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Prevention of heterologous Porcine Reproductive and Respiratory Syndrome Virus (PRRSv) infection using an antibody-virus complex vaccine

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**Prevention of heterologous Porcine Reproductive and Respiratory Syndrome Virus
(PRRSv) infection using an antibody-virus complex vaccine**

by

Khushboo Hemnani

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Immunobiology

Program of Study Committee:
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Michael Cho
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Ames, Iowa
2014

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DEDICATION

I dedicate this thesis to my family and friends for always being there for me.

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NOMENCLATURE

PRRSv	Porcine Reproductive and Respiratory Syndrome virus
Ab-virus complex	Antibody-virus complex
IDD	Immunodominant epitope
MAb	Monoclonal antibody
GP	Glycoprotein
MLV	Modified-live virus
IFN	Interferon
Nab	Neutralizing Antibody

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ABSTRACT

Porcine Reproductive and Respiratory Syndrome virus (PRRSv) continues to cause critical issues for the swine industry worldwide, with an estimated loss of \$1 billion annually in the United States. PRRSv is a genetically diverse RNA Arterivirus with up to 20% variability. Available vaccines, mainly modified-live and autogenous, provide limited protection against divergent strains. Additionally, PRRSv immune dysregulation, and immune evasion strategies pose huge challenges in the successful development of cross-protective vaccines. A vital immune evasion strategy utilized is termed deceptive imprinting. Many pathogens contain highly immunodominant, commonly variable, non-protective or partially protective antigenic determinants termed “decoys”. Decoys subvert host immune responses away from conserved, cross protective and critical viral determinants leading to poor vaccine performance. In attempt to subvert immunodominance of decoys, by masking decoys with PRRSv specific antibodies, we hypothesize virus-antibody complex vaccine can improve vaccine performance.

We present efficacy of preliminary and primary vaccine trials utilizing antibody-virus complex (Ab-virus) vaccines in prevention of homologous and heterologous PRRSv infection through assessment of viral loads as determined by real-time polymerase chain reaction and PRRSv associated pathology in the lung. Additionally, we determined the safety and immunogenicity of the Ab-virus complex. Antibody-virus complex vaccines were compared to autogenous and commercially available PRRSv vaccines. An initial study utilizing Ab-virus complex vaccine and heterologous

challenge, Ab-virus vaccinees developed Nab towards the vaccine and challenge strains earlier than controls and had reduced pulmonary pathology.

The primary vaccine trial demonstrated that a single Ab-virus complex vaccination was effective in preventing heterologous infection in thirty-three percent of vaccinees compared to only ten percent of pigs receiving autogenous vaccine. Vaccinees did not make pigs ill. Ab-virus complex vaccinees not protected from infection following heterologous challenge, had marked reduction in viremia as compared to autogenous and unvaccinated controls. Vaccinees that became infected, had significant reduction in pulmonary viral loads which was reflective of reduced pulmonary pathology. Future studies building on this research involve determination of the breadth of cross-protection afforded with these novel vaccines and the mechanism of immune refocusing.

CHAPTER I

INTRODUCTION

Statement of the problem and objective

Porcine reproductive and respiratory syndrome (PRRS) was first identified in the United States in 1987 and Europe in late 1990, though the etiology of the syndrome was unknown (Chapter 1, Jeffrey Zimmerman, 2003 PRRS Compendium Producer Edition). In 1991, PRRS virus (PRRSv) was identified as the etiological agent of “blue ear disease” or “mystery swine disease”, as the clinical disease manifestation was initially referred as (Chapter 1, Jeffrey Zimmerman, 2003 PRRS Compendium Producer Edition). Within a few years after discovery, the virus was pandemic (Chapter 2.8.7 OIE 2010). In the assessment done in 2009, PRRSv has become endemic in 49% of grower/finishing herds in the U.S. (USDA-APHIS VS CEAH info sheet January 2009 “PRRS seroprevalence on U.S. Swine operations). Economic losses due to PRRSv have been estimated to be \$1billion/year in the U.S. alone (Holtkamp D 2011). It is believed that globally, an estimated \$6-9 billion of losses can be attributed to PRRSv. The virus causes respiratory distress and pneumonia in piglets but the biggest economic impact is incurred due to poor growth performance and reduced average daily gain, resulting in decreased value to the producer. Another major implication of PRRSv infection is the increased susceptibility to secondary infections due to the immuno-suppressive behavior displayed by the virus. The virus manifests as reproductive disease in sows, causing late term abortion, stillbirth and infertility. Several abortion storms have been reported throughout the years since the discovery of PRRSv where incidence of abortions in a

herd have reached up to 50%, along with high mortality of sows and piglets (Halbur PG, Bush E 1997). In the light of the overall significant economic loss associated with PRRSv infection, it has become a top priority of the swine industry to strive for the eradication of the virus.

However, many hurdles have been recognized when attempting to eradicate PRRSv from larger pig populations. It has been reported that the virus can be transmitted over large distance for up to 9.1 km in the air, spreading the infection among herds (Otake S et al. 2010). Also, PRRSv has the highest mutation rate reported amongst any RNA virus, averaging 10^{-2} /site/year as compared 10^{-3} - 10^{-5} for other RNA viruses (Gojobori T et al. 2005). This high replication error rate has resulted in the circulation of PRRSv strains with a genetic divergence of up to 20% which often also associate with variability in pathogenicity. The virus displays multiple evasion strategies of both the innate and adaptive immune response, leading to persistent infections in hosts. Lack of a complete understanding of the virus pathogenesis makes the development of a vaccine a challenge as the viral targets are unknown. There are also considerable gaps in the knowledge of the host immune response to the virus, for example, the correlates of protection against natural infection or vaccine strains are unknown.

In order to be efficacious, a PRRSv vaccine would have to result in reduction of viral loads, clinical signs and pathology, and also the transmission of the virus from infected to naïve animals. Current licensed vaccines- such as attenuated modified live virus (MLV) vaccines provide partial to complete protection against homologous strain but efficacy of these vaccines towards heterologous strains is poor. As such, there is an

urgent need to design a vaccine that offers complete protection from not only the homologous strain but towards heterologous ones as well. The overall objective of this thesis was to evaluate the efficacy of an antibody-virus immune complex vaccine against infection with homologous and heterologous PRRSv strains.

Thesis organization

The thesis is organized in four chapters which includes the current section. Chapter II is a literature review summarizing pertinent information related to PRRSv and vaccines, purpose and rational of the research presented in the thesis. Chapter III presents results from a pilot animal study designed to evaluate the success of the candidate vaccine constructs and select an appropriate candidate for the subsequent animal study. Chapter IV is currently in preparation for publication in a peer reviewed scientific journal and presents the original research executed to evaluate the efficacy of the Ab-virus complex vaccine against heterologous PRRSv challenge in pigs. Lastly, chapter V provides a conclusion of the thesis.

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CHAPTER II

LITERATURE REVIEW

Porcine Reproductive and Respiratory Syndrome virus (PRRSv) is an Arterivirus, a positive sense, single-stranded enveloped RNA virus in the order Nidovirales which include members of the family *Coronaviridae*. Other members of the *Arteriviridae* family include Equine Arteritis virus (EAV), Lactate Dehydrogenase Elevating virus (LDV) of mice and Simian Hemorrhagic Fever virus (SHFV) endemic in some genera of African and Asian monkeys. While most arteriviruses were discovered 50-60 years ago, PRRSv was isolated simultaneously in Europe and North America in the late 1980s. PRRSv manifests in pigs in the form of respiratory distress and pneumonia in pigs of all ages and reproductive disorders including SMEDI (stillbirth, mummification, embryonic death and infertility) in breeding age females. Reports have also demonstrated the occurrence of “abortion storms” over the years where high incidences of mid- to late-term abortions are associated with sow and gilt mortality (Halbur PG 1997). Along with these main clinical manifestations of PRRSv infection, additional morbidity is often associated with secondary infections due to the immuno-suppressive behavior of PRRSv. Examples of some common pathogens known to be a part of porcine respiratory disease complex (PRDC) are PRRSv, porcine circovirus type 2 (PCV2), swine influenza virus, *Mycoplasma hyopneumoniae*, *Streptococcus suis*, *Haemophilus parasuis*, *Actinobacillus suis* etc. (Opriessnig T et al. 2011).

Since its initial discovery in 1991, PRRSv has today become the most economically significant pathogen of the swine industry worldwide. In the U.S. alone,

annual losses were estimated to be approximately \$1 billion, including indirect cost such as biosecurity measures, veterinary services etc. (Holtkamp D 2011). PRRSv is endemic in U.S., with 49.5% of unvaccinated grower/finisher pigs positive for PRRSv specific antibodies (USDA-APHIS VS CEAH info sheet January 2009). In Iowa, the top pork producing state in the U.S., approximately 30 million pigs are raised each year and the total value added by Iowa pork producers to the state is more than \$2.5 billion (Iowa Pork Producers Association, 2008). As of December 2011, USDA estimated a total of 65.9 million hogs and pigs in the U.S. (AgMRC Agricultural Marketing Resource Center, July 2012). If Iowa roughly accounts for half the value to the pork producing industry, the annual PRRSv-associated loss of \$1 billion roughly accounts for a fifth of \$5-6 billion/year industry.

PRRSv strains are genetically quite divergent with up to 40% variability between the two main genotypes, type 1 or the European (EU) genotype and type 2 or the North American (NA) genotype. Even within the NA genotype, up to 20% genetic variability has been recognized, which is further reflected in the variability in antigenicity, virulence and pathogenicity of PRRSv isolates within a genotype (Kim et al. 2007). The NA genotype can be further divided into nine distinct lineages based on phylogenetic analysis; the definition of “lineage” indicates separation by at least 10% from neighboring lineages (Shi M et al. 2010)

Virus structure and genomic organization

The size of PRRSv particles is 40-60 nm and they are spherical in shape. The positive-sense, single-stranded genome is 15 kb in length; it is polyadenylated on the 3'-

end and capped on the 5'-end (Snijder EJ and Meulenberg JJM. 1998). Seventy-five percent of the PRRSv genome from the 5'-end encodes for non-structural proteins such as the helicase and the polymerase. The rest of the genome encodes for structural proteins such as glycoproteins GP2, GP3, GP4 and GP5, nucleocapsid protein N, ion-channel like protein E and matrix protein M. GP5 and M proteins form a dimer and this dimer is present most abundantly on the surface of the virus (Dea S et al. 2000). All glycoproteins are differentially glycosylated, and this glycosylation is known to play a role in the modulation of the host's immune response (Ansari IH et al. 2006).

Virus transmission and replication

Aerosol route is the primary mode of transmission of PRRSv between pigs and herds (Linhares D et al. 2012) and it has been reported that PRRSv can remain infectious after being transported for up to 9.1 km in the air from infected farms (Otake S et al. 2010). All Arteriviruses are macrophage-tropic and the primary target cell for PRRSv is the porcine alveolar macrophage (PAM). Cancerous cell lines derived from monkey kidney cells such as MARC-145 are routinely used to propagate PRRSv. The viruses are taken up into the cells following receptor-binding and endocytosis in a clathrin-dependent manner. Once in the low-pH environment inside the endosome, conformational changes lead to the release of the genome into the cytoplasm (Snijder EJ & Meulenberg JJM 1998).

Newly formed virus particles are assembled by budding of preformed nucleocapsids in the lumen of the endoplasmic reticulum (ER) and are released by budding off the plasma membrane. The infection of primary cells and cell lines by

PRRSv is cytopathic and this apoptosis is characterized by rounding of the cells and detachment from the culture plate can be observed around 10-20 hours post infection (Snijder EJ & Meulenberg JJM 1998).

An alternative mechanism of virus uptake into the target host cell can occur via the Fc receptor-mediated endocytosis, and antibody-dependent enhancement (ADE). Several researchers, both involved with PRRSv or other viral pathogens such as in family Flavivirus, have established ADE as a common mechanism used by the virus to evade the host immune response and cause persistent infections (Yoon KJ 1996, Cancel-Tirado SM 2003). In a study with 17 PRRSv field strains, a strain-dependent susceptibility to disease enhancement or neutralization by antibodies was demonstrated (Yoon KJ 1997). The characteristics and specificity of the antibody molecules has a huge impact on the outcome of infection (Brady JL 2005), supporting the different variability on ADE amongst strains.

Immune response to PRRSv

The interaction between PRRSv and the host immune response is complex and gaps still exist in the knowledge base. However, it has now been widely accepted that PRRSv displays immuno-suppressive behavior, which weakens the innate immune response, ultimately affecting the adaptive immune response.

Innate immunity

The immuno-suppressive behavior of PRRSv can be detected as early as 2 days post infection. Several studies have shown that the hallmark of PRRSv infection is the downregulation of type I interferon response that is essential for anti-viral activity

(Albina E et al. 1998, Buddaert W et al. 1998, van Reeth K et al.1999, Murtaugh MP et al. 2002). In a recent study, it was demonstrated that pigs infected with PRRSv had 50% reduction in Natural Killer cell (NK) cytotoxicity (Dwivedi V et al. 2012). The ultimate implication of a weaker innate immune response is the low and delayed stimulation of the adaptive immune response, negatively impacting the outcome of infection.

Cell mediated immunity

Various PRRSv researchers have demonstrated the delayed onset of cell-mediated immunity in PRRSv infected pigs. The immune response in sows which were repeatedly immunized with a MLV vaccine was compared to that of naïve sows and the authors demonstrated that naïve pigs had a greater antigen-specific proliferation of CD8⁺ and CD4⁺ T cells compared to the immunized pigs (Bassaganya-Riera J et al. 2004). Similarly, it has been reported that virus-specific IFN- γ secreting cells in pig lymphocyte population remain low for up to 3 months in both MLV vaccinated or naturally infected animals; the levels gradually increased by six months (Meier WA et al. 2003). On the other hand, the IFN- γ response was not detected at all under field conditions (Dotti S et al. 2013).

It was also reported that the PRRSv-specific T-cell response is weak, highly variable among animals and is different between secondary lymphoid tissues within an animal. This weak response is seen even though macrophage abundance is not altered (Xiao Z et al. 2004).

Resolution of viremia has been known to occur in the absence of a T-cell response, as is also seen with LDV infection. This all suggests that the cell mediated

immunity may not correlate with protection and thus there might be another mechanism of resolving viral replication

Humoral immune response

Antibodies towards the N protein can be detected as early as 7 days post infection (p.i.); by 14 days p.i, sera is reactive towards M protein and by 72 days p.i, antibodies react with GP5 of the infecting strain or similar strains. The reactivity has been shown to diminish as the heterogeneity increases between strains (Delputte PL et al. 2004). The appearance of this robust immune response early in infection does not protect against subsequent infection as determined by passive transfer experiments (Lopez OJ and Osorio FA 2004). Also, as mentioned previously, the presence of these non-neutralizing antibodies has been implied in enhancing disease through ADE (Yoon 1996). Overall, it is widely assumed in the PRRSv scientific community that the early antibody response is non-protective.

Virus neutralizing antibodies, on the other hand, do not appear until 3-4 weeks after PRRSv infection and the titers typically remain low even later in infection. These neutralizing antibodies are commonly being determined by fluorescent focus neutralization assay on MARC-145 cells. Neutralizing antibodies (Nab) have been shown to protect PAM cells upon infection *in vitro* as well, at least with homologous strains (Delputte PL et al. 2004). Though the role of these Nab as correlates of protection from infection and viral clearance is still a controversial topic, several studies pinpoint to a key role of antibodies. It has been shown that passive transfer of PRRSv-specific Nab that appear late during PRRSv infection protects from infection in a dose-dependent

manner (Lopez OJ et al. 2007). Recently, it was demonstrated that the Nab levels were the only response able to predict the duration and level of PRRSv viremia based on samples from experimentally infected pigs (Molina RM et al. 2008). The specificity of antibodies plays a major impact on the outcome of PRRSv infection. This fact is also supported by a study demonstrating that use of certain proteins in immunizations leads to a protective immunity, which can be correlated with the appearance of Nab (Bastos RG et al. 2004). All these observation suggests that the elicitation of Nab is important for the success of vaccines to protect against PRRSv (Lopez OJ and Osorio FA 2004).

Current vaccines

Modified live virus (MLV) or attenuated vaccines

Commercial vaccines-

The amount of genetic, antigenic and biological differences among PRRSv strains makes it a challenge to access the efficacy of vaccines; and to predict the outcome of protection towards different strains. Several reports suggest that the efficacy of the vaccine depends on the antigenic and genetic diversity of the challenge strain, with complete protection elicited when challenged with homologous strain rather than heterologous strain (Laberque G et al. 2004). However, other studies have reported that genomic homology is not predictive of degree of protection conferred by a vaccine and thus the vaccine would have to be constantly reformulated (Prieto C et al. 2008).

MLV or attenuated vaccines are the most widely used vaccines available on the market today. It has been reported that these vaccines are efficacious in reducing PRRSv-induced clinical diseases even in the face of heterologous strain challenge;

though complete virological protection is not provided against homologous or heterologous strain (Charerntantanakul W et al. 2006, Okuda Yo et al. 2008, Labarque G et al. 2003). Complete protection induced by a MLV vaccine (Pyrsvac-183, Spain) was demonstrated in vaccinated pigs upon challenge with the heterologous strain LV (Zuckermann FA et al. 2007). This protection was seen even with low levels of Nab or PRRSv-antigen-specific IFN- γ secretion. Similar results were seen with an attenuated MLV vaccine based on a highly pathogenic (HP-PRRSv) strain in pigs challenged with the homologous low passage strain. Protection from clinical signs and reduction in viremia were observed even in the absence of Nab response, suggesting that it is not the only correlate of protection (Leng X et al. 2012).

Partial protection has also been reported in gilts vaccinated with MLV vaccine before breeding and challenged with heterologous strain during gestation, with vaccinated gilts showing better reproductive performance than challenge control gilts (Scortti M et al. 2006).

MLV vaccination is also used to reduce and prevent PRRSv transmission through semen into breeding herds. A reduction in level and duration of PRRSv viremia along with reduced seminal shedding was observed after challenge with a heterologous strain (Nielsen TL et al. 1997). While vaccinated boars can still transmit PRRSv to naïve animals through semen and other routes, the virus quantity in semen is reduced compared to natural infection

In the past, countries that utilized the Ingelvac® PRRSv-MLV vaccine have detected MLV-strain like viruses. Two PRRSv isolates were obtained from herds

vaccinated multiple times with the Ingelvac® MLV vaccine and were compared in the pig challenge model (Opriessnig T et al. 2002). Based on sequence, amino acid analysis of ORF5 and pathogenicity in the challenged pigs, it was determined that one of the isolates was a direct derivative of MLV vaccine strain and had reverted to virulence. In a study researchers were also able to identify mutations occurring spontaneously in VR2332 strain, the parent strain of the MLV vaccine and pathogenic isolates collected shortly after vaccination to determine the selection pressure on these strains (Nielsen HS et al. 2001). Thus, there are concerns of reversion or recombinations associated from the use of any MLV vaccines.

Promising experimental MLV vaccines-

Other experimental vaccine in literature that has showed considerable promise is an MLV vaccine (Ingelvac® PRRS MLV, Boehringer Ingelheim Vetmedica) with *Mycobacterium tuberculosis* whole cell lysate (MT-WCL) as an adjuvant. Pigs that receive this vaccine along intra-nasally and which were later challenged with heterologous strain MN184 had better correlates of protection such as higher Nab and a better cell-mediated response (CMI; higher frequency of INF- γ secreting cells, reduced secretion of immunosuppressive cytokines such as IL-10 and TGF- β , reduced frequency of T_{regs}, increased frequency of $\gamma\delta$ T-cells and CD4⁺CD8⁺ DP T-cells). This CMI response and the humoral response were concomitant with reduced viral loads and reduced clinical PRRSv signs in the vaccinated group as compared to the unvaccinated challenge control group (Dwivedi V et al. 2011).

Alternative vaccination strategies- Autogenous vaccine

Aside from traditional vaccine, alternative means of eliciting immunity mimicking the natural infection have also been utilized. These approaches include exposure of the whole herd or farm to a farm-specific strain of PRRSv to induce long-term immunity in the herd. This was initially achieved by mixing naïve pigs with infected or feeding naïve pigs aborted fetuses or infected tissues. Serum inoculation replaced the earlier practices and it has been reported that planned exposure of pigs with PRRSv positive serum from infected pigs can prevent clinical diseases associated with homologous challenge and partially protect against viremia (Opriessnig et al. 2007).

Killed/inactivated vaccines

It has been reported by several PRRSv researchers that the commercial inactivated vaccine such as Progressis (Merial Ltd, France) provide only limited protection against homologous or heterologous challenge and elicit low levels of Nab (Geldhof MF et al. 2012, Vanhee M et al. 2009). Low levels of Nab were recognized after pigs received three doses of an inactivated vaccine (Zuckermann FA et al. 2007). Upon challenge with Lelystad Virus (LV), prototype EU strain, an anamnestic humoral response was elicited in vaccinated pigs even though this response was not protective. Increased elicitation of Nab against homologous strain along with reduction in titer and duration of viremia in serum were seen when using UV or binary ethylenimine- (BEI) inactivated LV virus in combination with four different commonly used adjuvants (Vanhee M et al. 2009). Inactivation of field strains using BEI and an oil-in-water adjuvant was sufficient to prime the host's immune response to elicit a partially

protective response towards the homologous strain as determined by viremia reduction and induction of a Nab response. Some VN antibodies were also formed against the heterologous strain and this proves that inactivated vaccines can be used to prime the host's immune response. It should be noted that these autogenous inactivated vaccines had the same level of protection as the commercial attenuated vaccines in terms of reduction of viremia. Commercial attenuated vaccines elicited no or low Nab antibodies (Geldhof MF et al. 2012).

Scotti M et al. reported that the use of inactivated vaccine before breeding increases the reproductive performance of gilts upon challenge with heterologous strain even though protection from viremia and transplacental spread to piglets is not prevented. They attribute this increased performance on the appearance of Nab elicited faster due to the priming of the immune response by the inactivated vaccine (Scotti M et al. 2007).

In summary, over two decades have passed since the discovery of PRRSv but there is still no means to eradicate this costly virus. Thus there is an urgent need to develop an efficacious vaccine which, in order to be successful, would have to offer protection from constellation of heterologous strains circulating in the field. Considering all the investigated and tested vaccine platforms, the solution likely has to be novel and unconventional.

Evasion strategies and immunopathogenesis

It is well recognized that PRRSv infection increases the susceptibility to secondary bacterial and viral infections in pigs. Many factors are involved in the clinical

manifestation of PRRS. During PRRSv infection, the mucociliary transport system in the lungs is impaired which inhibits the removal of microorganisms, thereby increasing the incidence of secondary infection. PRRSv infected PAMs display reduced phagocytosis activity and there is a marked increase in apoptosis of these cells, directly impacting the adaptive immune response. Also, an imbalance is created between pro-and anti-inflammatory cytokines, all resulting in increased susceptibility to other infections. For example, TNF- α expression is greatly reduced in infected PAMs impacting viral clearance from the host. On the other hand, IL-10, a regulatory cytokine that inhibits the synthesis of pro-inflammatory cytokines, is enhanced (Gomez-Laguna J et al. 2013).

Besides the immunomodulatory mechanisms that allows for the evasion of the host immune response, PRRSv is also known to employ several other evasion strategies leading to persistent infection. Some of these evasion strategies are downregulation of major histocompatibility complex (MHC) in infected macrophages, lack of surface expression of viral proteins in infected cells and differential glycosylation of neutralizing epitopes (Kimman GT et al. 2009). The ultimate effect of these strategies is the reduced quality and breadth of adaptive immune response. Though extremely important, the reader is directed elsewhere for details of other evasion strategies. From here on, the focus will be entirely on one evasion strategy pertinent to the research hypothesis described in the thesis, deceptive imprinting.

Deceptive imprinting

The phenomena of deceptive imprinting is based on the observations of Original Antigenic Sin (OAS), a term first coined in 1953 by Thomas Francis Jr. based on

serological observations made during infection with influenza virus. OAS is a phenomenon where the immune response is directed towards variants of an epitope influenced by the sequential order in which the host is exposed to them (Chapter 6, Steven A Frank, Princeton University Press). Upon secondary exposure to an antigenically similar strain, the immune response is often with a higher affinity elicited towards antigens of the strain exposed previously. Hence, instead of a primary immune response towards epitopes of the new variant, there is a secondary response towards the initial variant of the epitope, at the cost of eliciting responses towards other epitopes. Most observations for OAS have been made with influenza virus A infections but studies on other viruses such as flaviviruses point to the fact that it is a general phenomenon (Morens DM et al. 2010). It is an understanding that the initial variants/epitopes elicit robust immune response towards them, albeit one that is type-specific or limited and one of the major implications is that they allow for viral escape due to high hypervariability. Thus these immunodominant epitopes act as decoys that direct the immune response away from more conserved, less immunodominant regions, by being more immunogenic and by sterically protecting the conserved functional sub-dominant epitopes that elicit protective immune response. Many pathogens have been used as models to illustrate the phenomenon of deceptive imprinting such as, Foot-and-Mouth disease virus (FMDV), human Immunodeficiency virus (HIV-1), human influenza virus and caprine arthritis encephalitis virus (CAEV). For example, five major antigenic sites/immunodominant regions have been recognized in hemagglutinin (HA) trimer of H3 subtype. Overlapping of HA from other influenza subtypes such as H1 and H7 has shown that

even though the amino acid sequence is variable, the 3D structure is well conserved. In case of FMDV, 4-5 major epitopes have been identified, with the highly variable GH-loop of VP1 being the most immunodominant (Tobin GT 2008).

Immune Refocusing

It has been demonstrated with human influenza virus, FMDV, HIV-1 and CAEV that modification of immunodominant epitopes (IDD) allows for the elicitation of immune response towards previously subdominant epitopes. This then leads to the generation of broad protection towards heterologous strains. These modifications to the immunodominant epitopes can be in the form of addition of N-linked glycans to mask some or all part of IDD, elimination/deletion or other substitutions. Immune refocusing is thus the means by which the immune response is shifted from type-specific or non-protective epitopes to subdominant or conserved epitopes which provides protection against heterologous strains.

One such pathogen in which the concept of immune refocusing has been proven to be successful is CAEV, as tested in a goat immunization trial (Trujillo JD et al. 2004). This virus is a macrophage trophic lentivirus and as the name suggests, causes arthritis in goats. Upon immunization or natural infection, most of the humoral immune response is directed to the surface glycoprotein (SU). However this response is mainly non-neutralizing and has been shown to be elicited towards immunodominant linear epitopes. To test the effectiveness of immune refocusing from linear IDD epitopes to subdominant protective ones, SU of strain CAEV-63 was modified and constructed by either insertion of two N-linked glycans (SU-M) or deletion of amino acids upstream of the targeted

epitope (SU-T) in the C-terminal domain. SU-M, SU-T and SU-W (unmodified wild-type) was then used as immunogen in an immunization trial with 3 booster shots in three groups of goats. Neutralizing antibodies were tested against homologous strain (CAEV-63) as well as two different heterologous strains (CAEV-Co and CAEV-1g5). Goats vaccinated with SU-M demonstrated 2.4-2.7-fold increase in titer of both type-specific and cross-reactive NA as well as reduced recognition of glycosylated target epitopes. However, goats immunized with SU-T showed altered recognition of linear epitope, with a shift in response towards a second linear IDD and 2.8-4.6-fold decrease in type-specific and cross-reactive NA titers. Moreover, vaccinated goats were also challenged with a heterologous strain to assess the protective nature of the shift in immune response and also to evaluate the persistence of immune refocusing. It was demonstrated that only goats that were vaccinated with SU-M recognized neutralizing epitopes upon challenge and thus recognized SU differently than SU-W or SU-T immunized goats. 42 weeks post challenge, sera from goats vaccinated with SU-M had 17% of SU antibodies represented as neutralizing compared to less than 1% in the other two groups.

This CAEV challenge trial serves as a proof-of-concept model that a vaccine generated using the concept of immune refocusing can be used to elicit neutralizing antibody responses against heterologous strains; and this ability to recognize neutralizing antibodies can persist for a long duration.

Current state of knowledge of immunodominance in PRRSv

To design immunogens to refocus the immune response from immunodominant epitopes to subdominant ones, the antigenic structure of all the glycoproteins on the

surface of virions has to be characterized along with the immune response it elicits. Unfortunately, the state of knowledge regarding the same is controversial and incomplete at best at this point in time.

Two epitopes on the ectodomain of GP5 protein were identified using a 12-mer phage-display library and swine sera from hyperimmunized mice. Epitope A was determined to be highly immunodominant early and through-out the infection whereas epitope B is conserved among several PRRSv isolates, and was recognized by both the monoclonal antibody (MAb) ISU25-C1 from mice and test sera from a different study known to contain high titer of Nab (Ostrowski M et al. 2002). This same region also has a counterpart in LDV; epitope A is located seven amino acids from epitope B but both are independent of each other and can be recognized simultaneously. However, Li and Murtaugh demonstrated that the ectodomain which contains epitopes A and B is accessible for host antibody recognition but is not associated with virus neutralization. Initially antibodies specific towards GP5-ectodomain, GP5-M ectodomain and M-ectodomain were purified using sepharose beads coupled with synthetic polypeptides. These purified antibodies had no Nab activity in sera previously shown to contain neutralizing activity against homologous strain. They also showed that these polypeptides did not interfere with PRRSv infection of PAMs. Thus with this work this group demonstrated that GP5/M surface proteins do not contain neutralizing epitopes and are not directly involved in viral entry in PAMs; even though these epitopes are immunogenic and elicit specific antibody response (Li & Murtaugh 2012).

Similar differing results are available for epitopes on other glycoproteins of PRRSv; a limitation of these studies is the use of PepScan analysis or isolation of specific antibodies utilizing linear polypeptides. Even though this technology would allow for the recognition of multiple epitopes on the same protein, it would not allow for the detection of conformational epitopes. Also, with the high amount of genetic variability observed between PRRSv strains, the use of synthetic polypeptides might lead to false negative results.

Thus using immune refocusing techniques for PRRSv might be unfeasible at the moment with the lack of significant information in PRRSv antigenic hierarchy. Towards this end, we have developed a novel vaccine strategy presented in chapter III to overcome the knowledge gap.

Conclusion

More than 25 years after the discovery of the etiological agent of PRRS, PRRSv still remains the most economically significant pathogen of the swine industry. Current vaccines offer limited or no protection towards heterologous strains, which is a major hindrance to the field considering the high mutation rate reported for PRRSv. Infection with PRRSv increases the susceptibility to secondary infections in animals, thereby adding to the cost associated with PRRSv. Development of an efficacious cross-protective vaccine is one of the top priorities for PRRSv researchers and the pig industry if any attempts of eradication of the virus are to be successful.

Pathogens have evolved a mechanism whereby the host immune response is directed towards type-specific, limited and hypervariable epitopes, allowing for

development of escape mutants and at the expense of generation of a protective immune response. This mechanism or phenomena used by pathogens as an evasion strategy is termed “deceptive imprinting”. Multiple studies have been conducted to demonstrate a shift towards a cross-protective immune response from a type-specific or non-protective immune response by modifications to the immunodominant epitopes (Tobin GT 2008). These modifications included addition of N-linked glycans, target deletion and others. The strategy of modifying the immune response is called “immune refocusing” and allows for the development of vaccines with better efficacy (Trujillo JD et al 2004). One hindrance using this approach for vaccine production is that thorough knowledge of the hierarchy and characterization of all antigens of the investigated virus is required. There are considerable knowledge gaps regarding the same in PRRSv biology due to the complexity of the virus and the limitations of current techniques used for the detection of antigens.

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CHAPTER III

IMMUNE-COMPLEX BASED VACCINE EFFICACY PILOT TRIALS IN PIGS CHALLENGED WITH A HETEROLOGOUS STRAIN OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS

The phenomenon of deceptive imprinting used by various pathogens as an immune evasion strategy is well recognized (Garrity RR et al. 1997). Before the phenomenon of deceptive imprinting and hierarchy of immunodominance was discovered, it was already recognized that the quality of the immune response is more relevant than the quantity for providing protection against pathogens. For example, P1 antigen of *Streptococcus mutans*, a predominant etiological agent of dental caries, allows for the attachment to the acquired pellicle of teeth. Different specificities of immune response against different determinants have been demonstrated between caries-susceptible and -resistant individuals. Previously five anti-P1 MAbs were screened for their immunomodulatory activity by complexing them with *S. mutans* followed by the intraperitoneal administration of the immune complex (IC) to mice (Oli MW et al. 2004). The sera obtained from the mice were then tested for their protective activity by accessing inhibition of adherence of *S. mutans* to immobilized human salivary agglutinin. Two out of five MAbs had a more effective adherence response; two others had a less effective response whereas one did not have any impact of adherence.

Much more work is needed for detailed elucidation of the working mechanisms of protective vs non-protective antibodies. The quantity, subclass of antibody, Fc modulation, direct or indirect interaction with the host cell surface receptor and others

might be some of the reasons for the variable outcome. From the standpoint of making better efficacious therapeutics against pathogens as well as to answer fundamental questions about the host immune system-pathogen interactions, it is essential to study and characterize these variable antibody responses towards various determinants in detail. This would then aid in the generation of a protective response from the host from a therapeutic point of view. The following section offers one way to shift the immune response from type-specific, non-protective to a one that offers cross-protection.

Antibody-virus complex

Immune refocusing requires thorough knowledge of the hierarchy of epitopes for the pathogen of interest. By modification of the right targets, the immune response can be subverted from immunodominant epitopes towards subdominant protective epitopes. As the location of most epitopes for PRRSv still needs to be determined, our lab has rationalized the design of the vaccine platform for the generation of an efficacious broadly protective PRRSv vaccine. Antibody-virus complex is generated using immunoglobulin; and here the IgG subtype is obtained from serum samples from pigs infected with PRRSv during the acute phase. These IgG are complexed with live replicating homologous PRRSv. The rationale for using IgG obtained during the acute stage of PRRSv infection is that the robust antibody response generated during early infection has been proven to be non-protective (Lopez OJ and Osorio FA 2004). Our hypotheses were that, firstly, the specificities of these antibody molecules obtained from sera during the early phase of PRRSv infection is towards the immunodominant epitopes. Secondly, when complexed with virus, these molecules would mask the

immunodominant epitopes, allowing for recognition of subdominant ones; ultimately eliciting a cross-protective immune response. It should be noted that the scope of the research presented in this thesis is limited to the evaluation of the efficacy of Ab-virus complex vaccine against heterologous challenge only. The working mechanism of the vaccine has yet to be determined.

It should also be noted that the use of antibody bound to antigen has been demonstrated in various pathogenicity models to have an immunomodulatory effect, depending on the particular antigen and antibody used. Examples include *S. mutans*, Hepatitis B virus and others (Bouige P et al. 1996, Oli MW 2004). For Hepatitis B, the researchers used MAb towards the S region of hepatitis B surface antigen (HBsAg) and demonstrated increased recognition of pre-S2 region after administering the immune complex in mice. They also tested other MAb and not all IC tested had an enhanced effect, which is similar to what has been observed with *S. mutans* study (Bouige P et al. 1996). Antigen-antibody immune complexes are also the basis of a licensed vaccine against infectious bursal disease virus (IBDV) where addition of polyclonal chicken serum samples to a vaccine has shown to offer better protection than the uncomplexed vaccine (Haddad EE et al. 1997).

The objective of this chapter was to test the efficacy of Ab-virus complex vaccine against heterologous challenge with PRRSV in pigs. Towards this end, we conducted small pilot vaccine efficacy trials to get an initial assessment of the efficacy of the Ab-virus complex vaccine.

Results of the Antibody-virus complex vaccine efficacy pilot studies for PRRSv

Two separate animal pilot studies were conducted to get an initial assessment of cross-protection offered by the Ab-virus complex PRRSv vaccine. In order to formulate the vaccine, another objective of these initial trials was to evaluate the infection stage of PRRSv in pigs from which the immunoglobulin would be isolated. The Ab-virus complex vaccine is a complex of live replicating PRRSv and immunoglobulin; we hypothesize that the immunoglobulins would mask the immunodominant epitopes allowing for the recognition of conserved protective subdominant epitopes.

In the first pilot study, two Ab-virus complex vaccine groups were included. Ab-virus complex-Vac I was a complex of PRRSv strain VR2332 and IgG purified during mid-infection (28 days post inoculation, dpi) whereas Ab-virus complex-Vac II used the same PRRSv strain but IgG from late PRRSv infection (84 dpi). Non-vaccinated pigs served as negative control and autogenous vaccinated pigs (pigs infected with VR2332) as positive controls; there were 3 pigs in each of the 4 groups. All treatments were given by the intramuscular route. All pigs were bled weekly and challenged with heterologous strain JA142 (9.5% divergent from VR2332 based on the GP5 sequence) at 63 days post vaccination; all animals were then euthanized 1 week post challenge (74 days post vaccination).

All vaccinated animals seroconverted to PRRSv 7-14 days after vaccination, indicating that the vaccine preparation were immunogenic. The antibodies generated in each group of pigs were also tested for their neutralizing activity against VR2332 (homologous strain) as well as three other heterologous strains. (NC16845 is 7%

divergent from VR2332 based on the GP5 sequence; JA142 is 9% divergent and VR2385 is 10% divergent). Pigs vaccinated with Ab-virus complex-Vac II elicited Nab towards the homologous strain a week prior to the pigs in the autogenous group and had 2-fold higher titer in subsequent weeks. No difference was seen in the elicitation of neutralizing antibodies in kinetics or titer against NC16845. When the sera from all pigs were tested against JA142 and VR2385, Ab-virus complex vaccinated pigs elicited Nab 3 weeks prior to those in the autogenous group. It should be noted that the titer of these antibodies remained low as has been documented for PRRSv by other researchers as well.

Viral RNA prevalence and load in serum and lung tissues and macroscopic and microscopic lung lesions were evaluated as end-points of protection. All non-vaccinated control pigs had 5-7 logs of PRRSv in serum. All pigs receiving the autogenous vaccine and 2/3 of the Ab-virus complex-Vac I (mid-complex Ab-virus complex) pigs had undetectable levels of virus and the third pig in this group had reduced PRRSv loads. Ab-virus complex-Vac II (late-complex Ab-virus complex) group had a reduction of PRRSv prevalence and viral load but due to high variability between animals this was not significant. Macroscopic and microscopic lung lesions followed the same trend as viral loads, demonstrating that in this trial both autogenous and mid-complex Ab-virus complex were efficacious in preventing infection against heterologous strain. Due to the small number of animals per group and the variability between animals in the same group, significant differences were not observed in any of the parameters evaluated.

In the second pilot study, IgG molecules were isolated from the early (14 dpi) and the late phase (62 dpi). The Ab-virus complex-Vac III was a complex of JA142 with serum obtained at 14 dpi whereas the Ab-virus complex-Vac IV vaccine was JA142 with serum obtained at 62 dpi. The efficacy of these Ab-virus complex vaccines was also compared side-by-side with that of a commercially licensed vaccine from Boehringer Ingelheim Vetmedica (Ingelvac® PRRSv), as well as with an autogenous vaccine based on JA142.

Eight pigs in each group were vaccinated with PRRSv strain JA142. Four pigs in each group were then challenged with the homologous strain (JA142) and four with heterologous strain IA2?1 (15% divergent from JA142 based on GP5 sequence) 56 days post vaccination. The pigs were bled weekly pigs were euthanized at 2 weeks after challenge (69 days post vaccination).

After challenge, PRRSv RNA was detected in one pig challenged with the homologous group in the Ab-virus complex-Vac III group and not in any pigs in autogenous vaccine group. Nab data also suggested a trend in early appearance of neutralizing antibodies compared to the autogenous group. The obtained lung lesions were mild in all groups.

Conclusion

The use of antigen-antibody complex or immune complex (IC) to enhance the beneficial immune response towards a pathogen is known for a long time. However, the immunomodulatory effect of IC, i.e. enhancement, suppression or no effect on beneficial protective responses depend on the particular combination of antigen and antibody used

in the IC. Though the exact mechanism of immunomodulation needs to be accurately characterized, it has been shown that the use of MAb or polyclonal antibodies against certain highly immunogenic epitopes allows for the recognition of previously unrecognized epitopes. This concept of the presence of epitopes with different immunogenicities and the beneficial or undesirable effect on the outcome of infection depending on which epitope elicits an immune response is gaining more importance in infectious disease research.

The use of IC for development of an efficacious cross-protective vaccine for PRRSv might be a good solution as it does not require extensive studies on antigen analysis and design. Ab-virus complex vaccine is an IC where the whole virion is complexed with immunoglobulin isolated from sera from a PRRSv infected animal. Since the immunomodulatory effect of IC depends on particular antigen as well as antibody, we tested IgG from multiple phases of PRRSv infection to isolate these. We conducted two pilot vaccine efficacy trials against homologous and heterologous challenge and looked at Nab activity, viral RNA in serum and tissues, and pathological lesions. Results from these pilot studies suggest that heterologous protection is provided by the Ab-virus complex vaccine but different from the control autogenous vaccine, as animals in the Ab-virus complex vaccine group elicited Nab 3-4 weeks earlier.

The working mechanism of Ab-virus complex vaccine needs further evaluation but in order to demonstrate reliable and reproducible efficacy, much larger trials have to be conducted. The results generated from these pilot studies can be used to answer some fundamental questions regarding PRRSv biology and its interaction with the host

immune response. Moreover, PRRSV can serve as a model pathogen for development of vaccines for other pathogens that have proved to be a burden to human medicine.

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CHAPTER IV

Written in preparation for publication

**EFFICACY OF AN IMMUNE COMPLEX-BASED VACCINE TO PROTECT
AGAINST HOMOLOGOUS AND HETEROLOGOUS PORCINE
REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS CHALLENGE**

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Abstract

More than three decades after its discovery, Porcine Reproductive and Respiratory Syndrome virus (PRRSv) remains economically the most important virus affecting the swine industry worldwide. Current lack of understanding of immune correlates of protection in naturally PRRSv infected or vaccinated pigs along with the presence of heterogeneous strains have hindered the development of an efficacious vaccine that offers sufficient protection against heterologous strains. We have developed a cross-protective, immune complex based PRRSv vaccine prototype by utilizing antibodies isolated from porcine sera from acute phase of PRRSv infection. Antibody-Immunogen Masking (Ab-virus complex) is hypothesized to work by blocking of highly variable immunodominant epitopes on the viral surface thereby enabling recognition of cross-protective subdominant neutralizing epitopes. To test the efficacy of Ab-virus complex vaccine in clearance and prevention of infection against heterologous strain in a validated challenge-animal model, 9 pigs were vaccinated with the experimental Ab-virus complex vaccine and 10 pigs with autogenous vaccine at day 0 while 10 pigs served as positive challenge controls. At day 28, all pigs were challenged with a

heterologous strain IA2?1. Three of 9 Ab-virus complex vaccinated pigs were able to clear virus infection in the serum (7 and 14 days post challenge) and the lungs (14 days post challenge) whereas only 2/10 pigs receiving the autogenous vaccine cleared the infection. All challenge-controls were infected. Reduction in virus loads was correlated with low PRRSV-specific lesions in the lungs. To develop an efficacious vaccine against a constellation of PRRSV strains, the Ab-virus complex vaccine might be an important tool worth further investigation.

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSv), an *Arteriviridae* virus in the order *Nidovirales*, is currently the most important infectious disease of pigs, causing late-term reproductive failure in sows and respiratory disease of variable severity levels and poor growth performance in growing pigs. Estimated economic losses due to PRRSV in the United States had increased from \$560 million/year in 1999-2005 to over \$1 billion/year during 2006-2011 (Holtkamp D et al. 2011 and Neumann EJ et al. 2005). PRRSV has now become endemic in the U.S., with 49.8% of unvaccinated pigs being seropositive (USDA-APHIS VS CEAH info sheet January 2009).

Two genotypes of PRRSV are recognized, North American (NA) and European (EU), which display 40-50% genotypic and antigenic variability. PRRSV has reportedly the highest mutation rate ($4.7-9.8 \times 10^{-2}$ /site/year) amongst RNA viruses, which further adds to the genetic variability observed between PRRSV isolates within a genotype (up to 20% variability within NA genotype) (Gojobori T et al. 2005). Current commercial available vaccines against PRRSV infection include modified live virus (MLV) vaccines and inactivated vaccines. In addition,

autogenous vaccines, which contain low doses of circulating PRRSv strains, are also frequently used to improve the herd-level immunity (Opriessnig T et al.2005). All of these types of vaccines have partially or completely failed to provide protection against infection with heterologous strains (Lager KM et al.1999, Labarque G et al. 2004, Delputte PL et al. 2004, Charentantanakul W et al. 2006, Okuda Y et al. 2008, Labarque G et al. 2003). One of the major factors for such observations is the heterogeneity displayed by PRRSv virus (Murtaugh 2011, Kimman GT et al. 2009). Thus development of an efficacious cross-protective vaccine for PRRSv is one of the priorities for the swine industry and presented here is a novel technology of developing such a vaccine based on the hypothesis of immune refocusing.

Though the correlates of protection from infection and viral clearance are still a controversial topic among PRRSv researchers, several studies pinpoint to a key role of antibodies. It has been shown that passive transfer of PRRSv-specific neutralizing antibodies (Nab) that appear late during PRRSv infection protects from infection in a dose-dependent manner (Lopez OJ et al.2007). Zimmerman et al. reported that Nab level were the only response able to predict the duration and level of PRRSv viremia based on samples from experimentally infected pigs; other responses measured were binding antibody response and IFN- γ secreting cells (Molina RM et al. 2008). This observation suggests that the elicitation of Nab is important for the success of vaccines to protect against PRRSv.

However, Nab against PRRSv typically only appears in low levels 3-4 weeks post-infection. Therefore our hypothesis was that the low Nab response during PRRSv infection is due to an immune evasion strategy referred to as deceptive imprinting. This phenomenon is

where a pathogen evades the oligoclonal immune response of the host by presenting highly variable immunodominant epitopes. The immune refocusing technology is a mean by which the immunodominant epitopes are mapped and modified in such a way as to mask or dampen their immunogenicity. Masking or removing of immunodominant epitopes has been proven to facilitate the host immune system recognition of more highly conserved epitopes that elicit a Nab response. Immune refocusing of immunodominant epitopes of available strains against Human Immunodeficiency Virus-1, Influenza A virus, Caprine Arthritis Encephalitis virus (CAEV) all have resulted in a broader immune response, containing antiviral activities of greater breadth when compared to the original unmodified strain in immunization trials in animals (Tobin GT et al.2008). Goats either naturally infected or immunized with CAEV elicited high binding antibody and low Nab responses against linear immunodominant epitope on the surface glycoprotein (SU). Masking of these target epitopes by addition of N-glycans and using these modified SU as immunogens significantly enhanced induction of Nab in goats post immunization. Immunized goats challenged with a heterologous CAEV strain had 17% Nab in their total antibody response compared to less than 1% in non-immunized challenge controls and CAEV wild-type immunized goats (Trujillo JD et al. 2004). Recently our research group has utilized a more rapid methodology to develop a vaccine based on immune refocusing. We are hypothesizing that the new technology of Antibody-Immunogen Masking (Ab-virus complex) employs naturally occurring antibodies to mask immunodominant, non-protective epitopes to allow the immune system to react against conserved, yet previously silent epitopes to generate more broadly protective responses. Purified non-neutralizing immunoglobulins obtained from animals during the acute stage of infection are utilized to

mask epitopes on the intact virion. In the past, the Ab-virus complex technology has been shown to be very effective in chicken and has led to a licensed vaccine against Infectious Bursal Disease Virus (IBDV), an economically significant pathogen in poultry industry (Haddad EE et al.1997). Pilot studies using the Ab-virus complex technology to prevent homologous and heterologous PRRSV infection following challenge showed a reduction of PRRSV load in serum by 3-5 logs compared to the controls. This reduction in PRRSV viremia also correlated with reduction in PRRSV-induced macroscopic and microscopic lesions in the lung.

The objective of this study was to compare the efficacy of the Ab-virus complex technology and a commercial available vaccine side by side in a heterologous pig challenge model. Our results showed that Ab-virus complex vaccinated pigs had lower viral loads and less severe lesions compared to the control group. In addition, complete PRRSV clearance was observed in 33% of the Ab-virus complex treated pigs compared to 0% in non-treated pigs.

Methods

All animal work was approved by Iowa State University Institutional Animal Care and Use Committee (IACUC) and the Iowa State University Institutional Biosafety Committee.

Cell and viruses- The MARC-145 cell line, derived from African green monkey kidney cells, was used to propagate and titer virus from lung tissue homogenates using Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated FBS, 0.25 µg/ml fungizone, 1000U penicillin, 1mg/ml streptomycin and 20mM L-glutamine in humidified incubator at 37° C with 5% CO₂. PRRSV strains for preparation of the

vaccine (JA142) and the challenge stock (IA2?1) were propagated on MARC-145 cells for 4 and 9 days, respectively. The lysate was collected by freeze-thawing infected flasks three times, clarified by centrifugation at 10,000 RPM for 7 min and filter sterilized using a 0.22 μ m filter. The 50% tissue culture infectious dose (TCID₅₀) was determined on MARC 145 cells using cell cytopathic effect (CPE) endpoint and the Reed and Muench algorithm. The two strains chosen for the study have 86% nucleotide identity in the ORF5 gene as determined by BLAST[®].

Vaccine formulation- Prior to the main animal study, sera were obtained from a small pilot study (data not shown) during different phases of PRRSV infection (acute dpi 7-14, chronic dpi 21-84) and evaluated for highest efficacy when complexed with the Ab-virus complex vaccine. Sera from acute infection (dpi 14) with high binding antibody response and no neutralizing antibody response were chosen for IgG purification. IgG was affinity purified using a Protein A column (Pierce). In brief, 5 ml of agarose was diluted in 10 ml PBS and loaded onto the 1.5x20 cm column and allowed to pack while running. This was followed with a washing step using 100 ml cold PBS. As part of the sample preparation, serum was centrifuged at 20,000xg for 10 min and the clarified serum was diluted 1:2 (v:v) with cold PBS. After two runs and two washing steps, all the flow-throughs were combined (flow 1). Subsequently, the column was washed with 100 ml cold PBS and IgG was eluted 3 times using 10 ml of filtered 100mM Glycine-HCL elution buffer (pH 2.6) into tubes containing 1 ml 1M Tris (pH 8.0). The elution fractions were concentrated using 50Kda cut off spin cartridges (Millipore) and the protein concentration was determined using the Bradford protein assay and SDS-PAGE

electrophoresis under reducing and denaturing conditions with coomassie staining. For the final Ab-virus complex vaccine complex, 1 mg of IgG was added to 1×10^3 TCID₅₀/ml of PRRSv JA142 in five steps keeping the virus and Ab-virus complex at 4°C.

Animals- 35 crossbreed pigs obtained from a PRRSV-free farm at weaned at 2 weeks of age and transported to the facility at Iowa State University.

Study design- Table 1 depicts the experimental design of the animal trial. Pigs were randomly divided into three rooms and 9-10 pigs per room. Vaccination of the different groups was carried out on Day 0, which corresponds to five weeks of age. All vaccines were formulated in a 2ml dose in DMEM media, administered half intra-muscularly (IM) and intra-nasally (IN). Group 1 (n=10) served as challenge controls and received sham vaccination (DMEM). Group 2 (n=9) was vaccinated with the Ab-virus complex vaccine at a dose of 1×10^3 TCID₅₀/ml of PRRSv strain JA142, complexed with Immunoglobulin G (IgG) isolated from serum of PRRSv-infected pigs early in infection. Group 3 (n=10) received an autogenous vaccination by using 1×10^3 TCID₅₀/ml of wild-type JA142. All groups were challenged with 2 ml of 1×10^4 TCID₅₀/ml of heterologous PRRSv strain IA2?1 IN 28 days after vaccination or sham vaccination. All pigs were humanely euthanized at day 42 after vaccination and necropsied. Blood samples were collected at days 0, 7 14, 21, 28, 35 and 42.

Serology- Heat-inactivated serum samples collected at days 0, 14 and 42 were tested for the presence of PRRSv-specific binding antibody response by a commercial PRRSv ELISA (Herdchek PRRS virus antibody test kit, IDEXX Laboratories). A serum sample with a sample-to-positive (S/P) ratio of or greater to 0.4 was considered positive.

Serum samples collected weekly were also screened for the presence of neutralizing activity by utilizing virus titer reduction assay. Briefly, 0.5 ml of 10^3 TCID₅₀/ml of homologous virus (JA142) or heterologous challenge virus (IA2?1) were incubated with 0.5 ml of neat serum samples for 1 hour at 37°C. Post incubation, the serum-virus mixture for each sample was two-fold serially diluted. 100µl of each dilution was then used to infect four wells of a 96 well plate seeded with MARC-145 cells at 80% confluency. After 1 hour incubation, media was replaced in infected 96-well plates with DMEM with 10% FBS, 0.25 µg/ml fungizone, 1000U penicillin, 1mg/ml streptomycin and 20mM L-glutamine and further incubated in humidified incubator at 37° C with 5% CO₂ for 7 days. The 50% tissue culture infectious dose (TCID₅₀) was determined on MARC 145 cells using cell cytopathic effect (CPE) endpoint and the Reed and Muench algorithm. Percent reduction in virus titer and percent neutralization were calculated for each serum sample. Positive and negative control sera along with virus and cell-only controls were run in triplicate in each assay.

RNA extraction- Briefly, RNA was isolated from 100 ul of each serum sample using the BioSprint 96 One-For-All Vet RNA extraction kit (Qiagen) following manufacturer's instructions. PCR grade water (Manufacturer) was used as negative control and 25ul of a positive control provided with the PCR kit was used as a positive control of extraction. In addition, RNA was also extracted from lymph node, spleen and lung obtained during necropsy on day 49. In brief, 1 gram of lymph node or spleen and 0.2 g of lung tissue from 5 different pre-defined sections of lung to represent the entire lung as demonstrated previously (Halbur et. al.1995) were thawed overnight. Minced tissue was added to 1 ml

of lysis buffer provided in the RNA extraction kit (Qiagen) along with a 5mm grinding ball. The tissues were then homogenized with Qiagen TissueLyser 4 times for 10 seconds at a frequency of 10. Proteinase K was then added to the lysed tissue, pulsed vortex and incubated overnight at 56°C with shaking at 26 rpm (Vortemp 56, Labnet). 100 ul of the supernatant was then used for RNA extraction as described for the serum samples.

Real-time RT-PCR- PRRSV RNA load was measured in the serum samples obtained on days 0-42 by using a commercial available one-step, reverse transcription assay (AnDiaTec AcuPig® PRRSV real time RT-PCR kit; AnDiaTec GmbH & Co. KG, Kornwestheim, Germany) following instructions provided by the manufacturer on 7500 Fast Real Time system (ABI).

Validation of the detection of both strains of PRRSV strains used in this study by the AnDiaTec RT-PCR kit was also performed. The results were compared with the kit commercially used in the U.S. for the detection of PRRSV (Tetracore).

For quantification, three standard curves were constructed from 10-fold serial dilution of type 2 PRRSV template RNA obtained from Applied Biosystems (TaqMan® NA and EU PRRSV Reagents, Ambion). The reproducible range for the detection of RNA was found to be 10^6 - 10^2 copy number. A sample with no amplification at a cycle threshold (Ct) of 34 was assigned negative. Samples were tested in three replicates and were considered positive if they were positive in at least two out the three replicates. In addition, samples resulting in 2 or three sigmoidal amplification curves but outside the range of detection were considered positive as well.

Pathology- Gross evaluation of lung tissues was performed in a to the treatment status blinded fashion, as previously described (Halbur et. al.1995). Briefly, each lung lobe was assigned a number that reflects an approximate volume percentage of the entire lung represented by that lobe. Gross lesions scores were then estimated for each lobe and added to get the percentage of the total lung surface affected by pneumonia. Microscopic examination of lung tissues was done on 5 pieces of lung as described previously (Halbur et al.1995). The tissues were fixed in 10% neutral-buffered formalin, embedded in paraffin in an automated tissue processor, cut into 6 μm sections and stained with Hematoxylin & Eosin (HE). A previously described scoring scheme ranging from 0= normal to 6= severe diffuse interstitial pneumonia was utilized.

Immunohistochemistry- Immunohistochemistry was utilized to visualize PRRSv antigen in lung sections as previously described with the following antisera: SDOW-17 and SR-30 (Rural Technologies). Tissues were scored for presence of PRRSv antigen ranging from 0= no PRRSv antigen detectable to 3= abundant PRRSv antigen present (Halbur et al. 1994).

Statistical analysis- Analysis for multiple comparisons of continuous data was done using ANOVA and if significant ($p < 0.05$) the Tukey-Kramer adjustment was used to determine which group mean differed. Nonparametric data were analyzed using the Wilcoxon score analysis.

Results

Strategy for vaccine design-

1-Vaccine formulation- Work from our laboratory (unpublished) and from several PRRSv researchers (Molina et al. 2008, Yoon KJ et al.1995) have demonstrated that high titers of binding antibodies are generated 10-14 days post vaccination whereas neutralizing antibodies are only generated 3-4 weeks post exposure in low titers. Fig. 1 shows the binding antibody and Nab response in pigs infected with JA142 strain of PRRSv, as detected by commercial indirect ELISA (Herdchek®) which uses antigens from the Nucleocapsid protein of both the EU and NA strains of PRRSv. This figure indicates that all animals infected with either strain had seroconverted by 14 days post infection whereas Nab was not developed until 35 days p.i.; similar results are obtained with PRRSv strains as well. As mentioned above, Antibody-Immunogen Masking or Ab-virus complex vaccine is a complex of polyclonal antibodies isolated from PRRSv-infected swine sera and whole PRRSv virions. We hypothesized that the binding antibodies from early infection may be specific towards non-conserved or type-specific immunodominant epitopes that consumes the host immune response and prevent the development of antibody response towards sub-dominant conserved protective epitopes. By complexing PRRSv virions with polyclonal antibodies from acute infection, we are hypothesizing that the immunodominant epitopes are being masked and upon vaccination with Ab-virus complex, a protective immune response is generated towards sub-dominant epitopes. Thus the binding antibody response shown in figure 1 agrees

with our rationalization in using IgG collected from acute PRRSv infection to generate the Ab-virus complex vaccine.

2) Swine IgG purification- Sera obtained during acute PRRSv infection were chosen because, as mentioned above, acute serum is known to contain high amount of binding antibodies and no neutralizing antibodies. Swine IgG was purified and eluted using a Protein A column and figure 1B and C is an SDS-PAGE coomassie gel and western blot stained with swine Anti-IgG (H+L) antibody showing banding pattern characteristic of swine IgG. Heavy chain was recognized at 50kDa and the light chain at 25kDa in lane 2 of the coomassie gel, as expected of the banding pattern of the IgG molecule. Non-reducing but denaturing conditions resulted in single band at 80kDa in lane 1 of the western blot, which might represent a monomer of H+L chain with disrupted disulfide bond between two L chains.

Humoral immune response-

To determine the efficacy of the Ab-virus complex vaccine and to compare it with that of the autogenous vaccine, a previously validated challenge control animal model was utilized. Again, Nab response has been suggested to be an important correlate of protection against PRRSv infection. As shown in Table 1, all animals were bled weekly starting from day 0 until day 42. At day 0, nine pigs were vaccinated with the Ab-virus complex vaccine, 10 with the autogenous vaccine, and 10 pigs served as non-vaccinated challenge controls. All pigs were challenged with a heterologous strain IA2?1 on day 28 and all pigs were necropsied day 42. Total antibody response was measured at days 0, 14 and 42.

1-Binding Antibody response-

Figure 2 shows the S/P ratios of commercial HerdChek® ELISA to quantify the total antibody response against vaccination and challenge. All pigs were negative for PRRSV-specific antibodies at the initiation of the study confirming that these pigs were not exposed to PRRSV before the vaccination and were thus naive. Two weeks post vaccination (day 14), all vaccinated pigs were positive for antibodies to PRRSV regardless of vaccine type as expected. Similarly, two weeks after challenge (day 42), all non-vaccinated pigs had seroconverted to PRRSV. Vaccinated pigs had higher levels of binding antibodies at day 42 compared to the non-vaccinated control, characteristic of an anamnestic response. This also indicates that as with the autogenous vaccine, the virus in the Ab-virus complex vaccine was also able to replicate and generate a systemic response in the host.

2-Neutralizing activity-

Figure 3 shows the percent virus titer reduction in homologous strain by sera from individual pigs from 0-42 days post vaccination in both the vaccine group. Positive challenge controls had no neutralizing activity (data not shown). As can be seen, two pigs in the ab-virus complex vaccine show marked reduction in virus titer 1-2 weeks earlier than the pigs in the autogenous vaccine group. Panel B shows the percent virus titer reduction in heterologous strain by sera from individual pigs from 28-42 days post vaccination in both the vaccine group. Again, there is more number of pigs in the ab-virus complex vaccine that have the capability to reduce percent virus titer of heterologous strain as compared to the autogenous strain.

Correlates of immunity- viral RNA in serum and tissues

PRRSv strains JA142 and IA2?1 were validated with the AnDiaTec kit and compared with the results obtained from the routinely used Tetracore kit. Table 2 shows the ct results.

1-Viral RNA copy number in serum post vaccination-

Viral RNA copy number in serum was determined by qPCR weekly starting from day of vaccination (day 0) to the termination of the study (day 42), also shown in figure 4. As expected, PRRSv RNA was not detected prior to day 35 in the non-vaccinated control group. RNA levels in serum for individual pigs in each group are presented in figure 3. The mean RNA copy number of 10 pigs in the autogenous vaccine group was $10^{8.02 \pm 0.55}$ copy number \pm standard deviation in serum 7 days post-vaccination. By day 28, all pigs except one had undetectable RNA copy number in the serum, with $10^{4.45}$ viral RNA copy number/ml of serum in the positive pig.

In the Ab-virus complex vaccine group, the mean of RNA copy number/ml \pm standard deviation of blood at day 7 in all 9 pigs was $10^{8.56 \pm 0.56}$. Similar to autogenous group, by day 28, only 4 pigs were positive with a mean of $10^{4.43 \pm 0.74}$ viral RNA copy number/ml serum \pm standard deviation. The results are consistent with what is observed in the field, with the viral replication moving away from systemic to hiding in the lungs.

2-Viral RNA copy number in serum and tissue post heterologous challenge-

Table 3 shows the individual RNA copy number/ml of serum in pigs from all three treatment groups at dpi 35 i.e. 7 days post challenge. Most of the pigs were positive for viral RNA in the positive challenge controls (nine of 10) as was expected, with a

mean of $10^{5.88 \pm 1.16}$ viral RNA. Six of 10 pigs were positive in the autogenous vaccine group, with a mean of $10^{5.6 \pm 0.73}$ viral RNA copy number/ml in serum. Only two of nine pigs were positive in the Ab-virus complex vaccine group, with the mean of the two pigs being $10^{4.62}$ RNA copy number/ml. Out of these two pigs, one had ct outside the linear curve of replication but had a sigmoidal amplification curve and thus was considered a suspect (data not shown). Thus, there was a considerable difference between the number of pigs positive for viral RNA between the Ab-virus complex and autogenous vaccine group at day 35.

Similarly, table 3 shows the viral RNA copy number/ml of serum at day 42 i.e. 2 weeks post challenge. High viral RNA was observed in five out of 10 pigs in the non-vaccinated control group. Only one of 10 pigs was positive in the autogenous vaccine group, whereas three of nine pigs were positive in the Ab-virus complex vaccine group.

In the target organ for PRRSv replication, i.e. lungs, Viral RNA was determined by extracting RNA from sections from different regions of the lung to represent the entire organ. Table 3 shows that nine out of 10 pigs were positive for viral RNA in lungs in the non-vaccinated control group, with a mean of $10^{5.60 \pm 0.47}$ RNA copy number/mg of tissue. In the Autogenous group, six out of 10 pigs and in the Ab-virus complex vaccine group, four out of nine pigs were positive of viral RNA. The mean viral RNA in tissue of positive pigs in both the groups was $10^{5.5 \pm 0.3}$.

Correlates of immunity- Pathology in the tissue

1-Macro- and microscopic lesions -As described in the material and method section, gross lesions are scores, on a scale of 1-100, that reflect the percentage of the entire lung

affected by pneumonia. Table 3 shows that the mean macroscopic lung lesion scores. Five out of 10 animals (50%) in the non-vaccinated control group had severe gross lesion scores whereas 1 out of 10 (10%) and 2 out of 9 (22%) animals had severe gross lesion scores in the autogenous and Ab-virus complex vaccine group, respectively. The low range of scores was perhaps attributed to the use of a low-pathogenic strain that was not able to provide us the best indication of protection from a heterologous strain. Similar trend was observed for the interstitial pneumonia, with five of 10 animals with moderate and severe scores in the non-vaccinated control group, two of 10 animals in both the autogenous and Ab-virus complex vaccine group with moderate and severe scores. No significant statistical difference was observed between any of the treatment groups for gross lesions and interstitial pneumonia.

2-Immunohistochemistry-The presence of PRRSV-specific antigen was also investigated in all tissues. As shown in table 3, three of 10 (30%) in the autogenous and two of nine (22%) in Ab-virus complex vaccine group were positive for IHC. With the exception of one pig, all other five pigs positive for IHC in the non-vaccinated control group were also positive for the presence of viral RNA in the lung as detected by RT-PCR. The four IHC negative pigs were positive for viral RNA in lung, making all 10 animals (100%) positive for PRRSV infection using both the techniques. This data is in accordance with the fact that RT-PCR is a more sensitive technique for the detection of PRRSV in lungs than IHC. In the autogenous group, all three pigs positive for IHC are also positive for RNA in lungs. Three additional animals are also positive for PRRSV RNA, with six of 10 animals (60%) positive for the presence of PRRSV as detected by either RT-PCR or

IHC. Similarly, the two IHC positive pigs in the Ab-virus complex vaccine group were also positive for PRRSv RNA in lungs, with the addition of two more animals positive for RNA, thus bringing the number of animals that are positive using both techniques to four of nine (44%).

Although no statistical significance was observed at any time point between any treatment groups, as shown in table 3, there were 3 pigs out of 9 (33%) in the Ab-virus complex vaccine group that had undetectable RNA post-vaccination in both serum and tissue as determined by both qPCR or IHC in lung tissue. Eight out of 10 pigs in the Autogenous group were positive at least one time point for viral RNA copy number in either serum or tissue, thus the efficacy of autogenous vaccine was 22%. Thus, Ab-virus complex vaccine had a 33% efficacy in prevention of infection from a heterologous challenge strain, which was higher compared to the efficacy provided by Autogenous vaccine.

Discussion

Current commercial vaccines available for PRRSv have so far showed inadequacy in protection from infection from heterologous strains, a caveat that result in a billion dollar loss in the US per year. Lack of an effective vaccine can be attributed to the high mutation rate of the virus, its immune-suppressive behavior and some key immune evasion strategies such as deceptive imprinting. Although a lot is now known about PRRSv biology and its important characteristics since its discovery, considerable gaps do still exist in its biology, evolution and transmission that hinder the eradication of the virus. The objective of this study was to evaluate the efficacy of antibody-virus complex

vaccine to protect against homologous and heterologous PRRSv challenge in a validated animal model and compare its efficacy with that of autogenous vaccine, a routinely used vaccine in field. Ab-virus complex is a complex of live replicating virus and immunoglobulins isolated during acute PRRSv infection, and the rationalization of using live replicating virus are the reports of failure of killed virus to generate protective responses. We defined the efficacy of the vaccine as clearance of infection at any time point post challenge in either tissues (serum and lungs). By that criterion, in this study, 33% of pigs that were vaccinated with Ab-virus complex vaccine were able to generate sterilizing immunity against heterologous strain of PRRSv, a result that has been unaccomplished so far by any other vaccine developed against PRRSv. Even though results were not statistically significant compared to autogenous and control group, there have been no reports of complete clearance of infection from a heterologous strain, more so with a single dose of the vaccine.

Mean gross lesion scores for the non-vaccinated control group was 34 ± 14.61 and both Ab-virus complex and autogenous vaccine groups had similar range of gross lesions, no significant difference is observed in lung lesions. Similar results were observed in interstitial pneumonia scoring as well. This might be due to a major limitation of the study such as the use of low pathogenic PRRSV strain to evaluate efficacy. PRRSv-specific IHC scores on the other hand showed that six control pigs were positive for PRRSv antigen in lungs as opposed to only two in Ab-virus complex vaccine group and three in autogenous vaccine group. Using both IHC and RT-PCR in

lung sections, 60% of pigs were positive in the autogenous group as opposed to only 40% in the Ab-virus complex vaccine group.

In this section, we begin by providing an explanation of deceptive imprinting as an immune evasion strategy used by PRRSv and other pathogen. Secondly, immune refocusing as an effective way to design a cross-protective vaccine is explicated followed by our rationalization in using an antibody-immune masking vaccine. Finally, we will discuss the significance of this study to the PRRSv field and other pathogens and as well as future direction of the research.

A large number of pathogens employ the strategy of deceptive imprinting to evade the host's immune response. This strategy involves the consumption of the host's immune response towards highly-variable, strain-specific immunodominant epitopes that lead to the elicitation of non-neutralizing antibodies. This consumption of the host's immune response prevents the recognition of cross-protective sub-dominant epitopes that if recognized, elicits neutralizing antibodies that are broadly protective in nature. Immune refocusing technology allows the modification of mapped immunodominant epitopes in order to mask or dampen their immunogenicity. Methods of immune refocusing entail site directed mutagenesis of the target gene encoding the viral protein that expresses the decoy epitope (Trujillo JD et al. 2004). The selected amino acids are either deleted, replaced by neutral or less charged ones and/or addition of an N-linked glycosylation signal (Garrity RR et al. 1997). These modifications are done to facilitate the recognition of more highly conserved sub-dominant epitopes by the host's immune system to enhance the development of Nab. This methodology has been used

successfully to elicit high titer Nab to CAEV following protein immunization with wild type replicating CAEV (Trujillo JD et al.2004, Garrity RR et al.1997). In addition to CAEV, immune refocusing of immunodominant epitopes in other virus models such as HIV-1, Influenza virus have also been tested for the elicitation of beneficial immune responses of greater breadth when compared to the original unmodified strain (Tobin GT et al. 2008).

Neutralizing antibodies play a major role and have impact on the outcome of PRRSv infection as well and have known to be elicited by all the major glycoproteins of PRRSv. However, they also suggest that the type of antibodies produced can have a significant effect on the outcome of the infection (Oli MW et al. 2004). Some immunodominant epitopes in PRRSv structural and nonstructural proteins have been characterized but the viral targets of protective Nab have not been characterized (Murtaugh PM & Gonzow M 2011, Chareerntantanakul W 2012), which makes it a challenge to employ the technology of immune refocusing in order to create a cross-protective vaccine.

Recently our research group has utilized a more rapid methodology to develop and investigate immune refocusing vaccines. The concept of Antibody-virus complex employs naturally occurring antibodies and is hypothesized to mask immunodominant, non-protective epitopes to allow the immune system to react against more highly conserved, yet previously silent epitopes to generate more broadly protective responses. The Ab-virus complex technology has been recently employed for modification of autologous PRRSv vaccination with moderate success in providing protection from

heterologous challenge. As is generally observed with PRRSv infection and supported by binding antibody ELISA generated in our lab, only non-neutralizing immunoglobulins are elicited during acute stage of infection. Our rationalization in using purified IgG from animals in acute infection to mask epitopes on the intact virion is that these non-neutralizing antibodies are specific towards the immunodominant epitopes. The Ab-virus complex technology has been shown to be effective in the design of a licensed vaccine against IBDV in chickens (Haddad EE et al. 1997).

Further investigation will address the limitations of the current study and will use a more stringent study design. There are multiple benefits of the Ab-virus complex vaccine, such as complete clearance of infection in some pigs, the ease of manufacturing the vaccine without the need to know the hierarchy of immunodominance in its entirety and no requirement for reformulation of the vaccine constantly. These benefits provide this vaccine an advantage from current vaccines and also a model that can be applied to other class-II pathogens for which no vaccines are available yet. One of the immediate goals would also be to test the heterologous breadth of the Ab-virus complex vaccine. Additionally, the biological generated from this study could be utilized to map epitopes (either immunodominant or sub-dominant) on PRRSv glycoproteins further filling the knowledge gap in PRRSv biology.

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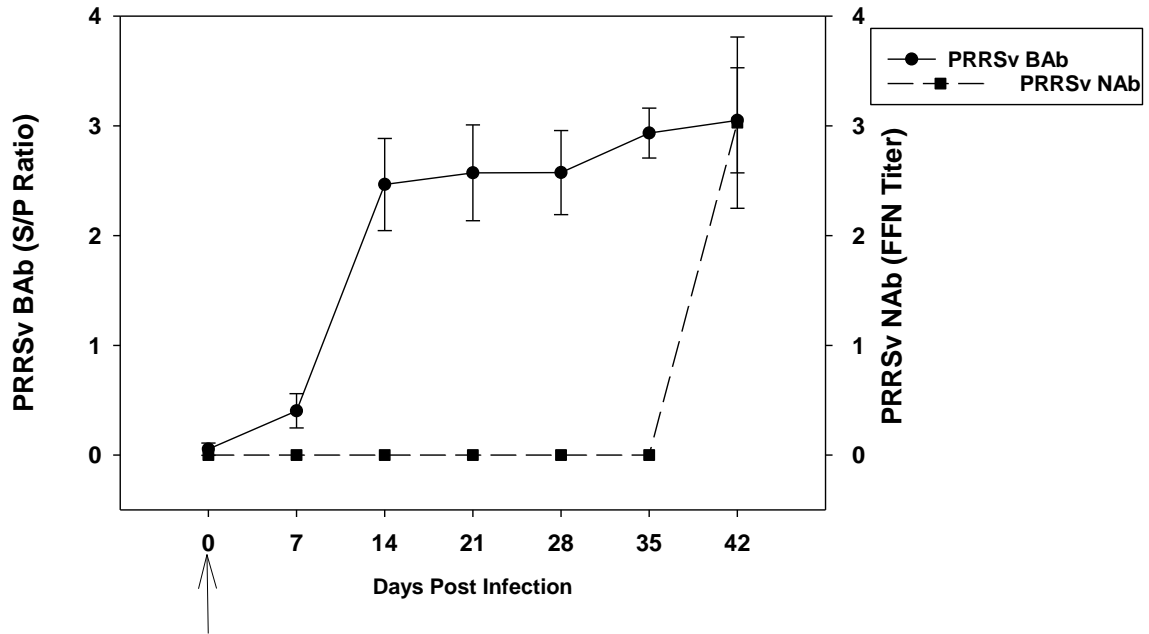
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Table 1- Immunization challenge strategy- two groups of pigs were immunized with either antibody-virus complex utilizing PRRSv strain JA142 or autogenous vaccine at Day 0. Positive control controls were kept separately and did not receive any treatment. At Day 28, pigs in all groups were challenged with heterologous strain IA2?1. Serum was banked at Day 0 and every week for 42 days. Study was terminated at Day 42 and necropsy was performed to evaluate end-point of pathology.

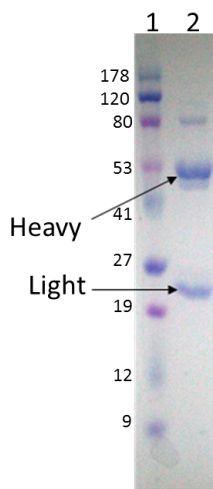
Group	No. of animals	Day 0 Vaccination	Day 28 PRRSv Challenge	Day 42 Terminate
Antibody-virus complex vaccine	9	JA142+IgG	IA2?1	Necropsy
Autogenous vaccine	10	JA142	IA2?1	Necropsy
Positive challenge control	10	-	IA2?1	Necropsy

Figure 1 Humoral immune response in pigs- pigs infected with JA142 strain of PRRSV and purification of IgG from Day 14 sera for antibody-virus complex vaccine using Protein A purification column. **A)** Demonstrates late appearance of Nab. Solid line represents the binding antibody response (BAb) in pigs exposed to JA142 strain as generated from commercial ELISA. Stripped-line shows the neutralizing antibody (Nab) titer as measured by Fluorescent Focus Neutralization assay. The results shown here are mean of 8 pigs \pm standard deviation. D0 (arrow) infection. Day 14 was used for vaccine design due to absence of Nab but predominance of BAb. **B)** Shows the SDS-PAGE gel under reducing conditions with coomassie staining; lane 1-marker, lane 2- elution fraction containing purified IgG. **C)** Shows western blot of purified IgG; lane 1- elution fraction under non-reducing conditions, lane 2- elution fraction under reducing conditions, lane 3- marker.

A



B



C

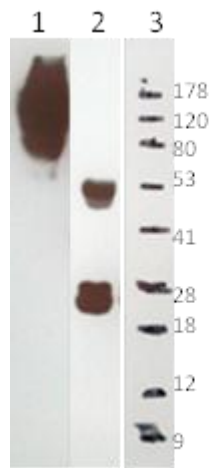


Figure 2- Seroconversion of pigs post vaccination and challenge. BAb response was measured using commercial ELISA on Day 0, Day 14 and Day 42 of the study. Data represents mean S/P ratio of all pigs in each group \pm 95% confidence interval. n=9 pigs in the Ab-virus complex vaccine; n=10 in autogenous vaccine and positive challenge control

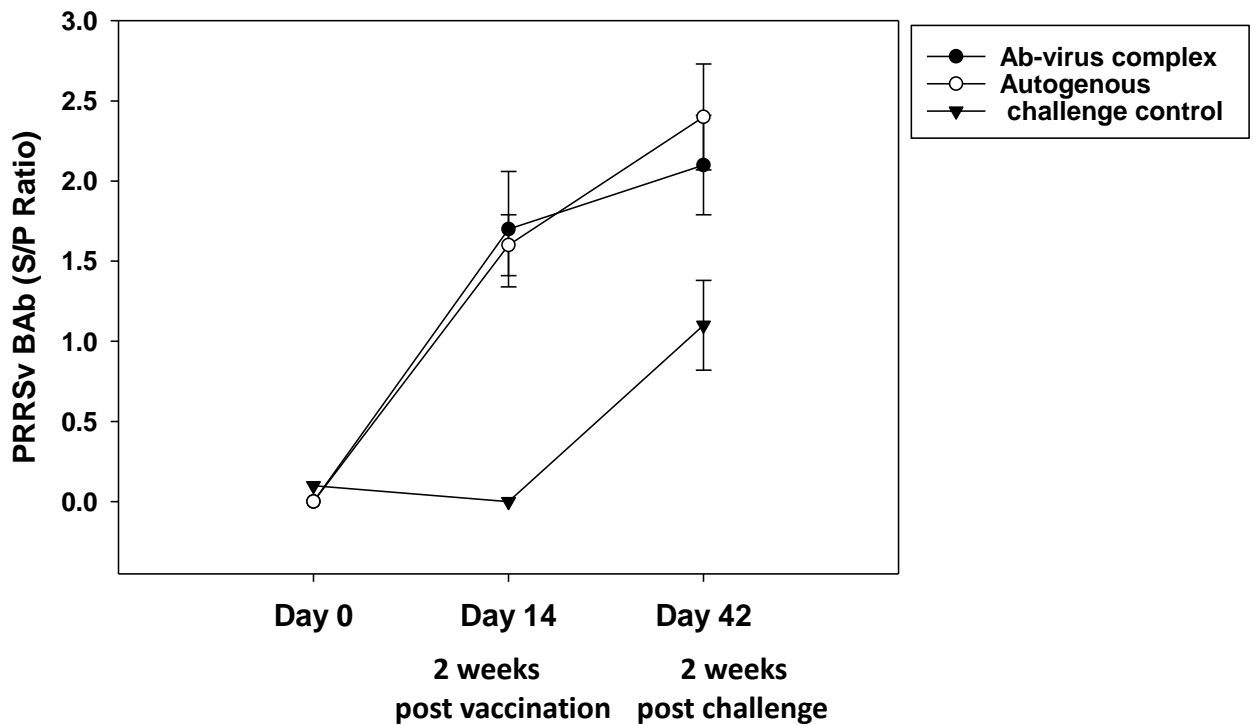
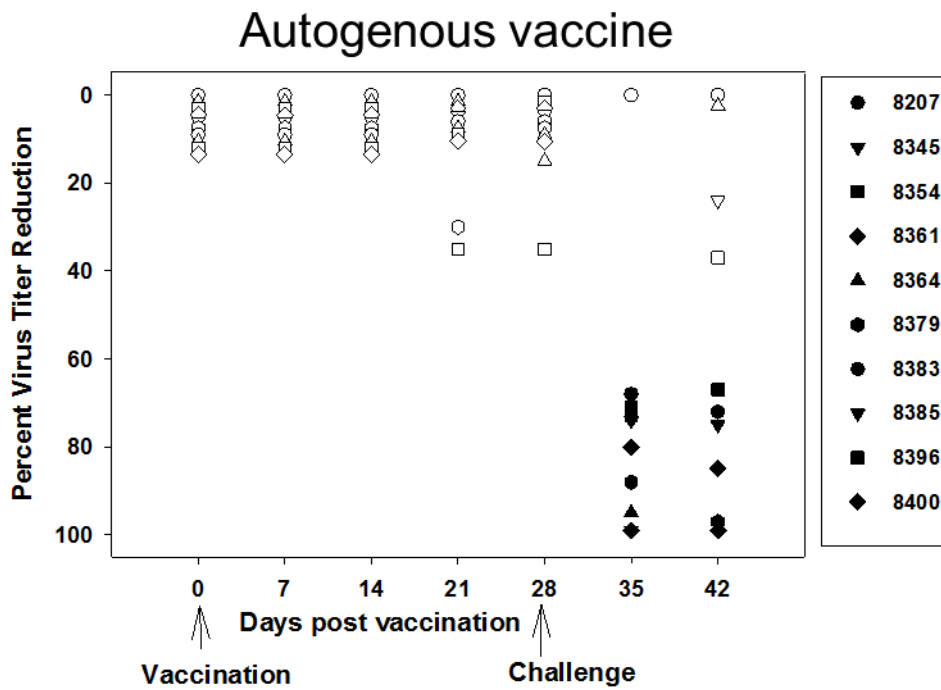
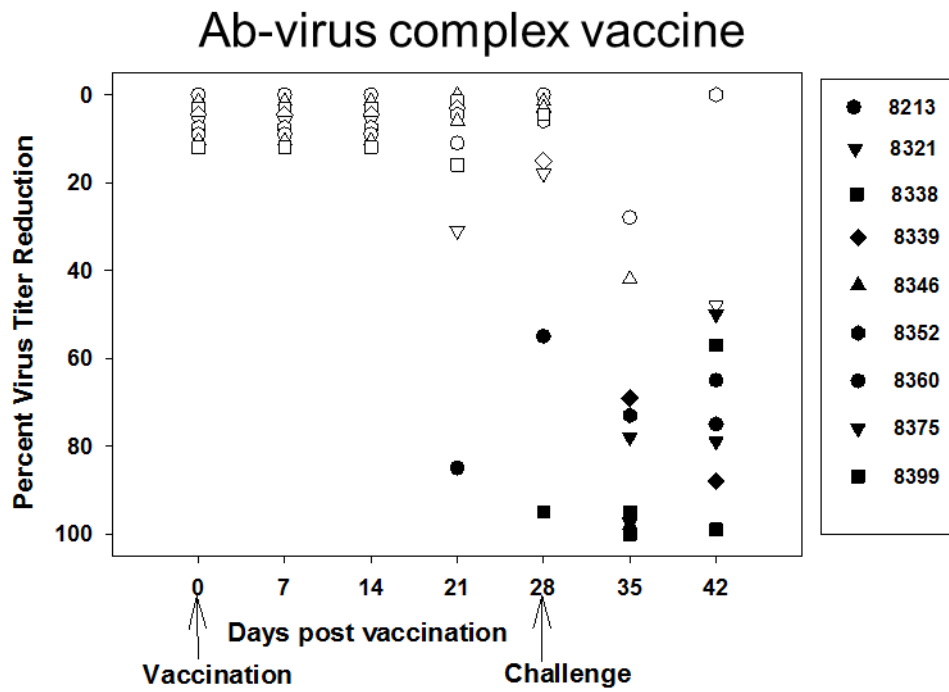


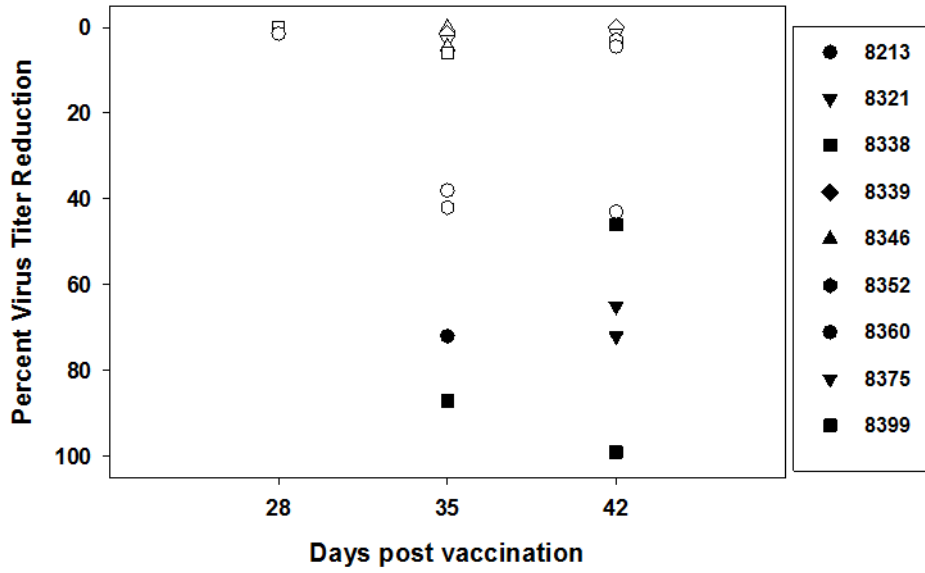
Figure 3- Neutralizing activity of sera from vaccinated pigs against homologous and heterologous PRRSv strains- **A)** neutralizing activity was measured by screening neat serum from individual pigs collected from 0-42 days post vaccination utilizing Virus titer reduction assay against homologous strain and **B)** by screening neat serum from individual pigs collected from 28-42 days post vaccination against heterologous strain. More than or equal to 50% reduction in virus titer was defined as positive. Open symbols represent sera with no neutralizing activity whereas closed symbols represent sera with neutralizing activity.

A



B

Ab-virus complex vaccine



Autogenous vaccine

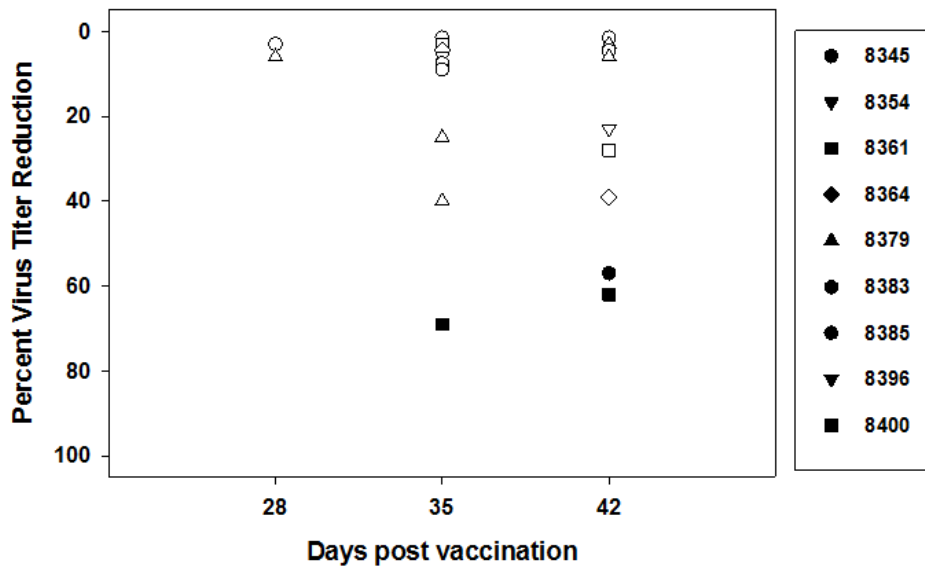
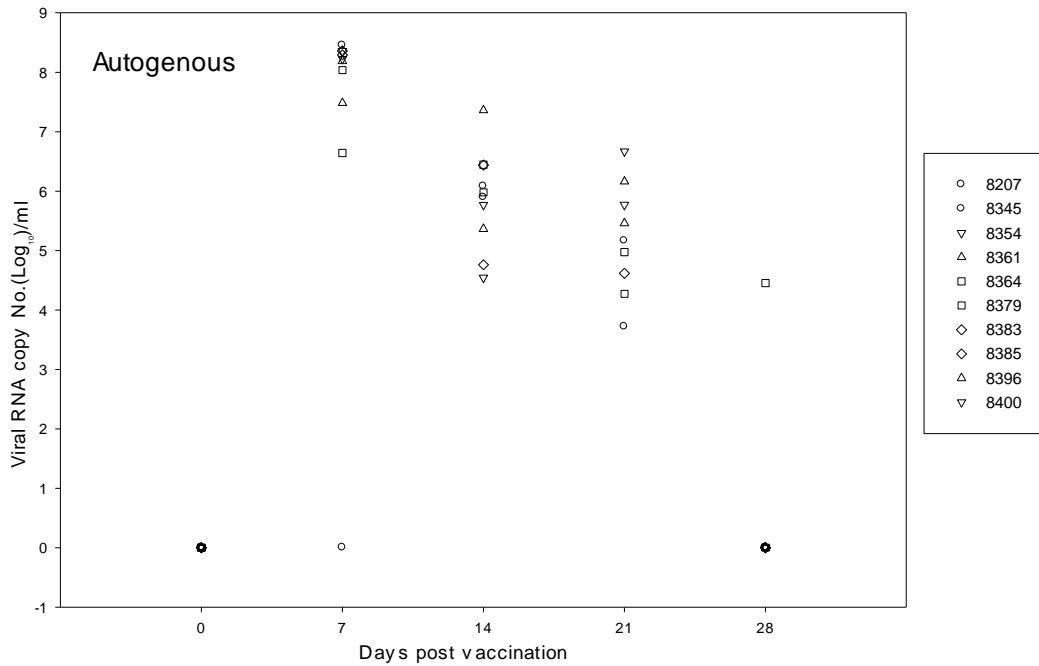


Table 2- Validation of detection of strains used in the study by the AnDiaTec AcuPig® PRRSV real time RT-PCR kit (AnDiaTec GmbH, Kornwestheim, Germany) by comparison of cycle threshold (C_T) with commercial kit used in the US, Tetracore (Tetracore, Rockville, MD)

	AnDiaTec	Tetracore
JA142	20.52	17.1
IA2?1	20.65	19.5

Figures 4- Viral RNA copy number/ml of serum for each pig in the Autogenous and Ab-virus complex group at different time points post-vaccination of the duration of the study



2D Graph 4

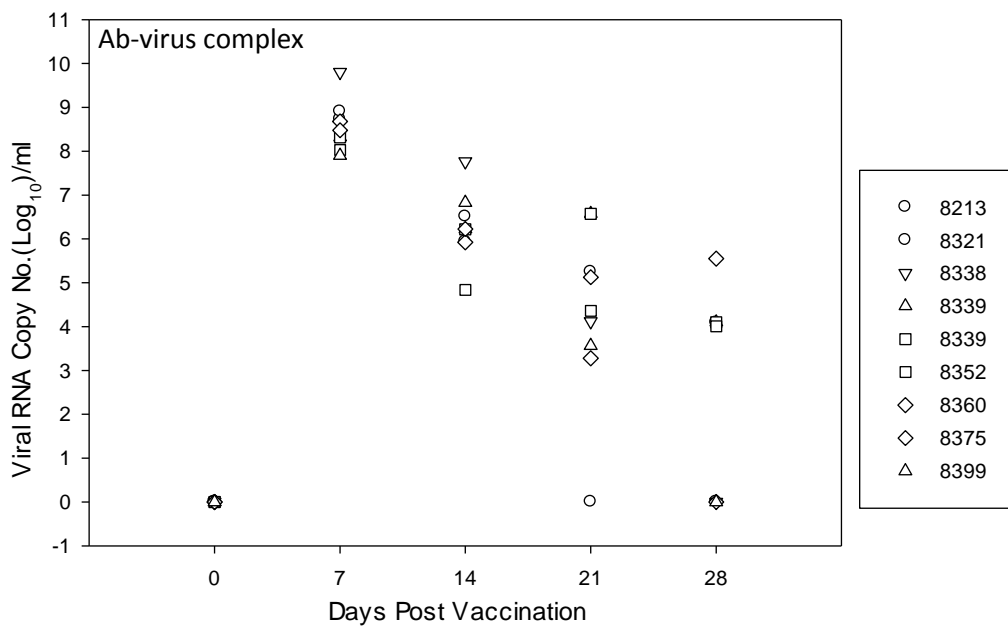


Table 3- Viral RNA copy number and pathological end-points for determination of vaccine efficacy- status of individual pig for presence of viral RNA in serum at 35 and 42 days post vaccination and lung tissue at day 42 are presented along with individual gross lesion scores, interstitial pneumonia scores and presence/absence of PRRSV antigen in lung tissue by immunohistochemistry (IHC). Pigs positive for viral RNA are represented as Pos, Sus represented those samples that were positive but above the limits of quantitation, ND is not-detected. Gross lesion scores of <20=normal, 25-35=mild, 36-45=moderate and >45=severe and diffuse. Similarly, interstitial pneumonia scores range from 1-2=normal-mild, 3-4=mild to moderate, 5-6 severe and diffuse.

Positive challenge Controls	D35 (7D)	D42 (14D)	Lung (D42)	Gross lesion	Interstitial pneumonia	IHC
8216	Pos	Sus	Pos	55	4	+
8239	Pos	ND	Pos	8	1	0
8242	Pos	Sus	Pos	19	3	0
8306	Pos	Pos	Pos	57	5	+
8318	Pos	Pos	Pos	31	4	+
8319	Pos	Pos	Pos	48	5	+
8320	Pos	ND	Pos	55	4	+
8334	Pos	Sus	pos	2	2	0
8390	pos	Sus	pos	60	3	0

Autogenous	D35 (7D)	D42 (14D)	Lung (D42)	Gross lesion	Interstitial pneumonia	IHC
8207	Pos	ND	Pos	21	2	0
8345	ND	Sus	Pos	28	4	+
8354	ND	ND	Pos	46	5	+
8361	Pos	ND	ND	17	3	0
8364	Pos	ND	Sus	37	3	0
8379	Pos	ND	Pos	20	3	+
8383	ND	ND	ND	9	2	0
8385	Pos	ND	ND	9	2	0
8396	Pos	Sus	Pos	26	2	0
8400	Pos	Sus	ND	7	2	0

Table 3- Viral RNA copy number and pathological end-points for determination of vaccine efficacy

Ab-Virus Complex	D35 (7D)	D42 (14D)	LUNG (D42)	Gross lesion	Interstitial pneumonia	IHC
8213	ND	ND	ND	10	2	0
8321	ND	Sus	Pos	80	5	+
8338	Pos	ND	Sus	19	2	0
8339	ND	ND	ND	8	2	0
8346	Pos	ND	ND	13	2	0
8352	ND	ND	ND	10	2	0
8360	ND	Sus	Pos	70	4	+
8375	ND	Sus	ND	40	2	0
8399	ND	ND	Sus	25	3	0

Table 3- Viral RNA copy number and pathological end-points for determination of vaccine efficacy

CHAPTER V

GENERAL CONCLUSIONS

The objective of the thesis presented here was to evaluate the efficacy of an immune complex vaccine in pigs challenged with a heterologous strain of Porcine Reproductive and Respiratory Syndrome virus (PRRSv). PRRSv, a virus with highest mutation rate reported amongst RNA virus, is the most economically significant pathogen of the swine industry, accounting for an annual loss of \$1 billion in the US. Clinical signs of the virus include respiratory distress and pneumonia in piglets; the virus also leads to poor growth performance and increased susceptibility to secondary infections in these pigs. In sows, reproductive failure with SMEDI (stillbirth, mummification, embryonic death and infertility) and sudden death may occur. High amount of genetic variability observed between isolates and gaps in knowledge in immunopathogenesis of the virus has posed major challenges in the development of a cross-protective vaccine.

Our research group has evaluated an immune complex vaccine utilizing antibodies from the early phase of PRRSv infection and the research sought to determine the efficacy of the vaccine in an animal trial. The use of immune complexes (IC) in modulating the host immune response has been well documented and is also a licensed vaccine against IBDV. But IC has never been tested for PRRSv. Our rationale in using antibodies isolated from pig sera collected from acute phase of PRRSv infection was two-fold. Firstly, we hypothesized that the specificities of these early antibodies is towards non-protective immunodominant epitopes. Secondly, when complexed with the

virus, we hypothesized that the antibodies would mask the immunodominant epitopes on the surface of the virus, allowing for the recognition of previously unrecognized epitopes. Thus, we would be able to shift the immune response from non-protective, highly variable immunodominant epitopes to conserved ones, leading to the generation of cross-protective immunity.

For the purpose of our study, we defined the efficacy of the vaccine as clearance of infection at any time point post challenge in either serum or lung tissue. Based on that definition, 33% of pigs that were vaccinated with the Ab-virus complex vaccine were able to generate sterilizing immunity against heterologous strain of PRRSv. Though the results are highly encouraging, further studies need to be done to elaborate on the heterologous breadth of protection offered by the vaccine while improving on the limitations of the current study presented. A major limitation of the study was the use of low pathogenic heterologous PRRSv strain that hindered true evaluation in reduction of pathology offered by the vaccine.

The mechanism of protection of the Ab-virus complex vaccine remains to be determined. Masking of the immunodominant epitope by antibodies isolated from PRRSv-infected sera might allow for the recognition for previous silent epitopes. Shift in the immune response from non-protective to protective due to the immuno-modulatory behavior of immune complexes is one hypothesis for the working mechanism of Ab-virus complex vaccine. Another mechanism might be Fc receptor modulation of the IC, resulting in a change in the immunogenicity of the virus. This can be due to differential

antigen uptake of the virus, changing the cytokine profile elicited by host cell or differential procession of antigen.

Further evaluation of the mechanism of Ab-virus complex vaccine efficacy might allow us to answer some important questions regarding the host-pathogen interactions. PRRSv might also serve as a model pathogen to understand the biology of other viruses important in human and veterinary medicine.