome virus of the American type collected from Danish swine herds." Arch Virol **143**(9): 1683-1700.
13. Meng, X. J., P. S. Paul, P. G. Halbur and M. A. Lum (1996). "Characterization of a high-virulence US isolate of porcine reproductive and respiratory syndrome virus in a continuous cell line, ATCC CRL11171." J Vet Diagn Invest **8**(3): 374-381.
14. Milich, D. R., A. McLachlan, G. B. Thornton and J. L. Hughes (1987). "Antibody production to the nucleocapsid and envelope of the hepatitis B virus primed by a single synthetic T cell site." Nature **329**(6139): 547-549.
15. Oleksiewicz, M. B., A. Botner, K. G. Madsen and T. Storgaard (1998). "Sensitive detection and typing of porcine reproductive and respiratory syndrome virus by RT-PCR amplification of whole viral genes." Vet Microbiol **64**(1): 7-22.
16. Snijder, E. J. and J. J. Meulenberg (1998). "The molecular biology of arteriviruses." J Gen Virol **79 (Pt 5)**: 961-979.
17. Terpstra, C., G. Wensvoort and J. M. Pol (1991). "Experimental reproduction of porcine epidemic abortion and respiratory syndrome (mystery swine disease) by infection with Lelystad virus: Koch's postulates fulfilled." Vet Q **13**(3): 131-136.
18. Tian, Z. J., T. Q. An, Y. J. Zhou, J. M. Peng, S. P. Hu, T. C. Wei, Y. F. Jiang, Y. Xiao and G. Z. Tong (2009). "An attenuated live vaccine based on highly pathogenic

porcine reproductive and respiratory syndrome virus (HP-PRRSV) protects piglets against HP-PRRS." Vet Microbiol **138**(1-2): 34-40.

19. Van Breedam, W., P. L. Delputte, H. Van Gorp, G. Misinzo, N. Vanderheijden, X. Duan and H. J. Nauwynck (2010). "Porcine reproductive and respiratory syndrome virus entry into the porcine macrophage." J Gen Virol **91**(Pt 7): 1659-1667.
20. Van Breedam, W., H. Van Gorp, J. Q. Zhang, P. R. Crocker, P. L. Delputte and H. J. Nauwynck (2010). "The M/GP(5) glycoprotein complex of porcine reproductive and respiratory syndrome virus binds the sialoadhesin receptor in a sialic acid-dependent manner." PLoS Pathog **6**(1): e1000730.
21. Zuckermann, F. A., E. A. Garcia, I. D. Luque, J. Christopher-Hennings, A. Doster, M. Brito and F. Osorio (2007). "Assessment of the efficacy of commercial porcine reproductive and respiratory syndrome virus (PRRSV) vaccines based on measurement of serologic response, frequency of gamma-IFN-producing cells and virological parameters of protection upon challenge." Vet Microbiol **123**(1-3): 69-85.

Chapter II: Literature Review

Porcine reproductive and respiratory syndrome virus (PRRSV) vaccines

PRRS has depraved swine industry, since its discovery in late 1980s. In the United States alone, this disease accounts for economic losses of more than 560 million dollars annually (Neumann et al., 2005). Currently, two types of commercial vaccines are available against PRRSV, namely, inactivated vaccines and modified live vaccines (MLV). Commercial inactivated vaccine under trademark PRRomiSe ™, Intervet was once available in US, but was discontinued since 2005. Nevertheless, inactivated vaccines are ineffective even against homologous strains (Kimman et al., 2009; Zuckermann et al., 2007). Several MLV vaccines, such as Ingelvac® PRRS MLV, ReproCyc® PRRS-PLE are licensed for use in United States. PRRSV MLV vaccines have myriad concerns with respect to efficacy and safety. First, vaccine protection is strain-specific rather than genotype-specific. Second, these vaccines render delayed protection, which is seen only after 3-4 weeks of vaccination(Zuckermann et al., 2007). Third, there is reversion of PRRSV vaccine to virulence (Botner et al., 1997; Storgaard et al., 1999), which of safety concern. Therefore, these efficacy and safety issues make PRRS vaccine development the topic of interest.

Experimental vaccines

DNA vaccine

DNA vaccine relies on reverse transcription of PRRSV RNA into cDNA with some genetic manipulation. This allows the host itself to produce viral proteins and present to immune system in a manner similar to that of natural infection. A plasmid containing GP5 under cytomegalovirus (CMV) promoter induced virus neutralizing antibody sufficient to protect animals from generalized viremia and macroscopic lung lesions (Pirzadeh and Dea, 1998).

GP5 was modified by insertion of Pan Dr T-helper cell epitope (PADRE) between the putative neutralizing epitope and a decoy epitope, which enhanced immunogenicity (Fang et al., 2006). Mutation of four potential N-glycosylation sites in GP5, rendered significantly immunogenic GP5 based DNA vaccine (Li et al., 2009a). Later a DNA vaccine expressing GP5 and M proteins was developed. It was shown that virus neutralizing antibody response and lymphocyte proliferation responses were increased when GP5/M heterodimers were formed, rather than GP5 or M protein alone (Jiang et al., 2006b). GP5 gene was codon optimized and was expressed along with swine ubiquitin. This improved efficacy, when compared to other vaccines, reduced viremia levels significantly (Hou et al., 2008). DNA vaccines encoding either ORF4, ORF5, ORF6, or ORF7 were able to elicit both humoral and cell mediated immune response in pigs (Kwang et al., 1999). Lack of sufficient protection, complexity, cost and the labor are the major drawbacks of DNA vaccine strategy.

PRRSV recombinant vector vaccines

Viral and bacterial vectors were used to express a gene or group of genes from PRRSV. Vectors were either replicating or non replicating, which infected host to facilitate PRRSV gene expression. In some cases the vector itself is able to express the PRRSV genes outside of any assistance from the host while other vectors require assistance from the host to express the proteins of interest. The two major advantages of this strategy are the safety of the vector and its inherent ability to be differentiated from a wild type exposure. The availability of a universally protective PRRSV antigen that could be expressed in a vector system would illustrate the potential usefulness of this strategy

Bacterial vectors

A recombinant *Mycobacterium bovis* BCG (BCG) that expressed truncated PRRSV GP5 and the entire PRRSV M protein was generated (Bastos et al., 2002). These proteins were expressed in such a way, that they were located either in cytoplasm or on surface of BCG. These BCG constructs were successful in eliciting antibodies in mice. Later, the same BCG vector vaccine was tested in pig, conferred partial protection from virulent challenge with a reduction in viremia, pyrexia and viral load in bronchial lymph nodes but did not prevent these parameters (Bastos et al., 2004). Attenuated live *Salmonella typhimurium aroA* isolate was transformed with a PRRSV GP5 DNA vaccine vector, which showed significant level of antibody titer when injected to mice (Jiang et al., 2004). Although good expression of viral protein was achieved using prokaryotic expression vectors, it lacked glycosylation, which could pose problem in antigen recognition by host immune system.

Pseudorabies virus vectors

Recombinant adenoviruses expressing the PRRSV GP5 and M proteins were tested individually or in combination in mice (Jiang et al., 2006a). The virus co-expressing the Gp5 and M proteins provided a superior response, as indicated by significantly higher virus neutralizing antibody titers and stronger lymphocyte proliferation responses. Adenoviruses were even used to express GP3 and truncated GP3, termed as modified GP3. Modified GP3 provided enhanced immunogenicity when compared to that of unmodified GP3, when tested in mice (Jiang et al., 2007). Adenovirus comprising GP3, GP4 and GP5 gene fusions of varying combinations were tested on mice for neutralizing antibody production and cell-mediated immune responses. Viral vectors containing fusion proteins imparted higher response when compared to that of individual genes (Jiang et al., 2008). Both PRRSV GP3

and GP5 proteins were fused to the *Haemophilus parasuis* heat shock protein 70 (HSP70) with different linkers and expressed in adenoviruses (Li et al., 2009b). These vaccines, upon injection into pigs, provided partial protection, but there was no prevention of clinical signs, lung lesions and viremia when compared to the unvaccinated animals. Adenoviruses expressing either PRRSV GP3, GP5 alone or with co-expressed swine granulocyte-macrophage colony stimulating factor (GM-CSF) were evaluated for protection from the PRRS (Wang et al., 2009). The level of protection was superior in the group administrated with vaccines co-expressing PRRSV GP3, GP5 and the GM-CSF, with significantly decreased lung lesions and clinical signs.

Other viral vectors

A couple of other viral vectors were used as a prototype vector vaccine for PRRSV. PRRSV ORF2 gene was expressed in vaccinia virus and injected into piglets, which resulted in significant virus neutralizing antibody titer (Rogan, 2000) . Mice immunization studies were carried out using recombinant vaccinia virus constructs, expressing GP5 and M proteins in different arrangements. Vector that expressed these two proteins under different promoters resulted in the greatest virus neutralizing antibody response and cellular immune response (Zheng et al., 2007). PRRSV ORF5 was expressed in transmissible gastroenteritis coronavirus (TGEV) minigenome, which upon vaccination induced significant humoral immune response (Alonso et al., 2002). Semliki forest virus (SFV) expression system was used for PRRSV ORF5 antigen production, with an objective of using the recombinant virus particles in a vaccine prototype (Meulenberg and Petersen-den Besten, 1996; Meulenberg et al., 1997). PRRSV GP3 and GP5 proteins either with or without porcine IL-18 were co expressed in fowlpox viruses (Shen et al., 2007). This provided some protection after a

virulent PRRSV challenge with the same isolate which was used to generate the recombinant vector vaccine. Reduction in viremia and fewer virus isolations from the bronchial lymph nodes in the vaccinated animals were seen when compared to the control animals. Modified recombinant baculovirus gene delivery vector was used to express both PRRSV GP5 and M proteins under the control of independent CMV and vaccinated into mice (Wang et al., 2007). There was enhanced production of VN antibodies and a significantly increased level of INF-γ production, even at its lowest dose. This depicted that baculovirus vector vaccine surpassed DNA vaccine encoding the same antigens. Killed TGEV vector prototype vaccine that expressed the PRRSV ORF5 and ORF6 gene products was able to induce only partial protection from challenge as illustrated by reduced viremia and faster antibody production in the vaccinated animals, but interstitial pneumonia was still detected in the vaccinated animals.

Plant based PRRSV subunit vaccines

Plant expression systems offers myriad advantages for vaccine production such as low cost of production, ease of storage and transportation, reduced risk associated with mammalian viral contamination and no needle associated injury risk. It was known that mucosa of respiratory and reproductive tracts were the major route of PRRSV infection (Van Reeth, 1997). Therefore, it was recommended to activate common mucosal immunity from the first line of defense. Transgenic tobacco plant that expressed GP5 of PRRSV was the first oral transgenic plant based PRRSV vaccines (Chia et al., 2010). Pigs fed with the transgenic leaves, developed specific mucosal and even systemic humoral and cell mediated immune responses against PRRSV. However the neutralizing antibody titers were low, which might be due to the low GP5 expression level, which was 0.011% of the total soluble protein. In order to enhance the immune response, GP5 was fused with a mucosal adjuvant, heat-labile

enterotoxin B subunit (LTB) from *E.coli* and expressed in tobacco leaves (Chia et al., 2011). Pigs fed with these transgenic tobacco leaves exhibited added immune response, when compared to that of GP5 alone. However tobacco farming has been forbidden in many countries, as they contain nicotine and other harmful chemicals. In order to subdue this, corn and potato were regarded as more suitable for PRRSV oral vaccine production. PRRSV GP5 was expressed in potato and was orally administered into mice, which generated both serum and gut mucosal specific antibodies, but low levels of neutralizing antibodies (Chen and Liu, 2011). PRRSV M protein was expressed in corn and was fed into mice, which induced serum and intestine mucosal antigen-specific antibodies with neutralization activity. The transgenic corn even produced cellular immune responses and in specific IFN-γ, which is regarded as crucial for protection against PRRSV (Hu et al., 2012).

Approaches to universal PRRSV vaccine

PRRSV's ability to escape or suppress the host innate and adaptive immune response make the development of vaccine against them very challenging. Efficacy, universality, safety and ability to differentiate vaccinated from infected animals are regarded as necessities for a vaccine to eradicate PRRSV.

Multivalent vaccines

Multivalent vaccines, which are designed to immunize against two or more distinct strains, is a promising procedure to universal vaccine development. As not much is known about PRRSV complementing strains, it is yet to be examined whether universal multivalent PRRSV vaccine can be designed (Kimman et al., 2009). It was reported that single vaccine, which comprised five attenuated strains of PRRSV only provided partial protection, as that of

single strain virus (Mengeling et al., 2003). Recently, a method based on graph theory that quantifies the relative importance of viral variants was demonstrated. This method depends on network structure of PRRSV, where it identifies subsets of viral variants that are most important to overall viral diversity. This method yielded 22 sequences that were represented in top 100 sequences. Out of these, one had 48% of putative epitopes derived from the known diversity of PRRSV (Anderson et al., 2012). This approach makes minimal assumptions and is very useful in development of multi strain vaccines against PRRSV.

Conserved region vaccine

Immunity against one PRRSV strain does not confer protective immunity against different strain. This necessitates the PRRSV vaccines to display immunogens from the most conserved regions or the different conserved regions across viral proteome. It is known that GP5 T- and B- epitopes could elicit T-cell responses and neutralizing antibodies against all, or at least a majority of PRRSV strains.

This method was highly supportive to develop universal vaccines against influenza and HIV viruses, which are highly virulent and known for its genetic variability. Universal Influenza A virus vaccine was developed using Hepatitis B virus core protein, which displayed the conserved extracellular domain of the M2 protein (23 amino acids) (Neirynck et al., 1999). This upon mice administration conferred 90% to 100% protection against lethal virus confrontation. In case of HIV multi-clade immunogen was derived from highly conserved regions of the HIV-1 consensus proteome, which comprised the four HIV-1 clades (A, B, C and D). The gene coding for the HIV_{CONSV} protein was expressed in the three most studied vaccine vectors, plasmid DNA, human adenovirus serotype 5 and modified vaccine virus

Ankara (MVA) (Letourneau et al., 2007). This induced HIV-1-specific T cell responses in mice and these conserved regions prime $CD8^+$ and $CD4^+$ T cell to highly conserved epitopes in humans and also generate memory T cells in patients during natural HIV-1 infection (Letourneau et al., 2007). Therefore identification of such conserved regions plays a major role in development of PRRSV vaccines.

Polyepitope vaccines

Use of epitopes identified from pathogens as target antigens is an important strategy for developing universal vaccines. These highly conserved epitopes are synthesized artificially and are expressed as polypeptides or in DNA or viral vectors. This is of prime importance in PRRS vaccine development as conserved B and T cell epitopes are present within viral proteins.

Plagemann identified neutralization epitope located in the middle of GP5 sequence among North American PRRSV isolates in 2004. This corresponded to B cell epitope and was conserved among North American PRRSV isolates (Plagemann, 2004). Later, two independent epitopes were identified at the N-terminal of GP5. Epitope A was immunodominant and induced a rapid rise of antibodies with no neutralizing activity. Epitope B was conserved and was a neutralizing epitope (Ostrowski et al., 2002). Two more pentadecapeptide (^{117}LAALICFVIRLAKNC^{131} and ^{149}KGRLYRWRSPVIIEK^{163}) spanning GP5 were identified as immunodominant T cell epitopes. These epitopes were regarded as relatively conserved with at most two amino acid variations and were known to elicit a recall interferon-gamma response from peripheral blood mononuclear cells (Vashisht et al., 2008).

Conclusion

Numerous efforts such as use of several adjuvants, use of mixed strains of PRRSV, generation of alternative vaccines like DNA vaccine, subunit vaccine, viral vector vaccine and plant derived vaccines have been made to develop universal vaccine against PRRSV. Development of an ideal PRRS vaccine requires complete understanding on the viral strategies to suppress and evade host innate and adaptive immune responses, virus epitopes responsible for such suppression and the conserved epitopes that can confer broad range protection. Much more efforts are required to broaden these gaps of knowledge and to address these questions in order to develop an ideal PRRS vaccine.

References

1. Alonso, S., Sola, I., Teifke, J.P., Reimann, I., Izeta, A., Balasch, M., Plana-Duran, J., Moormann, R.J., Enjuanes, L., 2002. In vitro and in vivo expression of foreign genes by transmissible gastroenteritis coronavirus-derived minigenomes. The Journal of general virology 83, 567-579.
2. Anderson, T.K., Laegreid, W.W., Cerutti, F., Osorio, F.A., Nelson, E.A., Christopher-Hennings, J., Goldberg, T.L., 2012. Ranking viruses: measures of positional importance within networks define core viruses for rational polyvalent vaccine development. Bioinformatics 28, 1624-1632.
3. Bastos, R.G., Dellagostin, O.A., Barletta, R.G., Doster, A.R., Nelson, E., Osorio, F.A., 2002. Construction and immunogenicity of recombinant Mycobacterium bovis BCG expressing GP5 and M protein of porcine reproductive respiratory syndrome virus. Vaccine 21, 21-29.
4. Bastos, R.G., Dellagostin, O.A., Barletta, R.G., Doster, A.R., Nelson, E., Zuckermann, F., Osorio, F.A., 2004. Immune response of pigs inoculated with Mycobacterium bovis BCG expressing a truncated form of GP5 and M protein of porcine reproductive and respiratory syndrome virus. Vaccine 22, 467-474.

5. Botner, A., Strandbygaard, B., Sorensen, K.J., Have, P., Madsen, K.G., Madsen, E.S., Alexandersen, S., 1997. Appearance of acute PRRS-like symptoms in sow herds after vaccination with a modified live PRRS vaccine. The Veterinary record 141, 497-499.
6. Chen, X., Liu, J., 2011. Generation and immunogenicity of transgenic potato expressing the GP5 protein of porcine reproductive and respiratory syndrome virus. Journal of virological methods 173, 153-158.
7. Chia, M.Y., Hsiao, S.H., Chan, H.T., Do, Y.Y., Huang, P.L., Chang, H.W., Tsai, Y.C., Lin, C.M., Pang, V.F., Jeng, C.R., 2010. Immunogenicity of recombinant GP5 protein of porcine reproductive and respiratory syndrome virus expressed in tobacco plant. Veterinary immunology and immunopathology 135, 234-242.
8. Chia, M.Y., Hsiao, S.H., Chan, H.T., Do, Y.Y., Huang, P.L., Chang, H.W., Tsai, Y.C., Lin, C.M., Pang, V.F., Jeng, C.R., 2011. Evaluation of the immunogenicity of a transgenic tobacco plant expressing the recombinant fusion protein of GP5 of porcine reproductive and respiratory syndrome virus and B subunit of Escherichia coli heat-labile enterotoxin in pigs. Veterinary immunology and immunopathology 140, 215-225.
9. Fang, L., Jiang, Y., Xiao, S., Niu, C., Zhang, H., Chen, H., 2006. Enhanced immunogenicity of the modified GP5 of porcine reproductive and respiratory syndrome virus. Virus genes 32, 5-11.
10. Hou, Y.H., Chen, J., Tong, G.Z., Tian, Z.J., Zhou, Y.J., Li, G.X., Li, X., Peng, J.M., An, T.Q., Yang, H.C., 2008. A recombinant plasmid co-expressing swine ubiquitin and the GP5 encoding-gene of porcine reproductive and respiratory syndrome virus induces protective immunity in piglets. Vaccine 26, 1438-1449.
11. Hu, J., Ni, Y., Dryman, B.A., Meng, X.J., Zhang, C., 2012. Immunogenicity study of plant-made oral subunit vaccine against porcine reproductive and respiratory syndrome virus (PRRSV). Vaccine 30, 2068-2074.
12. Jiang, P., Jiang, W., Li, Y., Wu, S., Xu, J., 2004. Humoral immune response induced by oral administration of S. typhimurium containing a DNA vaccine against porcine reproductive and respiratory syndrome virus. Veterinary immunology and immunopathology 102, 321-328.
13. Jiang, W., Jiang, P., Li, Y., Tang, J., Wang, X., Ma, S., 2006a. Recombinant adenovirus expressing GP5 and M fusion proteins of porcine reproductive and respiratory syndrome virus induce both humoral and cell-mediated immune responses in mice. Veterinary immunology and immunopathology 113, 169-180.

14. Jiang, W., Jiang, P., Li, Y., Wang, X., Du, Y., 2007. Analysis of immunogenicity of minor envelope protein GP3 of porcine reproductive and respiratory syndrome virus in mice. Virus genes 35, 695-704.
15. Jiang, W., Jiang, P., Wang, X., Li, Y., Du, Y., Wang, X., 2008. Enhanced immune responses of mice inoculated recombinant adenoviruses expressing GP5 by fusion with GP3 and/or GP4 of PRRS virus. Virus research 136, 50-57.
16. Jiang, Y., Xiao, S., Fang, L., Yu, X., Song, Y., Niu, C., Chen, H., 2006b. DNA vaccines co-expressing GP5 and M proteins of porcine reproductive and respiratory syndrome virus (PRRSV) display enhanced immunogenicity. Vaccine 24, 2869-2879.
17. Kimman, T.G., Cornelissen, L.A., Moormann, R.J., Rebel, J.M., Stockhofe-Zurwieden, N., 2009. Challenges for porcine reproductive and respiratory syndrome virus (PRRSV) vaccinology. Vaccine 27, 3704-3718.
18. Kwang, J., Zuckermann, F., Ross, G., Yang, S., Osorio, F., Liu, W., Low, S., 1999. Antibody and cellular immune responses of swine following immunisation with plasmid DNA encoding the PRRS virus ORF's 4, 5, 6 and 7. Research in veterinary science 67, 199-201.
19. Letourneau, S., Im, E.J., Mashishi, T., Brereton, C., Bridgeman, A., Yang, H., Dorrell, L., Dong, T., Korber, B., McMichael, A.J., Hanke, T., 2007. Design and pre-clinical evaluation of a universal HIV-1 vaccine. PloS one 2, e984.
20. Li, B., Xiao, S., Wang, Y., Xu, S., Jiang, Y., Chen, H., Fang, L., 2009a. Immunogenicity of the highly pathogenic porcine reproductive and respiratory syndrome virus GP5 protein encoded by a synthetic ORF5 gene. Vaccine 27, 1957-1963.
21. Li, J., Jiang, P., Li, Y., Wang, X., Cao, J., Wang, X., Zeshan, B., 2009b. HSP70 fused with GP3 and GP5 of porcine reproductive and respiratory syndrome virus enhanced the immune responses and protective efficacy against virulent PRRSV challenge in pigs. Vaccine 27, 825-832.
22. Mengeling, W.L., Lager, K.M., Vorwald, A.C., Clouser, D.F., 2003. Comparative safety and efficacy of attenuated single-strain and multi-strain vaccines for porcine reproductive and respiratory syndrome. Veterinary microbiology 93, 25-38.
23. Meulenberg, J.J., Petersen-den Besten, A., 1996. Identification and characterization of a sixth structural protein of Lelystad virus: the glycoprotein GP2 encoded by ORF2 is incorporated in virus particles. Virology 225, 44-51.

24. Meulenberg, J.J., van Nieuwstadt, A.P., van Essen-Zandbergen, A., Langeveld, J.P., 1997. Posttranslational processing and identification of a neutralization domain of the GP4 protein encoded by ORF4 of Lelystad virus. Journal of virology 71, 6061-6067.
25. Neirynck, S., Deroo, T., Saelens, X., Vanlandschoot, P., Jou, W.M., Fiers, W., 1999. A universal influenza A vaccine based on the extracellular domain of the M2 protein. Nature medicine 5, 1157-1163.
26. Neumann, E.J., Kliebenstein, J.B., Johnson, C.D., Mabry, J.W., Bush, E.J., Seitzinger, A.H., Green, A.L., Zimmerman, J.J., 2005. Assessment of the economic impact of porcine reproductive and respiratory syndrome on swine production in the United States. Journal of the American Veterinary Medical Association 227, 385-392.
27. Ostrowski, M., Galeota, J.A., Jar, A.M., Platt, K.B., Osorio, F.A., Lopez, O.J., 2002. Identification of neutralizing and nonneutralizing epitopes in the porcine reproductive and respiratory syndrome virus GP5 ectodomain. Journal of virology 76, 4241-4250.
28. Pirzadeh, B., Dea, S., 1998. Immune response in pigs vaccinated with plasmid DNA encoding ORF5 of porcine reproductive and respiratory syndrome virus. The Journal of general virology 79 (Pt 5), 989-999.
29. Plagemann, P.G., 2004. The primary GP5 neutralization epitope of North American isolates of porcine reproductive and respiratory syndrome virus. Veterinary immunology and immunopathology 102, 263-275.
30. Rogan, D.J., Levere., 2000. Immune response for the open reading frame 2 (ORF2) membrane glycoprotein of porcine reproductive and respiratory syndrome virus
31. (PRRSV) delivered in pigs by recombinant Vaccinia virus, 16th International Pig Veterinary Society Congress, p. 643.
32. Shen, G., Jin, N., Ma, M., Jin, K., Zheng, M., Zhuang, T., Lu, H., Zhu, G., Jin, H., Jin, M., Huo, X., Qin, X., Yin, R., Li, C., Li, H., Li, Y., Han, Z., Chen, Y., Jin, M., 2007. Immune responses of pigs inoculated with a recombinant fowlpox virus coexpressing GP5/GP3 of porcine reproductive and respiratory syndrome virus and swine IL-18. Vaccine 25, 4193-4202.
33. Storgaard, T., Oleksiewicz, M., Botner, A., 1999. Examination of the selective pressures on a live PRRS vaccine virus. Archives of virology 144, 2389-2401.
34. Van Reeth, K., 1997. Pathogenesis and clinical aspects of a respiratory porcine reproductive and respiratory syndrome virus infection. Veterinary microbiology 55, 223-230.

35. Vashisht, K., Goldberg, T.L., Husmann, R.J., Schnitzlein, W., Zuckermann, F.A., 2008. Identification of immunodominant T-cell epitopes present in glycoprotein 5 of the North American genotype of porcine reproductive and respiratory syndrome virus. Vaccine 26, 4747-4753.
36. Wang, S., Fang, L., Fan, H., Jiang, Y., Pan, Y., Luo, R., Zhao, Q., Chen, H., Xiao, S., 2007. Construction and immunogenicity of pseudotype baculovirus expressing GP5 and M protein of porcine reproductive and respiratory syndrome virus. Vaccine 25, 8220-8227.
37. Wang, X., Li, J., Jiang, P., Li, Y., Zeshan, B., Cao, J., Wang, X., 2009. GM-CSF fused with GP3 and GP5 of porcine reproductive and respiratory syndrome virus increased the immune responses and protective efficacy against virulent PRRSV challenge. Virus research 143, 24-32.
38. Zheng, Q., Chen, D., Li, P., Bi, Z., Cao, R., Zhou, B., Chen, P., 2007. Co-expressing GP5 and M proteins under different promoters in recombinant modified vaccinia virus ankara (rMVA)-based vaccine vector enhanced the humoral and cellular immune responses of porcine reproductive and respiratory syndrome virus (PRRSV). Virus genes 35, 585-595.
39. Zuckermann, F.A., Garcia, E.A., Luque, I.D., Christopher-Hennings, J., Doster, A., Brito, M., Osorio, F., 2007. Assessment of the efficacy of commercial porcine reproductive and respiratory syndrome virus (PRRSV) vaccines based on measurement of serologic response, frequency of gamma-IFN-producing cells and virological parameters of protection upon challenge. Veterinary microbiology 123, 69-85.

Chapter III

Virus-like particles as a vaccine against porcine reproductive and respiratory syndrome virus (PRRSV)

M.V.Ambika[1], Yanyan. Ni[2], X.J.Meng[2], Chenming Zhang[1]

[1]Department of Biological Systems Engineering, Virginia Tech,

200 Seitz Hall, Blacksburg, VA 24061, USA

[2]Center for Molecular Medicine and Infectious Disease, Department of Biomedical Sciences & Pathobiology, College of Veterinary Medicine, Virginia Tech,

Blacksburg, VA 24060-0342, USA

* Corresponding author. Tel: +1 540 231 7601; fax: 540 231 3199.

Email address: chzhang2@vt.edu (C. Zhang)

Abstract

Porcine reproductive and respiratory syndrome (PRRS) is the most significant infectious disease currently affecting the swine industry worldwide. Several inactivated and modified live vaccines (MLV) have been developed to curb PRRSV infections. The unsatisfactory efficacy and safety of these vaccines, drives for development of new generation PRRS universal vaccines. Virus-like particles (VLPs) based vaccines are gaining increasing acceptance compared to subunit vaccines, as they present the antigens in more veritable conformation and are even readily recognized by the immune system. Hepatitis B virus (HBV) core antigen (HBcAg) is very well studied and has been successfully used as a carrier for more than 100 other sequences. In this study, hybrid HBcAg VLPs are generated by fusion of the conserved protective epitopes of PRRSV and expressed in *E. coli*. An optimized purification protocol that overcomes issues from ultracentrifugation is developed to obtain hybrid HBcAg VLP protein from the inclusion bodies. This hybrid HBcAg VLP protein self assembled to 23 nm VLPs that were shown to block virus infection of susceptible cells when tested on MARC 145 cells. Therefore, the safety of non-infectious and non-replicable VLPs and production through low-cost *E. coli* fermentation may make this vaccine competitive against current vaccines on both efficacy and cost.

Keywords: Porcine reproductive and respiratory syndrome virus, PRRSV, vaccine, VLP, inclusion bodies.

Introduction

Porcine reproductive and respiratory syndrome (PRRS), also known as blue-ear pig disease, emerged as a disastrous disease in late 1980's. It was first identified in 1987 in US and in 1990 in Europe (Keffaber, 1989; Loula, 1991). The causative agent, Porcine Reproductive and Respiratory Virus (PRRSV) accounts for more than 600 million dollars annual loss, for swine industry in United States alone as estimated by US national breeding and growing pig herd. It causes severe reproductive problems like poor farrowing rates, premature farrowings and increased stillbirths in sows and respiratory distress such as pneumonia in piglets and growing pigs (Cho and Dee, 2006).

PRRSV is single stranded enveloped, RNA virus of order *Nidovirales* and family *Artiviridae.* PRRSV can be grouped into two genotypes, namely European (Type 1) and North American (Type 2)(Meng et al., 1996). At genomic sequence level they differ approximately by 40% and they differ serologically. PRRSV has a genome size of about 15kb, where genomic RNA is capped and polyadenylated. It comprises of 10 Open Reading Frames (ORF1a, ORF1b, ORF2, ORF2a, ORF3, ORF4, ORF5a, and ORF5 - ORF7). ORF 1a and 1b accounts to about 75 % of viral genome and are translated into two large polyproteins by ribosomal frameshifting (Snijder and Meulenberg, 1998). These proteins are cleaved into 14 non-structural proteins (nsp), either co- or post- translationally modified by four putative viral proteases. ORF2a, ORF3, ORF4 and ORF5 encodes membrane associated N-glycosylated structural proteins GP2a, GP3, GP4 and GP5 respectively. Recently, ORF5a was discovered, which encodes ORF5a protein comprising 51 amino acids, of unknown function (Johnson et al., 2011). ORF2b and ORF6 encode E and M proteins respectively, which are non-glycosylated membrane protein. Nucleocapsid protein, N, is encoded by ORF7. GP5 and M

proteins forms heterodimeric complexes via disulfide bonds in PRRSV infected cells and are indispensable for the formation of PRRSV particles. GP and M proteins interacts with cellular receptors, sialoadhesin and heparan sulfate respectively and are associated with virus attachment and internalization (Delputte et al., 2002; Dokland, 2010; Van Breedam et al., 2010a; Van Breedam et al., 2010b).

Although, several structural proteins of PRRSV are known to induce neutralizing antibodies, GP5 induced neutralization antibodies perform vital role in protection against infection (Gonin et al., 1999; Weiland et al., 1999). Plagemann (2004) identified the neutralization epitope, which is located in the middle of GP5 sequence among North American PRRSV isolates. This neutralization epitope corresponded to B cell epitope (^{37}SHLQLIYNL^{46}) and is conserved among North American PRRSV isolates (Plagemann, 2004). Two more pentadecapeptide (^{117}LAALICFVIRLAKNC^{131} and ^{149}KGRLYRWRSPVIIEK^{163}) spanning GP5 are identified as immunodominant T cell epitopes. These T cell epitopes are relatively conserved with at most two amino acid variations and are known to elicit an interferon-gamma response from peripheral blood mononuclear cells (Vashisht et al., 2008).

Wide variety of inactivated and modified live vaccines (MLV) has been developed to prevent PRRSV infections. Inactivated PRRSV vaccines are considered ineffective, as they fail to prevent clinical signs and viremia caused by virus challenge even with homologous strains (Kimman et al., 2009; Zuckermann et al., 2007). Although MLV PRRSV vaccines are regarded more effective when compared to inactivated vaccines in reduction of clinical signs associated with PRRSV, it has several disadvantages. First, it confers partial protection against heterologous strains. Second, they were unstable, which resulted in intrinsic risk of reversion of the vaccine virus to virulence under farm conditions. Third, vaccinated pigs shed infective virus, which demands vaccination of all pigs in the pen at the same time (Botner et al., 1997; Madsen et al., 1998; Oleksiewicz et al., 1998). Therefore, development of new

generation PRRSV vaccines vaccine that confers protection against PRRS field virus is more claiming.

VLPs are gaining increasing acceptance as a potential vaccine candidate, as they overcome the limitations associated with inactivated vaccines and MLVs (Ludwig and Wagner, 2007). VLPs are expedient candidate for vaccine design, as they mimic the 3D structure of native viruses and are devoid of viral genome. These VLPs are known to stimulate B cell mediated response, CD4 cell proliferating response and cytotoxic T Lymphocyte (CTL) responses. VLPs cross-link the B-cell receptors and efficiently reach the MHC class I pathway (Bachmann and Jennings, 2010). VLPs can even target dendritic cells (DC), which is essential to invoke both humoral and cell mediated immunity, making VLP based vaccine more attractive (Aguilar and Rodriguez, 2007). VLPs formed by Hepatitis B core antigen (HBcAg) acts as an efficient carrier platform as they can display antigens (such as epitopes) unrelated to the VLP itself (Bachmann and Jennings, 2010). It has been rendered that poorly immunogenic B-cell and T-cell epitopes have been converted into a highly immunogenic malaria vaccine candidate by linkage of these epitopes to HBcAg VLPs (Birkett et al., 2002; Milich et al., 2001). Furthermore, a universal influenza A vaccine fused to a viral conserved 23-amino-acid M2 peptide (M2e) with HBcAg has successfully completed phase I clinical trial (De Filette et al., 2006).

Until now, development of VLP based PRRS vaccine using *E.coli* expression system has not been reported. This study is the first to describe development of versatile hybrid HBcAg VLPs by fusion of the conserved protective epitopes of PRRSV. After hybrid HBcAg VLP production, they were tested for blocking virus infection in MARC 145 cells.

Materials and methods

Construction of recombinant plasmids

The conserved protective B-cell and T-cell epitopes were fused in between amino acid 48 and 49 of Hepatitis B virus core protein. The nucleotide sequence of length 585 bp, coding for this construct was artificially synthesized and cloned into pUC57 vector at Bam HI and Hind III by Genscript Corporation, Piscataway, NJ. The pUC57 plasmid was digested by Nhe I and Hind III enzymes and inserted into pET28a vector (Novagen). The constructed plasmid (pET28a/VLP) was transformed into *E.coli* DH5 alpha cells by electroporation. The presence of 585 bp insert in the constructed plasmid (pET28a/VLP) was confirmed by restriction digestion and the confirmed plasmid was transformed into *E.coli* BL21 (DE3).

Expression and solubility test

Pre inoculum of recombinant plasmid (pET28a/VLP) was prepared in 50 ml of 2X YT media supplemented with 50 µg/ml kanamycin. The culture was incubated at 37 ºC and 200 rpm for 16 hours. The inoculum was prepared in 2 liter flasks using 0.02% of pre inoculum and was incubated at 37 ºC and 200 rpm, till the exponential growth phase was reached (Optical density at 600 nm is 0.6-0.7). The culture was induced with 1 mM IPTG and was incubated for additional three and half hours at 37 ºC and 180 rpm.

Cells were harvested by centrifugation at 8000 rpm for 10 mins and were resuspended using lysis buffer (50 mM sodium phosphate, 500 mM sodium chloride, pH 8.2). Cells were lysed by ultrasonication with 30% amplitude at 4 ºC for about 55 - 60 cycles. Each cycle had pulse of seven second succeeded by rest time of five second. Total cell extract was subjected to centrifugation at 13000 rpm for 10 mins to separate soluble and insoluble fractions. All these fractions were analyzed on SDS gel using un induced as control.

Solubilization of inclusion bodies

Insoluble fraction was accrued and washed thrice with washing buffer (50 mM sodium phosphate, 500 mM sodium chloride, 0.1% sarkosyl, pH 8.2). An additional three washes with distilled water was given to obtain pure inclusion bodies. This pure inclusion bodies were resuspended in IB solubilizing buffer (50 mM sodium phosphate, 500 mM sodium chloride, 0.9% sarkosyl, pH 8.2). MgCl2 (100 mM), DNase (200 µg/ml), RNase (200 µg/ml) and PMSF (100 µg/ml) were added and were incubated at room temperature for three hours with frequent vortexing. This was subjected to centrifugation at 13000 rpm for 10 mins and the supernatant was collected. The supernatant was precipitated with 30% $(NH4)_2SO4$ and the precipitate was subjected to centrifugation at 13000 rpm for 10 mins. The precipitate was dissolved in column loading buffer (50 mM sodium phosphate, 500 mM sodium chloride, 0.9% sarkosyl, pH 8.2).

Immobilized metal ion chromatography (IMAC)

ÄKTA™ purifier fast performance liquid chromatography (FPLC) system (GE Healthcare, Uppsala, Sweden) was used for protein purification experiments. IMAC Sepharose 6 Fast Flow column (GE Healthcare, Uppsala, Sweden) containing 5ml of resin were prepared as per manufacturer's instructions. The resins were charged with 0.1 M NiSO4 and equilibrated with 10 column volumes (CV) of column loading buffer. 5 ml of the precipitate dissolved in column loading buffer was loaded on to column. Unbound proteins were removed by 10 CV of loading buffer and finally the bound proteins were eluted by elution buffer (50 mM sodium phosphate, 500 mM sodium chloride, 0.9% sarkosyl, 300 mM imidazole, pH 8.2).

Refolding of recombinant fusion proteins

IMAC elutes were pooled and dialyzed at room temperature against refolding buffer using Slide-A-Lyzer Dialysis Cassettes with a molecular weight cut-off of 3.5K (Thermo Scientific, Rockford, IL, USA). Denatured fusion proteins in the IMAC elutes were dialyzed against 0.5 %, 0.25 %, 0.05 % sarkosyl with 25 mM sodium phosphate and 100 mM sodium chloride (pH 8.2). Protein samples were dialyzed with each concentration of sarkosyl for 8 hours.

Anion exchange chromatography (AEX)

Final polishing of refolded recombinant fusion protein was performed using anion exchange chromatography. 5 ml of DEAE sepharose fast flow resin (GE Healthcare, Uppsala, Sweden) was packed into column as per manufacturer's instructions. The column was equilibrated with 10 CV of AEX binding buffer (25 mM sodium phosphate, 100 mM sodium chloride, 0.05 % sarkosyl, pH 8.2) and 5 ml of refolded protein sample was loaded onto column. Unbound proteins were removed by washing with 5 CV of AEX binding buffer. Elution of bound proteins were performed using NaCl gradient starting with 100 mM to 500 mM along with 25 mM sodium phosphate and 0.05% sarkosyl. Finally column was regenerated using 1 M NaCl and 25 mM sodium phosphate, pH 8.2.

SDS PAGE and Western Blotting

SDS PAGE was performed as described elsewhere. For western blot experiment, the protein separated by 12% NuPAGE Bis-Tris gel was transferred on to a nitrocellulose membrane (Whatman, Germany) as per manufacturer's instruction. The membrane was blocked with 5% nonfat dry milk in TBS (Tris Buffered Saline) for 1 hour at room temperature. It was then

incubated with primary antibody, anti-His monoclonal antibody Ig G (Genscript Corporation, Piscataway, NJ, USA) 1:2000 dilution, in TBS containing 5% nonfat dry milk for 1 hour at room temperature. The membrane was then washed thrice with TBST (TBS buffer with 0.02% of Tween 20) for 10 min each. Later the membrane was incubated with secondary antibody, Horse radish peroxidase (HRP) goat anti-mouse Ig G antibody (Bethyl Laboratories Inc., Montgomery, TX, USA) 1:20,000 dilution, in TBS containing 5% nonfat dry milk for 1 hour at room temperature. The membrane was then washed thrice with TBST (TBS buffer with 0.02% of Tween 20) for 10 min each. Later the membrane was incubated in a 1:1 mixture of HRP luminal/enhancer solution and peroxide buffer (BioRad, Hercules, USA) for 5 min. The membrane was visualized by a ChemiDoc XRS molecular imager (BioRad, Hercules, USA).

Electron microscopy

Purified VLP's (2-3 µg) were adsorbed on fresh carbon coated grid. Excess sample was blotted by filter paper and allowed to stand for 1 min. Later the sample was stained with freshly prepared 1% aqueous uranyl acetate. Excess stain was blotted using filter paper and samples were allowed to dry. For transmission electron microscopy JEM 1400 manufactured by JEOL was used. Micrographs were taken under 20,000 X magnification.

Virus blocking assay

To analyze the function of purified hybrid HBcAg VLPs to block PRRSV infecting susceptible cells, an indirect immune-fluorescence test was performed as described elsewhere with some modifications (Hu et al., 2012). Purified hybrid HBcAg VLPs were added into wells of a plate coated with confluent MARC-145 cells. After incubation for 1 h, PRRSV dilutions with 2000 $TCID_{50}$/ml was added to each well and incubated for 15 min at 37 ^{o}C.

The inoculums were then removed and 100 µl of fresh DMEM supplemented with 2% fetal bovine serum, was added to each well. After incubation for 24 h at 37 ºC in a humidified atmosphere of 5% CO_2, the plates were fixed with 80% acetone for 15 min at room temperature. After extensive wash with PBST, 50 µl of fluorescein isothiocyanate conjugated anti-PRRSV monoclonal antibody, SDOW17-F (Rural Technologies, Brookings, SD, USA), diluted 1:100 in PBST with 2% BSA was added to each well. After an hour of incubation, the cells were washed four times with PBS and the number of fluorescent foci in each well was counted. The virus inhibition rate was expressed as a ratio of reduced fluorescent foci number by a protein sample over the number of fluorescent foci of the negative control (all assay components without hybrid HBcAg VLPs).

Results

Construction of hybrid HBcAg VLPs

The conserved protective epitopes were fused at a region located at the tip of the core particle surface spikes of HBcAg as shown in fig 1. It has been reported that region between amino acid 48 and 49 form the tip of the core particle (Bottcher et al., 1997). A lysine (K) residue was added to the C-teminus of the T2 epitope (LAALICFVIRLAKNC) for its efficient processing (Livingston et al., 2001). The nucleotide sequence coding for this construct was commercially synthesized and cloned in pUC57 vector at Bam HI and Hind III sites by Genscript Corporation, Piscataway, NJ. Digestion with Nhe I and Hind III enzymes released 585 bp insert, as Nhe I was incorporated at the N terminal end of the sequence, to ease the cloning in pET28a vector. The 585 bp insert release as shown in lane 2 of Fig 2was gel eluted and cloned into gel eluted Nhe I and Hind III digested pET28a vector (lane 5, fig 2) using ligase enzyme. The clone was confirmed using double digestion, as in Fig 3, where insert

release of 585 bp product is seen (lane 2 and 3) . No insert release was seen in vector alone in lane 6 which served as a control.

Expression and solubility check of hybrid HBcAg VLPs

Kanamycin resistant clone were examined for hybrid HBcAg VLP protein expression under IPTG induction. Different concentrations of IPTG ranging from 0.3 mM to 1 mM were used to obtain satisfactory expression profiles. *E.coli* cells induced with 1 mM IPTG gave maximum expression with post induction temperature of 37 ºC for 3 and half hours. Comparing expression profiles of induced samples with that of uninduced samples as shown in Fig 4 ratified this. In figure 4, lane 2 corresponds to total uninduced protein fraction and lane 1, represents the total induced fraction, where prominent protein band corresponding to 21 kDa expressing protein of interest was seen. Unfortunately, all expressed proteins were in pellet fraction (lane 3, fig 4). Several methods like different growth media for *E.coli*, lowering post induction temperature, different IPTG concentration, different buffers with different pH range for protein extraction were tried to make hybrid HBcAg VLP proteins soluble. But none of these methods were successful in obtaining desired protein in soluble fraction.

Purification and refolding of HBcAg VLP proteins from inclusion bodies

After cell growth and lysis, inclusion bodies were solubilized in IB buffer containing 0.9% sarkosyl. As the HBcAg VLP proteins had His tag at its N terminal, immobilized metal ion chromatography (IMAC) was chosen for protein purification. Since, IMAC efficiency depends on imidazole concentration in binding buffer, it was found 30 mM imidazole was optimal binding concentration. 30 mM imidazole was able to remove most of the impurities bound to the column. Elution peak in lane 7 of figure contains pure protein, with minimal

impurities. Elution peak fractions were collected and protein was refolded by stepwise dialysis. Refolding efficiency was found to be 80%. IMAC chromatography profile is depicted in figure 5. Other method like urea denaturation was used to extract proteins from inclusion bodies. Although, purified proteins were obtained by using 8 M urea, refolding was not successful. Proteins were precipitated during stepwise dialysis for urea removal. Even on column protein refolding failed to give pure protein of interest. Therefore, using 0.9% sarkosyl in IB buffer appears to be the optimized method for this particular protein purification protocol. Refolded HBcAg VLP proteins were subjected to anion exchange chromatography, to even get rid of the minimal impurities.

Polishing of purified HBcAg VLP protein

Chromatographic profile of anion exchange chromatography is as depicted in figure. Elution peak was collected and analyzed on SDS-PAGE. A clear single band corresponding to 21 kDa is seen in lane 8 of Figure 5. A clear signal of size 21 kDa is even obtained by western blot figure 6) with anti His monoclonal antibodies. A single clear band, with no traces of multiple bands, on both SDS PAGE and western blot corroborates the protein purity.

TEM and virus blocking assay

Hybrid HBcAg VLP proteins were allowed to stand overnight and were examined under transmission electron microscopy. VLPs of size corresponding around 23 nm was visualized as shown in figure 7, with significant homogeneity. This confirmed the self-assembly of hybrid HBcAg VLP protein into VLPs. It has been even reported earlier that purification of protein with ionexchange chromatography can lead to successful self assembly of protein into VLPs without ultra centrifugation process (Koho et al., 2012). These VLPs even block virus

very efficiently in concentration dependent fashion, as shown in figure 8. Therefore, an optimized process is developed for Hybrid HBcAg VLP production as shown in figure 9.

Discussion

PRRSV is highly pathogenic and causes huge economic loses to the pig industry worldwide. Vaccination appears to be the cost effective and feasible method to control PRRSV. Several approaches have been reported, including live attenuated vaccines, inactivated vaccines, mixed strains vaccines (Mengeling et al., 2003a; Mengeling et al., 2003b), recombinant subunit vaccines (DNA (Barfoed et al., 2004; Rompato et al., 2006), bacteria (Bastos et al., 2004), adenovirus (Cai et al., 2010; Jiang et al., 2008; Zhou et al., 2010), poxvirus (Shen et al., 2007; Zheng et al., 2007), alphavirus (Mogler, 2009), baculovirus (Plana Duran et al., 1997) and gastroenteritis virus (Cruz et al., 2010) bearing different PRRSV structural proteins), plant derived vaccines (Chen and Liu, 2011; Chia et al., 2010) and synthetic peptide vaccines (Charerntantanakul et al., 2006). These vaccines can confer protection at its best, but do not posses characteristics of an universal PRRS vaccine.

VLP based vaccine are one of the most exciting and emerging vaccine technologies as they present epitopes in similar pattern as that of native virus. VLPs are supra-molecular assemblages that mimic the structure of authentic viruses, without being infectious. HBcAg VLPs are considered very flexible, as they allow wide array of foreign insertions (Karpenko et al., 2000). As HBcAg VLPs even elicit strong B-cell and T-cell responses (Jegerlehner et al., 2002), they are regarded as one of the most promising VLP based vaccine platforms. During the last decade, two prophylactic VLP vaccines for prevention of Hepatitis B virus and Human papillomavirus infections have been registered for human use, while another 12 vaccines have entered clinical development (Kirnbauer et al., 1992; Lee et al., 2011). Wide variety of VLP based vaccines have been even developed for animals (Brun et al., 2011).

However, studies on VLP based PRRSV vaccines are very limited. Recently, VLPs comprised of GP5 and M proteins of PRRSV were generated in insect cells and were shown to induce both neutralizing antibodies and IFN-γ response in mice (Nam et al., 2013). However, the existence of adverse factors within viral proteins such as non-conserved epitope which is an immunodominant decoy epitope in viral GP5 protein and glycan shielding of neutralization epitopes can mislead the immune system to non-conserved epitopes and/or diminish the immune responsiveness of the conserved protective epitopes (Li et al., 2009; Lopez and Osorio, 2004). This may explain the poor heterologous protection of current commercial and experimental PRRS vaccines. Therefore, we aim at establishing a method to potentiate the immune response to the conserved protective epitopes of PRRSV and to focus the immune system on these epitopes by effective antigen presentation and removal of the adverse factors within the viral proteins, which is the key to a universal PRRS vaccine. Our study is the first to explore the novel antigen presentation method by fusing the conserved protective epitopes of PRRSV with HBcAg in *E.coli*, which will self-assemble to form VLPs.

Encouragingly, although it is partial or weak, heterologous protection does exist and common epitopes are likely to be involved in protection in different strains (Mateu and Diaz, 2008). Three conserved protective epitopes (one B-cell epitope and two T-cell epitopes) are used in our study. B-cell epitope is conserved among isolates, and neutralizing antibodies are mainly directed against this epitope (Lopez and Osorio, 2004; Ostrowski et al., 2002; Plagemann, 2004). The T-cell epitopes (^{117}LAALICFVIRLAKNC^{131} and ^{149}KGRLYRWRSPVIIEK^{163}) are also conserved and can recall IFN-γ response (Vashisht et al., 2008). Epitope based vaccines are captivating because of its advantages, such as, high degree of specificity and their ease and safety of use (Franke et al., 1999). In this study, hybrid HBcAg VLP proteins were generated by the fusion of three conserved epitopes gene into HBcAg protein gene at major immunodominant region (MIR) site and its expression in *E.coli.* Hybrid HBcAg VLP

proteins were expressed in *E.coli* inclusion bodies. After successful purification, renaturation and polishing of hybrid HBcAg VLP proteins, they self assembled to VLP of size 23nm. These hybrid HBcAg VLPs were shown to block virus effectively when tested on MARC 145 cells in-vitro. As MIR of HBcAg is the most exposed region of the assembled VLP, the epitopes fused were more likely to be presented on the surface of each hybrid HBcAg VLP, which might even increase the avidity for the target epitope. Therefore, hybrid HBcAg VLPs can be used as an effective epitope based PRRSV vaccine, which might be even administered without using an adjuvant.

A limit of the current study is the lack of in-vivo studies, that is to check the effectiveness of hybrid HBcAg VLPs in mice and in pigs. It has been reported recently that B cell and T cell epitopes of GP5 along with adjuvant Gp96N, could elicit strong immune response in mice (Chen et al., 2013). It was revealed that Gp96N activated PRRSV specific humoral responses elicited by B cell epitope peptides and even promoted the PRRSV specific cellular immunity elicited by T cell epitope peptides (Chen et al., 2013). Therefore, hybrid HBcAg VLPs generated in this study have greater chances of eliciting both humoral and cell mediated immunity in mice and pigs, as VLPs have been claimed effective adjuvant by themselves (Ludwig and Wagner, 2007).

Conclusion

We have expressed and purified hybrid HBcAg VLP protein consisting of conserved epitopes of PRRSV. An optimized protocol was developed to obtain pure hybrid HBcAg VLPs from *E. coli* inclusion bodies. This process can produce approximately 3 mg of HBcAg VLPs from 1litre fermentation broth and even overcomes the issues of using ultra centrifugation for pure VLP production. For the first time, we attempt to develop VLP based PRRSV epitope

vaccine from low cost *E.coli* fermentation. As hybrid HBcAg VLPs block virus infection of susceptible cells *in vitro*, this could serve as universal vaccine against PRRSV.

Acknowledgment

We thank CALS integrated research program and Department of States, USA for funding this project. We would like to thank Kathy Lowe for her assistance in TEM.

Figures

Figure 1: Construction of hybrid HbcAg VLP

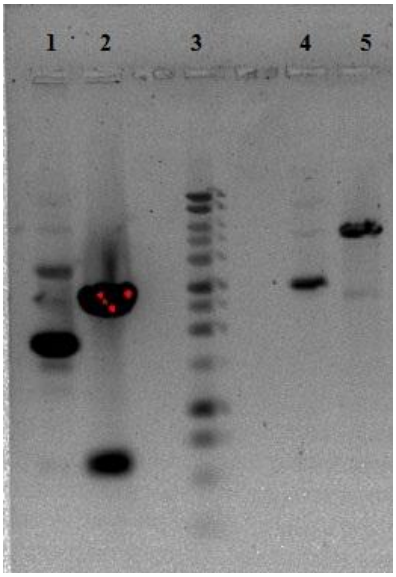

Figure 2: Agarose Gel Electrophoresis (AGE) depicting release of nucleotide sequence coding for (pUC57/VLP) , from pUC57 vector

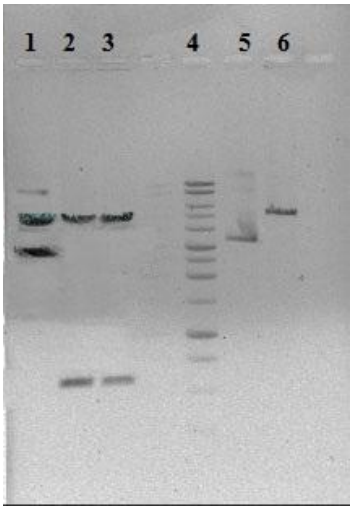

Figure 3: Agarose Gel Electrophoresis (AGE) for confirmation of presence of hybrid HBcAg VLP nucleotide sequence in (pET28a/VLP)

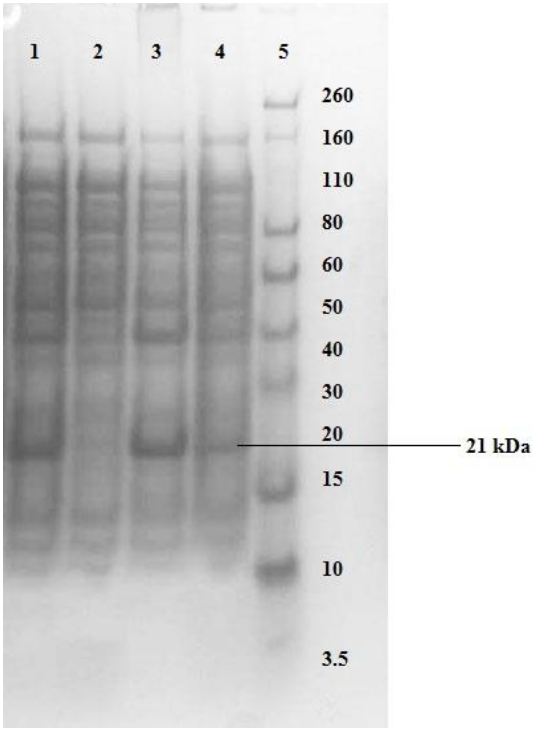

Figure 4: Expression and solubility profile of hybrid HBcAg VLP protein

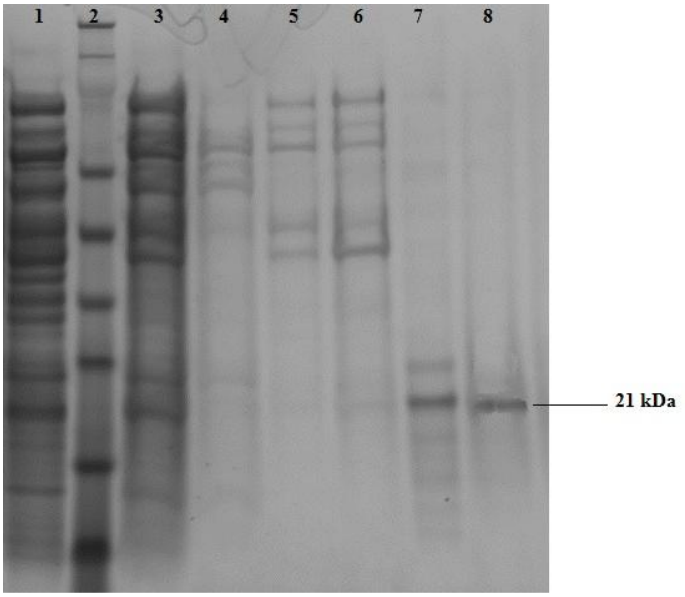

Figure 5:Purification profile of hybrid HBcAg VLP protein

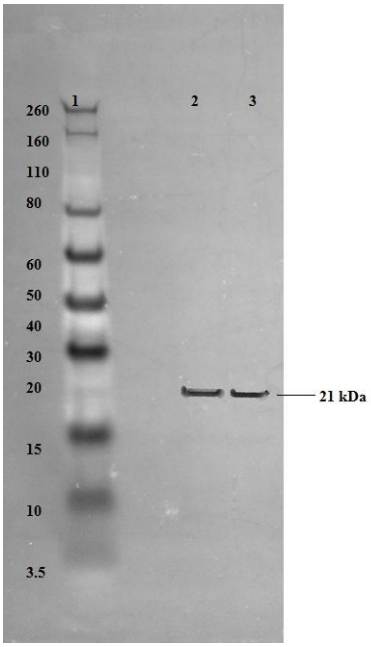

Figure 6:Western blot analysis of hybrid HBcAg VLP protein

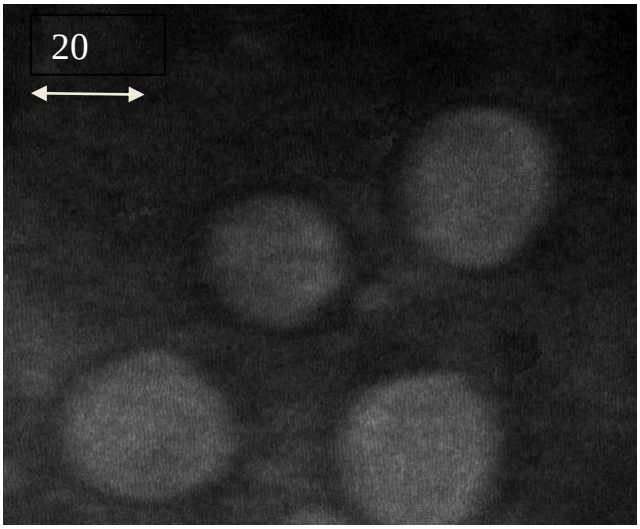

Figure 7:Examination of hybrid HBcAg VLP protein by transmission electron microscopy**.**

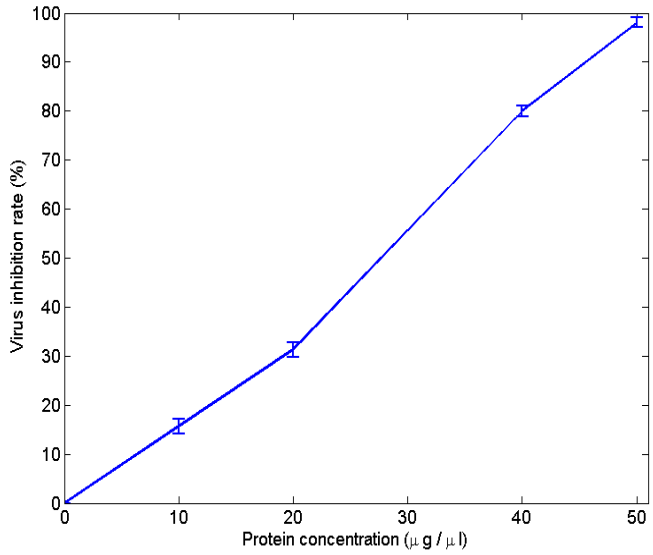

Figure 8:Virus blocking assay of hybrid HBcAg VLPs

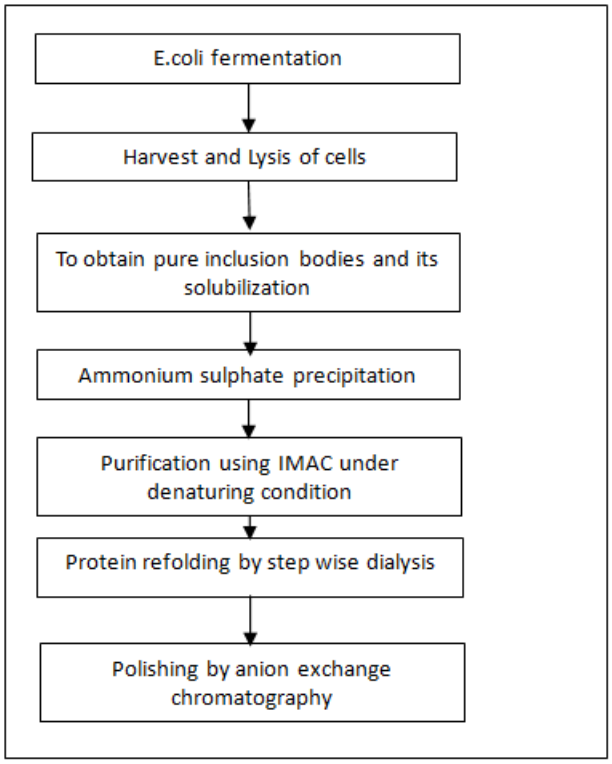

Figure 9:Hybrid HBcAg VLP production process

Legends for figures

Figure1. Construction of hybrid HbcAg VLP. B (green color) represents the linear and conserved protective B cell epitope SHLQLIYNL. T1 represents the conserved protective T cell epitope KGRLYRWRSPVIIEK. T2 represents the conserved protective T cell epitope LAALICFVIRLAKNC

Figure2. Agarose Gel Electrophoresis (AGE) depicting release of nucleotide sequence coding for (pUC57/VLP), from pUC57 vector. Lane 1: Uncut pUC 57 vector. Lane 2: pUC57 vector

with Nhe I and Hind III. Lane 3: 1 kb marker. Lane 4: uncut pET28a vector. Lane 5: pET28a vector linearized with Nhe I and Hind III enzymes.

Figure3: Agarose Gel Electrophoresis (AGE) for confirmation of presence of hybrid HBcAg VLP nucleotide sequence in (pET28a/VLP).

Lane1: Uncut (pET28a/VLP), lane2 : pET28a HBcAg VLP with Nhe I and Hind III enzymes. Lane 3: 1 kb marker. Lane 4: Uncut pET28a vector, lane 5: pET28a vector with Nhe I and Hind III enzymes.

Figure 4: Expression and solubility profile of hybrid HBcAg VLP protein. Lane 1: total protein fraction, lane 2: uninduced protein fraction, lane 3: protein fraction in pellet, lane 4: soluble protein fraction, lane 5: pre-stained Novagen marker

Figure 5: Purification profile of hybrid HBcAg VLP protein. Lane 1:IB solubilized protein fraction, lane 2: ammonium sulphate precipitated protein fraction applied onto IMAC column. lane 3: prestained Novex protein marker, lane 4: unbound protein fraction, lane 5: 10mM imidazole wash, lane 5: 30mM imidazole wash. Lane 6: elute from IMAC which is refolded by dialysis. Lane 7: Elute fraction from AEX chromatography.

Figure 6: Western blot analysis of hybrid HBcAg VLP protein. Lane 1: prestained Novex protein marker, lane 2 : refolded hybrid HBcAg VLP protein, lane 3: Elute fraction from AEX chromatography.

Figure 7: Examination of hybrid HBcAg VLP protein by transmission electron microscopy.

Figure 8: Virus blocking assay of hybrid HBcAg VLPs

Figure 9: Hybrid HBcAg VLP production process

References

1. Aguilar, J.C., Rodriguez, E.G., 2007. Vaccine adjuvants revisited. Vaccine 25, 3752-3762.
2. Bachmann, M.F., Jennings, G.T., 2010. Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns. Nature reviews. Immunology 10, 787-796.
3. Barfoed, A.M., Blixenkrone-Moller, M., Jensen, M.H., Botner, A., Kamstrup, S., 2004. DNA vaccination of pigs with open reading frame 1-7 of PRRS virus. Vaccine 22, 3628-3641.
4. Bastos, R.G., Dellagostin, O.A., Barletta, R.G., Doster, A.R., Nelson, E., Zuckermann, F., Osorio, F.A., 2004. Immune response of pigs inoculated with Mycobacterium bovis BCG expressing a truncated form of GP5 and M protein of porcine reproductive and respiratory syndrome virus. Vaccine 22, 467-474.
5. Birkett, A., Lyons, K., Schmidt, A., Boyd, D., Oliveira, G.A., Siddique, A., Nussenzweig, R., Calvo-Calle, J.M., Nardin, E., 2002. A modified hepatitis B virus core particle containing multiple epitopes of the Plasmodium falciparum circumsporozoite protein provides a highly immunogenic malaria vaccine in preclinical analyses in rodent and primate hosts. Infection and immunity 70, 6860-6870.
6. Botner, A., Strandbygaard, B., Sorensen, K.J., Have, P., Madsen, K.G., Madsen, E.S., Alexandersen, S., 1997. Appearance of acute PRRS-like symptoms in sow herds after vaccination with a modified live PRRS vaccine. The Veterinary record 141, 497-499.
7. Bottcher, B., Wynne, S.A., Crowther, R.A., 1997. Determination of the fold of the core protein of hepatitis B virus by electron cryomicroscopy. Nature 386, 88-91.

8. Brun, A., Barcena, J., Blanco, E., Borrego, B., Dory, D., Escribano, J.M., Le Gall-Recule, G., Ortego, J., Dixon, L.K., 2011. Current strategies for subunit and genetic viral veterinary vaccine development. Virus research 157, 1-12.
9. Cai, J., Ma, Y., Li, J., Yan, C., Hu, R., Zhang, J., 2010. Construction and characterization of a recombinant canine adenovirus expressing GP5 and M proteins of porcine reproductive and respiratory syndrome virus. The Journal of veterinary medical science / the Japanese Society of Veterinary Science 72, 1035-1040.
10. Charerntantanakul, W., Platt, R., Johnson, W., Roof, M., Vaughn, E., Roth, J.A., 2006. Immune responses and protection by vaccine and various vaccine adjuvant candidates to virulent porcine reproductive and respiratory syndrome virus. Veterinary immunology and immunopathology 109, 99-115.
11. Chen, C., Li, J., Bi, Y., Yang, L., Meng, S., Zhou, Y., Jia, X., Meng, S., Sun, L., Liu, W., 2013. Synthetic B- and T-cell epitope peptides of porcine reproductive and respiratory syndrome virus with Gp96 as adjuvant induced humoral and cell-mediated immunity. Vaccine 31, 1838-1847.
12. Chen, X., Liu, J., 2011. Generation and immunogenicity of transgenic potato expressing the GP5 protein of porcine reproductive and respiratory syndrome virus. Journal of virological methods 173, 153-158.
13. Chia, M.Y., Hsiao, S.H., Chan, H.T., Do, Y.Y., Huang, P.L., Chang, H.W., Tsai, Y.C., Lin, C.M., Pang, V.F., Jeng, C.R., 2010. Immunogenicity of recombinant GP5 protein of porcine reproductive and respiratory syndrome virus expressed in tobacco plant. Veterinary immunology and immunopathology 135, 234-242.
14. Cho, J.G., Dee, S.A., 2006. Porcine reproductive and respiratory syndrome virus. Theriogenology 66, 655-662.
15. Cruz, J.L., Zuniga, S., Becares, M., Sola, I., Ceriani, J.E., Juanola, S., Plana, J., Enjuanes, L., 2010. Vectored vaccines to protect against PRRSV. Virus research 154, 150-160.
16. De Filette, M., Ramne, A., Birkett, A., Lycke, N., Lowenadler, B., Min Jou, W., Saelens, X., Fiers, W., 2006. The universal influenza vaccine M2e-HBc administered intranasally in combination with the adjuvant CTA1-DD provides complete protection. Vaccine 24, 544-551.
17. Delputte, P.L., Vanderheijden, N., Nauwynck, H.J., Pensaert, M.B., 2002. Involvement of the matrix protein in attachment of porcine reproductive and

respiratory syndrome virus to a heparinlike receptor on porcine alveolar macrophages. Journal of virology 76, 4312-4320.

18. Dokland, T., 2010. The structural biology of PRRSV. Virus research 154, 86-97.
19. Franke, E.D., Hoffman, S.L., Sacci, J.B., Jr., Wang, R., Charoenvit, Y., Appella, E., Chesnut, R., Alexander, J., Del Guercio, M.F., Sette, A., 1999. Pan DR binding sequence provides T-cell help for induction of protective antibodies against Plasmodium yoelii sporozoites. Vaccine 17, 1201-1205.
20. Gonin, P., Pirzadeh, B., Gagnon, C.A., Dea, S., 1999. Seroneutralization of porcine reproductive and respiratory syndrome virus correlates with antibody response to the GP5 major envelope glycoprotein. Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc 11, 20-26.
21. Hu, J., Ni, Y., Dryman, B.A., Meng, X.J., Zhang, C., 2012. Immunogenicity study of plant-made oral subunit vaccine against porcine reproductive and respiratory syndrome virus (PRRSV). Vaccine 30, 2068-2074.
22. Jegerlehner, A., Storni, T., Lipowsky, G., Schmid, M., Pumpens, P., Bachmann, M.F., 2002. Regulation of IgG antibody responses by epitope density and CD21-mediated costimulation. European journal of immunology 32, 3305-3314.
23. Jiang, W., Jiang, P., Wang, X., Li, Y., Du, Y., Wang, X., 2008. Enhanced immune responses of mice inoculated recombinant adenoviruses expressing GP5 by fusion with GP3 and/or GP4 of PRRS virus. Virus research 136, 50-57.
24. Johnson, C.R., Griggs, T.F., Gnanandarajah, J., Murtaugh, M.P., 2011. Novel structural protein in porcine reproductive and respiratory syndrome virus encoded by an alternative ORF5 present in all arteriviruses. The Journal of general virology 92, 1107-1116.
25. Karpenko, L.I., Ivanisenko, V.A., Pika, I.A., Chikaev, N.A., Eroshkin, A.M., Veremeiko, T.A., Ilyichev, A.A., 2000. Insertion of foreign epitopes in HBcAg: how to make the chimeric particle assemble. Amino acids 18, 329-337.
26. Keffaber, K., 1989. Am. Assoc. Swine Pract. Newslett 2.
27. Kimman, T.G., Cornelissen, L.A., Moormann, R.J., Rebel, J.M., Stockhofe-Zurwieden, N., 2009. Challenges for porcine reproductive and respiratory syndrome virus (PRRSV) vaccinology. Vaccine 27, 3704-3718.
28. Kirnbauer, R., Booy, F., Cheng, N., Lowy, D.R., Schiller, J.T., 1992. Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly

immunogenic. Proceedings of the National Academy of Sciences of the United States of America 89, 12180-12184.

29. Koho, T., Mantyla, T., Laurinmaki, P., Huhti, L., Butcher, S.J., Vesikari, T., Kulomaa, M.S., Hytonen, V.P., 2012. Purification of norovirus-like particles (VLPs) by ion exchange chromatography. Journal of virological methods 181, 6-11.
30. Lee, D.H., Park, J.K., Lee, Y.N., Song, J.M., Kang, S.M., Lee, J.B., Park, S.Y., Choi, I.S., Song, C.S., 2011. H9N2 avian influenza virus-like particle vaccine provides protective immunity and a strategy for the differentiation of infected from vaccinated animals. Vaccine 29, 4003-4007.
31. Li, B., Xiao, S., Wang, Y., Xu, S., Jiang, Y., Chen, H., Fang, L., 2009. Immunogenicity of the highly pathogenic porcine reproductive and respiratory syndrome virus GP5 protein encoded by a synthetic ORF5 gene. Vaccine 27, 1957-1963.
32. Livingston, B.D., Newman, M., Crimi, C., McKinney, D., Chesnut, R., Sette, A., 2001. Optimization of epitope processing enhances immunogenicity of multiepitope DNA vaccines. Vaccine 19, 4652-4660.
33. Lopez, O.J., Osorio, F.A., 2004. Role of neutralizing antibodies in PRRSV protective immunity. Veterinary immunology and immunopathology 102, 155-163.
34. Loula, T., 1991. Agri-practice 12.
35. Ludwig, C., Wagner, R., 2007. Virus-like particles-universal molecular toolboxes. Current opinion in biotechnology 18, 537-545.
36. Madsen, K.G., Hansen, C.M., Madsen, E.S., Strandbygaard, B., Botner, A., Sorensen, K.J., 1998. Sequence analysis of porcine reproductive and respiratory syndrome virus of the American type collected from Danish swine herds. Archives of virology 143, 1683-1700.
37. Mateu, E., Diaz, I., 2008. The challenge of PRRS immunology. Veterinary journal 177, 345-351.
38. Meng, X.J., Paul, P.S., Halbur, P.G., Lum, M.A., 1996. Characterization of a high-virulence US isolate of porcine reproductive and respiratory syndrome virus in a continuous cell line, ATCC CRL11171. Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc 8, 374-381.

39. Mengeling, W.L., Lager, K.M., Vorwald, A.C., Clouser, D.F., 2003a. Comparative safety and efficacy of attenuated single-strain and multi-strain vaccines for porcine reproductive and respiratory syndrome. Veterinary microbiology 93, 25-38.
40. Mengeling, W.L., Lager, K.M., Vorwald, A.C., Koehler, K.J., 2003b. Strain specificity of the immune response of pigs following vaccination with various strains of porcine reproductive and respiratory syndrome virus. Veterinary microbiology 93, 13-24.
41. Milich, D.R., Hughes, J., Jones, J., Sallberg, M., Phillips, T.R., 2001. Conversion of poorly immunogenic malaria repeat sequences into a highly immunogenic vaccine candidate. Vaccine 20, 771-788.
42. Mogler, M.A.V.V., R. L. ; Erdman, M.M. ; Harris, D.L., 2009. Replicon particles expressing PRRSV GP5 and Matrix reduce
43. viremia following homologous and heterologous challenge, International PRRS Symposium, p. 107.
44. Nam, H.M., Chae, K.S., Song, Y.J., Lee, N.H., Lee, J.B., Park, S.Y., Song, C.S., Seo, K.H., Kang, S.M., Kim, M.C., Choi, I.S., 2013. Immune responses in mice vaccinated with virus-like particles composed of the GP5 and M proteins of porcine reproductive and respiratory syndrome virus. Archives of virology.
45. Oleksiewicz, M.B., Botner, A., Madsen, K.G., Storgaard, T., 1998. Sensitive detection and typing of porcine reproductive and respiratory syndrome virus by RT-PCR amplification of whole viral genes. Veterinary microbiology 64, 7-22.
46. Ostrowski, M., Galeota, J.A., Jar, A.M., Platt, K.B., Osorio, F.A., Lopez, O.J., 2002. Identification of neutralizing and nonneutralizing epitopes in the porcine reproductive and respiratory syndrome virus GP5 ectodomain. Journal of virology 76, 4241-4250.
47. Plagemann, P.G., 2004. The primary GP5 neutralization epitope of North American isolates of porcine reproductive and respiratory syndrome virus. Veterinary immunology and immunopathology 102, 263-275.
48. Plana Duran, J., Climent, I., Sarraseca, J., Urniza, A., Cortes, E., Vela, C., Casal, J.I., 1997. Baculovirus expression of proteins of porcine reproductive and respiratory syndrome virus strain Olot/91. Involvement of ORF3 and ORF5 proteins in protection. Virus genes 14, 19-29.
49. Rompato, G., Ling, E., Chen, Z., Van Kruiningen, H., Garmendia, A.E., 2006. Positive inductive effect of IL-2 on virus-specific cellular responses elicited by a

PRRSV-ORF7 DNA vaccine in swine. Veterinary immunology and immunopathology 109, 151-160.

50. Shen, G., Jin, N., Ma, M., Jin, K., Zheng, M., Zhuang, T., Lu, H., Zhu, G., Jin, H., Jin, M., Huo, X., Qin, X., Yin, R., Li, C., Li, H., Li, Y., Han, Z., Chen, Y., Jin, M., 2007. Immune responses of pigs inoculated with a recombinant fowlpox virus coexpressing GP5/GP3 of porcine reproductive and respiratory syndrome virus and swine IL-18. Vaccine 25, 4193-4202.
51. Snijder, E.J., Meulenberg, J.J., 1998. The molecular biology of arteriviruses. The Journal of general virology 79 (Pt 5), 961-979.
52. Van Breedam, W., Delputte, P.L., Van Gorp, H., Misinzo, G., Vanderheijden, N., Duan, X., Nauwynck, H.J., 2010a. Porcine reproductive and respiratory syndrome virus entry into the porcine macrophage. The Journal of general virology 91, 1659-1667.
53. Van Breedam, W., Van Gorp, H., Zhang, J.Q., Crocker, P.R., Delputte, P.L., Nauwynck, H.J., 2010b. The M/GP(5) glycoprotein complex of porcine reproductive and respiratory syndrome virus binds the sialoadhesin receptor in a sialic acid-dependent manner. PLoS pathogens 6, e1000730.
54. Vashisht, K., Goldberg, T.L., Husmann, R.J., Schnitzlein, W., Zuckermann, F.A., 2008. Identification of immunodominant T-cell epitopes present in glycoprotein 5 of the North American genotype of porcine reproductive and respiratory syndrome virus. Vaccine 26, 4747-4753.
55. Weiland, E., Wieczorek-Krohmer, M., Kohl, D., Conzelmann, K.K., Weiland, F., 1999. Monoclonal antibodies to the GP5 of porcine reproductive and respiratory syndrome virus are more effective in virus neutralization than monoclonal antibodies to the GP4. Veterinary microbiology 66, 171-186.
56. Zheng, Q., Chen, D., Li, P., Bi, Z., Cao, R., Zhou, B., Chen, P., 2007. Co-expressing GP5 and M proteins under different promoters in recombinant modified vaccinia virus ankara (rMVA)-based vaccine vector enhanced the humoral and cellular immune responses of porcine reproductive and respiratory syndrome virus (PRRSV). Virus genes 35, 585-595.
57. Zhou, J.X., Xue, J.D., Yu, T., Zhang, J.B., Liu, Y., Jiang, N., Li, Y.L., Hu, R.L., 2010. Immune responses in pigs induced by recombinant canine adenovirus 2 expressing the glycoprotein 5 of porcine reproductive and respiratory syndrome virus. Veterinary research communications 34, 371-380.

58. Zuckermann, F.A., Garcia, E.A., Luque, I.D., Christopher-Hennings, J., Doster, A., Brito, M., Osorio, F., 2007. Assessment of the efficacy of commercial porcine reproductive and respiratory syndrome virus (PRRSV) vaccines based on measurement of serologic response, frequency of gamma-IFN-producing cells and virological parameters of protection upon challenge. Veterinary microbiology 123, 69-85.