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Isotyping of porcine reproductive and respiratory syndrome (PRRS) virus antibody response and its diagnostic use

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by

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CHAPTER 1.INTRODUCTION

Thesis Organization

This thesis is comprised of four chapters. Chapter 1 is the literature review describing past, present and future perspective of PRRSV serodiagnostics and general overview of humoral immune response to PRRSV. Chapter 2 is a research paper describing isotype-specific and neutralizing antibody response in pigs experimentally challenged with PRRSV VR-2332 strain and evaluating the diagnostic performance of specific IgG, IgM and IgA response to PRRSV infection. In chapter 3, isotype-specific response is evaluated in pigs vaccinated with a novel experimental modified live vaccine and development of neutralizing antibody against viruses homologous and heterologous to the vaccine virus is illustrated and compared to each isotype-specific response. Chapter 4 summarizes research observations and their implications and proposes future studies.

Literature Review. PRRSV Serodiagnostics: Past, Present and Future

Historical aspect

First reported in 1987 in the United States, porcine reproductive and respiratory syndrome (PRRS) remains to be one of the most economically significant diseases in the swine industry worldwide. Economic loss due to PRRS in the United States has been recently estimated to be from 560 million to 664 million USD annually (Holtkamp et al., 2011, Neumann et al., 2005). Possible import of a North American PRRS virus to Europe could potentially result in 1.5 billion USD annual losses to the European Union swine industry (Kristensen et al., 2013).

PRRS was initially named “Mystery Swine Disease” (Hill, 1990) or occasionally “Blue ear pig disease “due to specific skin lesions (Keffaber et al., 1989). The disease rapidly spread throughout Europe and North America in the 1990s. Porcine reproductive and respiratory syndrome virus (PRRSV), the causative agent of PRRS, was identified as an enveloped RNA virus in Netherlands in 1991 and was named “Lelystad virus” (Wensvoort et al, 1991). When first isolated in 1992 in the United States, the virus was named ‘Swine Infertility and Respiratory Syndrome (SIRS) virus and later characterized to be an enveloped RNA virus with average diameter of 62 nm (Benfield et al., 1992, Collins et al., 1992). Since its initial identification, PRRS has been reported in all major pig producing regions throughout the world.

Clinical disease

PRRS can manifest itself from asymptomatic to acute severe disease and is often characterized by reproductive failure in breeding swine and respiratory distress in pigs of all ages. Abortion, still-births, mummified pigs, premature farrowing and increased number of weak piglets are common manifestations of reproductive form of the disease while sneezing, increased nasal discharge, breading difficulty, and coughing are common clinical signs of respiratory distress (OIE 2008, Zimmerman et al., 1997). The virus strain, management and immune status of the herd can influence the clinical disease caused by PRRSV (OIE, 2008). Clinical signs of PRRSV infection resemble that of several other pathogens, some of which include: classical swine fever virus, porcine parvovirus, swine influenza virus and Haemophilus parasuis and need to be considered in differential diagnosis of PRRSV (OIE, 2008).

Concurrent infection by pathogens, such as streptococcus suis and Mycoplasma hyopneumoniae, can increase the severity of disease by PRRSV infection (Galina et al., 1994, Thacker et al., 1999); however, there are other reports which indicate a non-significant

relationship between co-infection with Mycoplasma hyopneumoniae and increased viral shedding in aerosols (Cho et al., 2006). Antibody dependent enhancement (ADE) of infection has also been reported to occur during PRRSV infection (Yoon et al, 1996). Sub-neutralizing levels of antibody attached to viral particles can facilitate binding to Fc receptors on macrophages and increase viral entry. The phenomenon is known as antibody dependent enhancement (ADE) of infection or disease although under experimental conditions ADE has not been proven to increase the severity of disease caused by PRRSV (Yoon et al., 1996, Yoon et al., 1997). It has been reported that specific epitopes on PRRSV nucleocapid and GP5 protein may be associated with ADE of PRRSV infection (Cancel-Tirado et al., 2004).

PRRSV

The virus belongs to the genus Arterivirus of the family Arteriviridae in the order Nidovirales (Cavanagh, 1997). Other viruses in this family include lactate dehydrogenase elevating virus (LDV), equine arthritis virus (EAV) and simian haemorhagic fever virus (SHFV) (Conzelmann et al., 1993, Plagemann et al 1992). Arteriviruses have a preference for replication in macrophages and can establish a persistent infection in their host (Plagemann et al 1992, Wills et al 1997).

The PRRS virion contains positive-sense single-stranded RNA with capped 5' end and polyadenylated 3' end. The viral genome is approximately 15 Kb in length and comprises of 9 different open reading frames (Allende et al., 1999, Dea et al., 2000). ORF 1a and 1b comprise 2/3 of the genome and encode at least 12 non- structural proteins (Nsps) which form replicase components of the virus and play a critical role in virus replication (Allende et al., 1999). The 3' end of PRRS genome (almost 3 kb) is where open reading frames (ORF) 2-7 are located. These ORFs encode structural proteins of the virus and are expressed through a co-3' terminal nested

set of 6 subgenomic mRNAs. ORF 2a, 3 and 4 encode minor envelop N-glycosylated protein GP2a, GP3 and GP4, respectively (Wissink et al., 2005). These proteins are approximately 256, 254 and 178 amino acids in length respectively and form a heterotrimer through disulfide bond (Dea et al., 2000, Murtagh et al., 1995). A non-glycosylated envelope protein called E protein is encoded by ORF2b which is entirely embedded in ORF2 (Wu et al 2001). ORF5 encodes an approximately 200 amino acid-long major envelope glycoprotein GP5 which has been reported to be the main protein to elicit neutralizing antibody (Gonin et al., 1999, Pirzadeh et al., 1998). A novel ORF5a region has recently been reported which was shown to have a role in virus viability and elicit specific but non-neutralizng antibody. (Johnson et al., 2011, Robinson et al., 2013, Sun et al., 2013). ORF6 encodes matrix (M) protein (around 174 amino acids in length), GP5 and M protein also form a heterodimer (Mardassi et al., 1996). ORF7 encodes the nucleocapsid (N) protein which is approximately 123 amino acids long. Since N protein is highly immunogenic and elicits a large amount of non-neutralizing antibody in infected pigs, it has been used as the basis of most serologic tests (Dea et al., 2000, Kim et al 2008).

There are two distinct genotypes of PRRSV: European genotype 1 represented by Lelystad virus strain and North American genotype 2 by VR-2332 strain (Wensvoort et al., 1992).The two genotypes share only 60-70% similarity in their entire genome (Dea et al, 2000, Kapur et al., 1996, Meng et al., 1995, Murtagh et al., 1995, Nelsen et al.,1999). VR-2332 and Lelystad strains have the least similarity of 72% in their ORF3 and highest similarity of 91% in their ORF6 amino acid sequence (Murtagh et al., 1995).

Current PRRSV diagnostics

Detection of PRRSV infection is based on either demonstration of viral antigen or genome by virus isolation technique, polymerase chain reaction (PCR) based assay, and tissue fluorescent

antibody test and immunohistochemistry staining in clinical specimens or virus-specific serum antibody by enzyme-linked immunosorbent assay, virus neutralization test, indirect fluorescent antibody test and immunoperoxidase monolayer assay (Albina et al., 1992, Batista et al., 2004, Brown et al., 2009, Christopher-Hennings et al., 1995, Christopher-Hennings et al., 2002, Egli et al., 2001, Harmon et al., 2012, OIE manual 2008, Sorensen et al., 1997, Yoon et al., 1994).

Virus isolation

PRRSV can be isolated and grown on cells of macrophage lineage, specifically porcine alveolar macrophages (PAMs) or a highly permissive clone of African Monkey kidney cell line such as MARC-145 cells and CL-2621 (Kim et al., 1993, Wissink et al., 2003).

Cells expressing CD-163, CD151 and sialoadhesin have been shown to be permissive to PRRSV; therefore, these cell surface proteins are considered the major receptors for PRRSV (Kim et al., 2006, Vanderheijden et al., 2001, Vanderheijden et al., 2003, Van Gorp et al., 2008). Cells originally non-permissive to PRRSV can be genetically modified to express CD163 on their surface, rendering these cells suitable and permissive to PRRSV propagation (Lee et al., 2010).

Although virus isolation is considered the “Gold Standard” in PRRSV diagnostics, some of its disadvantages include being laborious and low recovery rate of isolates. Furthermore a successful virus isolation procedure is typically done in several days and requires more than one passage, i.e., slow turnaround time.

Antigen detection methods

Fluorescent antibody (FA) test and immunohistochemistry (IHC) have been employed. These techniques are used for detection of viral antigen(s) in frozen (FA) or formalin-fixed (IHC)

tissues. If coupled with lesions, these tests can be causality indicators. However, these tests possess a relatively low sensitivity and the results can be affected by sample quality (Yoon et al., 2003). The performance of these tests is hinged on the quality of antibodies (polyclonal or monoclonal antibody) used with the test. The potential for misdiagnosis when relying on a monoclonal antibody due to rapid genetic mutations of PRRSV leading to antigenic change has been reported (Yoon et al 1995).

Nucleic acid based assays

Detection of PRRSV genetic material by reverse transcriptase PCR is reported to be highly sensitive and specific and is also suitable for high-throughput testing (Christopher-Hennings et al., 1995, Mardassi et al., 1994, Wasilk et al., 2004). Several different PCR platforms have been developed for PRRSV detection with different gene targets although most primer and probes are designed to amplify conserved regions in ORF6 and ORF7 of PRRSV. A conventional nested RT-PCR targeting ORF1b and ORF7 was developed and used to detect PRRSV in boar semen (Christopher-Hennings et al., 1995). There have been PCR assays designed to differentiate North American (type 2) and European (type 1) PRRSV based on the size of ORF7 and specific primers for ORF5 (Oleksiewicz et al., 1998). A multiplex TaqMan real time PCR was also established based on ORF7 using a different probe for each of the North American and European strains (Egli et al., 2000, Kleiboeker et al., 2005). The multiplex real-time PCR with dual-labeled probes was reported to be 100% sensitive and 96.6% specific (Kleiboeker et al., 2005).

SYBR green real-time PCR tests capable of differentiating between strains have been recently introduced and were reported to detect PRRSV genome as early as 2-3 days post infection (dpi) (Chai et al., 2013, Martinez et al., 2008). Performance of PCR assay can be affected by a high rate of PRRSV genetic changes and also by sample matrix. False negative

results can be then the main obstacle to deal with in case of PRRSV PCR assays. The problem was pointed out in a ring trial evaluation of 4 different commercial real-time PCR kits and was shown to be more pronounced for detection of European strains rather than North American strains (Wernike et al., 2012). A recent comparison between two commercially available PRRS real-time PCR assays indicated disagreement in terms of false negative results (Harmon et al., 2012). In general, high level of diversity in PRRSV genome resulting in even a small mis-matching of primer and/or probes, pooling of samples and lack of sufficient assay optimization can lead to false negative results.

Antibody detection methods (Serology)

Enzyme-linked immunosorbent assay (ELISA)

Indirect ELISA for PRRSV was first introduced by Albina et al in 1992. Several groups have developed ELISA tests based on the most abundant and highly immunogenic PRRSV nucleocapsid protein (Dea et al., 2000, Sorrensen et al., 1997, Seuberlich et al., 2002, Witte et al., 2000). Protein-specific ELISA tests based on nsp2 and GP5 (5' and 3' end) have also been experimentally designed; it appeared that antibody response to nsp2-based and N-based ELISA are similar to each other (Mulpuri et al., 2008). PRRSV Nsp1, Nsp2 and Nsp7 proteins were reported to be highly immunogenic. A Nsp7-based ELISA test was proven to correlate well with IDEXX PRRS 2X ELISA in terms of repeatability, sensitivity and specificity. The assay was reported to be 99.8% sensitive and 99.3% specific in detecting pigs exposed to North American PRRSV (Brown et al., 2009).

IDEXX's commercial PRRS ELISA kit (Herdchek® PRRS X3) is currently the most commonly used serological test to screen herds for PRRSV infection/exposure with reported sensitivity of 97.4 % and specificity of 99.6 % by the manufacturer (Herdchek PRRS package

insert; IDEXX Laboratories, Inc., Westbrook, ME). A lower sensitivity (88.1%) has been reported by others when evaluating the performance of the commercial ELISA on serum samples collected from pigs experimentally inoculated with PRRSV and followed for 193 dpi (Molina et al., 2008). Singleton positives detected by this test need to be further confirmed by other tests such as indirect fluorescent antibody test or serum-virus neutralization test. A blocking ELISA has also been suggested as a confirmatory follow-up test on suspect positives by the IDEXX PRRS ELISA (Ferrin et al., 2004). It was reported that the blocking ELISA developed with plasmid expressed N protein and SDOW17 monoclonal antibody has a sensitivity and specificity of 97.8 % and 100 % respectively (Ferrin et al., 2004).

Recently commercial ELISA has been adapted to detecting different antibody isotypes in oral fluids. With sample to positive ratio cut-off of > 0.4 this assay was reported to be 94.7 % sensitive and 100 % specific (Kittawornrat et al., 2012).

Immunoperoxidase monolayer assay (IPMA)

IPMA is a cell-culture based assay based on the reactivity of serum antibodies with whole viral antigen. Antigen-antibody reaction in this assay is visualized with a horseradish peroxidase system instead of a fluorescent dye being used in an indirect fluorescent antibody test. Although IPMA has some advantages including the ability to detect antibodies both very early and late in the course of PRRSV infection, it has not been used in North American veterinary diagnostic laboratories (Christopher-Hennings et al, 2001, Yoon et al., 1995). IPMA also possesses all disadvantages of cell-culture based assays including being laborious and subjective interpretation of the results.

Indirect fluorescent antibody (IFA) test

First developed in 1992, PRRSV IFA test is another cell-culture based serologic technique based on the reactivity of serum antibodies with whole virus antigens (Yoon et al., 1992). The test is an equivalent of IPMA except that antigen-antibody reaction is detected with a fluorescent system rather than HRP system. IFA can detect IgG at 7-11 dpi and IgM antibody as early as 7 dpi. The test is laborious and prone to subjective interpretation. Another disadvantage of the test is its being incapable of detecting antibodies to strains antigenically distinct from the strain utilized in the assay.

Serum-virus neutralization (SVN) test

SVN test is an immunity indicator as it measures functional antibody and is based on reactivity with membrane-associated proteins. SVN test has a lower sensitivity compared to IFA and ELISA. Antibody titers measured by SVN test as well as IPMA and IFA test can be affected by strain difference (Christopher-Hennings et al, 2001). In general SVN test is used more commonly for research purpose than routine serodiagnosis.

Fluorescent microsphere immunoassay (FMIA)

FMIA is a relatively new test for antibody detection using Luminex® platform and has been applied to PRRSV serology. A PRRSV-specific FMIA was developed based on multiple fluorescent beads conjugated to recombinant Nsp7 and N proteins of both type 1 and 2 PRRSV strains and evaluated for antibody detection in serum and oral fluid sample matrices (Langenhorst et al., 2012). The sensitivity and specificity of the same assay on sera was estimated to be more than 98% and 95%, respectively, while the estimated sensitivity and specificity of FMIA on oral fluids was more than 90%. FMIA was able to detect antibodies at 7

dpi up to 202 dpi. N-protein based and nsp7 protein based FMIA of both type I and II were successfully combined in to a multiplex assay with high correlation to each individual assay.

Humoral immune response to PRRSV infection

Innate and adaptive immune response to PRRSV has been studied extensively. Several studies have attempted to illustrate antibody response to the virus (Joo et al., 1997, Murtaugh et al., 2002, Mulpuri et al., 2008, Loemba et al., 1996, Labarque et al., 2000, Yoon et al., 1995). However, kinetics of antibody response to PRRSV was only partially described. The sequence of events after exposure to PRRSV typically starts with an acute infection and high level of viremia. It can take up to 150 days or more for the virus to completely clear from pigs body, i.e., persistent infection. It has been postulated that one of the reasons PRRSV can establish a persistent infection is the inability of the host to elicit adequate and timely protective immunity (Lopez et al., 2004). PRRSV-specific IgG measured by IFA appear as early as 7-9 days post inoculation; however, these antibodies have been identified as non-neutralizing (Yoon et al., 1994, Loemba et al., 1996). On the other hand, neutralizing antibodies have been able to clear viremia although SVN antibody develops slowly (Labarque et al., 2000, Pirzadeh et al., 1998).

Kinetics of antibody response to PRRSV can vary depending upon serologic assays. Antibody response against PRRSV (VR-2402 strain) was assessed using IFA test, IPMA, ELISA and SVN test each of which were able to detect virus-specific antibodies by 9-11, 5-9, 9-13 and 9-28 dpi, respectively (Yoon et al., 1995). Using a regression analysis, the antibodies detected by these tests were estimated to decline to undetectable levels by 158, 324, 137 and 356 dpi, respectively (Yoon et al., 1995). In experimentally infected pigs followed for 202 dpi, IgG antibody response was measured in serum and muscle transudate (“meat juice”) with a commercial ELISA and fluorescent focus neutralization (FFN) test. Pig serum samples were

positive by the commercial ELISA from dpi 28 till the end of the study. In contrast, 42 dpi was the first time point in which neutralizing antibody was detected in serum samples. FFN antibody was detectable until dpi 202, although the highest FFN titer (1:9.3 on average) was only seen at dpi 56 (Molina et al., 2008). Other studies reported that neutralizing antibody to PRRSV VR-2332 strain was detectable as early as 14 dpi; most experimental studies have reported the peak neutralizing response to be around 70 dpi (Batista et al., 2004, Loemba et al., 1996, Murtagh et al., 2002).

Different PRRS viral proteins play different roles in antibody production. Antibody response assessed by a peptide-specific ELISA which was based on chimeric polypeptide GP5-M correlated with neutralizing antibody response, indicating that these proteins may contain neutralizing epitopes (Molina et al., 2008). In comparison, ELISA antibody response assessed in the same study using a commercial kit (IDEXX HerdCheck® PRRS) strongly correlated with the N protein-specific ELISA. As mentioned earlier, IDEXX's PRRS ELISA kit commonly used for PRRSV serology in veterinary diagnostic laboratories is based on the highly immunogentic N protein of PRRSV. Another attempt to evaluate a protein-specific ELISA showed that non-structural proteins Nsp1, Nsp2 and Nsp7 are highly immunogenic and was capable of detecting antibody specific for these proteins up to 202 dpi, while using other PRRS viral proteins as antigen such as GPs, M and N could only detect antibody up to approximately 126 dpi (Brown et al., 2009).

PRRSV GP5 has been demonstrated to contain major neutralizing epitopes (Pirzadeh et al., 1998). Other neutralizing epitopes of PRRSV have been reported to exist on GP3, GP4 and M protein (Dea et al., 2000, Jiang et al, 2008). The neutralizing epitope of GP5 is known to be located in the middle of GP5 ectodomain corresponding to amino acids 37-45 (Ostrowski et al.,

2002) and is commonly referred to as epitope B. A non-neutralizing but immunodominant epitope has been reported to exist in the amino acid location 27-31 of GP5. This epitope is designated as epitope A and has been postulated to act as a decoy to disrupt the function of epitope B (Ostrowski et al., 2002, Lopez et al., 2994). The studies revealed that antibodies against epitope A were produced early in the course of infection, while neutralizing antibodies against epitope B did not appear until 30 dpi. Not only the amino acid sequence of PRRSV neutralizing epitopes but also their glycosylation pattern and conformational changes can influence neutralizing antibody response. For instance, a natural mutant of the PRRSV VR-2332 strain lacking two N-glycosylated sites in ORF5 induced 10-fold higher SVN antibody compared to the VR-2332 strain showing glycosylation of neutralizing epitopes disrupts development of neutralizing antibodies (Ansari et al., 2006).

In 3- to 10-week old pigs and sows infected with PRRSV MN-1b, MN-H or modified live virus vaccine strain, IgM responses detectable by IFA test differed slightly with the age of pigs (Joo et al., 1997). IgM was detected between 5-28 dpi in 3-week old pigs, 10-28 dpi in 10-week old pigs, and 7-21 dpi in sows. IgG antibody was, on the other hand, detected by IFA test in pigs of all ages between 9 to 14 dpi and lasted until the end of study regardless of strains given. This study was one of the first suggesting utility of IgM detection as an indicator of recent exposure to PRRSV.

When cesarean-derived colostrum-deprived pigs were infected with PRRSV type 1 (i.e., Lelystad virus) and tested for virus-specific antibody isotypes in the bronchoalveolar lavage fluid (BAL) and sera using IMPA, IgG1 and IgM were the first isotypes detected in those pigs as early as 7 dpi. By 20 dpi, IgM antibody titer declined to undetectable in both serum and BAL samples. IgG1 and IgG2 remained high until 52 dpi when the study was terminated although IgG1 levels

were consistently higher than IgG2. IgA was at its highest level on 14 dpi and was detected until 35 dpi (Labarque et al., 2000)

IgG and IgM responses to Nsp2, N, and GP5 were studied by using a peptide-specific ELISA (Mulpuri et al., 2008, Molina et al., 2008). IgM response to Nsp2 and N appeared at 7 dpi and declined to non-detectable levels by 28 dpi. No IgM response to GP5 endodomain was seen; however, the response to GP5 ectodomain harboring neutralizing epitope started at 14 dpi, peaked at 28 dpi and then declined. IgG response to Nsp2 and N was similar, starting at 7 dpi and peaking at 35 dpi. The only difference was that anti-N IgG declined more gradually than anti-Nsp2 IgG. Anti-GP5 ectodomain and endodomain IgG responses were first detected at 21 dpi. The peak response to GP5 endodomain was at 28-42 dpi. The peak response to GP5 ectodomain was seen at 35 dpi with a high degree of variation between animals (Mulupuri et al., 2008).

Viral strain can greatly affect the immune response to PRRSV (Diaz et al., 2012, Kim et al., 2007). Humoral immune response to virulent PRRS viruses measured by ELISA S/P ratio and IFA titer was higher and appeared earlier than attenuated viruses. IFA responses to homologous and heterologous viruses were not significantly different from each other in most cases. On the other hand, SVN titers against homologous virus were significantly different among different virus strains and were not affected by level of virulence. More importantly there was a minimum cross neutralizing activity to heterologous strains. Degree of amino acid sequence identity of ORF 4 and 5 between 2 viruses were shown to correlate with cross neutralizing antibody titer against the 2 viruses (Kim et al., 2007).

In two separate experiments, pigs were intranasally inoculated with one of type 1 PRRSV strains 3262 and 3267 at 0 dpi. At dpi 84 pigs, the pigs were divided into two groups and challenged intranasally with homologous or heterologous strain. Pigs inoculated with the strain 3262 at dpi 0 showed no homologous neutralizing antibody response. After re-inoculation with either homologous or heterologous strain at dpi 84, still no neutralizing antibody against the 3262 strain (i.e., homologous) was developed. Surprisingly the same pigs were able to mount cross-neutralizing antibody against the heterologous strain 3267 (titer ranging from 1:2 to 1:8), and the titer was boosted up after second inoculation. In pigs inoculated with the strain 3267, on the other hand, neutralizing antibody was raised against the homologous strain ranging from 1:8 to 1:16 while cross-neutralizing antibody titer against the strain 3262 was very low ranging from 1:2 to 1:8 after first and second inoculation. The difference between the two strains was attributed to the difference in glycosylation pattern (3262 was more glycosylated around putative neutralizing epitopes) and quantity of neutralizing epitopes present on GP3, GP4 and GP5 (Diaz et al., 2012).

Future steps in PRRSV diagnostics

Given the drawbacks associated with PRRSV diagnostic assays, from the laborious nature of cell-culture based assays to dealing with ELISA false positive result and PCR false negative results, it is often suggested to use a combination of assays for accurate detection of PRRSV infection/exposure. For screening purposes in 9-16 week old pigs, it was shown that ELISA and confirmatory RT-PCR is the most efficient protocol for PRRS diagnosis (Duinhof et al., 2011). Other studies have suggested use of IFA as a confirmatory test on ELISA-suspect positive samples (Kim et al., 2007). Nevertheless, effective use of serology to determine the stage of PRRSV infection is still one of challenges for prevention and control of PRRS. Isotype-specific

serology, as indicated by the literature, might be a promising area for future research. A complete picture of isotype-specific response to PRRSV infection and comparison with neutralizing antibody response is not available. Once available, the potential value of isotype-specific serology can be elucidated.

The use of oral fluids for detection of PRRSV-specific antibodies has been recently proposed as alternative sample matrix for surveillance. Oral fluid- based diagnostic assays are under development or have been developed and shown to be successful surveillance diagnostic tools (Kittawornrat et al., 2010, Prickett et al., 2008). Isotype-specific ELISA on oral fluid samples collected from vaccinated pigs under experimental conditions, using an optimized protocol for oral fluids, produced similar results to titers and kinetics obtained from serum samples (Kittawornrat et al., 2012). IgG antibody peaked at 15 dpi and then very gradually declined, but remained at positive levels until the end of the study. IgM response peaked between 9-15 dpi and declined by day 28, whereas IgA response peaked at 11 dpi and continued to be detected by 126 dpi. While experimental data show a promise of oral fluid based diagnostics for disease surveillance, sampling strategies, any pre-treatments for better outcome of testing on oral fluid samples remain to be further investigated along with advance in diagnostic tools and technologies.

Thesis Objectives

Despite the previous research on humoral immune response to PRRSV infection, there are very few studies which fully characterized antibody response to the virus for a long period of time after infection. Due to the fact that current serological methods routinely used for PRRSV serodiagnosis and surveillance in swine herds are based on IgG response, it was recognized the ontogeny and isotype kinetics of antibody response to PRRSV infection needs to be further

characterized for better understanding of humoral immunity for the virus. The other aim of study was to investigate if the ontogeny and isotype profile of antibody response differ when pigs are exposed to an attenuated virus as compared to a wild type.

CHAPTER 2. ISOTYPE PROFILE OF PPRSV NUCLEOCAPSID-SPECIFIC ANTIBODY RESPONSE IN PIGS AFTER EXPERIMENTAL INFECTION

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Abstract

Effective use of serology to determine the stage of porcine reproductive and respiratory syndrome (PRRS) virus infection is one of challenges for prevention and control of PRRS. The objective of this study was to characterize kinetics of virus-specific IgG, IgM and IgA in pigs after experimental infection with VR2332 strain. Sera were collected from 27 pigs in a 2-week interval from 0 to 202 day post inoculation (dpi) and tested for various antibody isotypes specific for viral nucleocapsid on a commercial PRRS ELISA kit. The pigs comprised 4 groups based on the presence and absence of inoculation at 0 dpi and re-inoculation at193 dpi: 1) inoculated/re-inoculated; 2) inoculated/not re-inoculated; 3) not inoculated/inoculated; and 4) negative control. All samples were randomized and, after 1:5 dilution, tested on IDEXX HerdCheck® PRRS X3 kits using goat anti-swine IgG-, IgM- or IgA antibody labeled with HRP and TMB substrate. Optical density (OD) was measured at 450nm. A cut-off OD and corresponding sensitivity and specificity was determined for each assay based on Receiver Operator Curve analysis and applied to interpretation of ELISA results. Group 4 remained seronegative until the end of the study (202 dpi). After inoculation, pigs developed PRRSV-specific IgG by 7-14 dpi. The IgG lasted at a high level until 202 dpi and was not boosted up by re-inoculation, suggesting that IgG is a good indicator of exposure. PRRSV-specific IgM appeared by 7 dpi and then rapidly

declined. By 28 dpi, no IgM was detected. Re-inoculation did not elicit IgM response whereas Group 3 developed IgM antibody sharply, demonstrating that IgM response can be indicative of first or recent exposure in naïve pigs. PRRSV-specific IgA appeared by 14 dpi, started to decline after 70 dpi to the negative level by182 dpi, and was boosted up by re-inoculation. First neutralizing antibody response measured by fluorescent focus neutralization (FFN) assay was detected at 28 dpi and was boosted up by re-inoculation. The highest neutralizing titer detected during the study was 1:32. Overall FFN antibody and IgA responses appeared to follow the same kinetic pattern. In conclusion, isotype-specific serologic assessment can be useful to determine infection and immune status of pigs.

Introduction

Porcine reproductive and respiratory syndrome (PRRS) virus is an enveloped, positive-sense, single-stranded RNA virus belonging to the family Arteriviridae (Cavanagh, 1997). PRRS virus (PRRSV) preferably replicates in cells of macrophage lineage and can establish a persistent infection in pigs (Plagemann et al 1992, Wills et al 1997). The virus causes reproductive failure in breeding swine and respiratory distress in pigs of all ages, as well as systemic disease in young pigs (Zimmerman et al., 1997). PRRS remains to be one of the most economically significant diseases in the swine industry worldwide. In North America, annual economic loss due to PRRS has recently been estimated to be approximately 660 million USD (Holtkamp et al 2011). In response to economic importance of PRRS, a significant amount of efforts have been mounted into prevention and control including herd management, surveillance testing, vaccination and regional eradication Yet, suboptimal understanding of protective immunity against and transmission dynamics of PRRSV has been an impediment to effective prevention and control of PRRS (Yoon et al., 2003).

Humoral immune response to PRRSV has been studied by several research groups. Yoon et al (1995) used indirect fluorescent antibody (IFA) test, immunoperoxidase monolayer assay (IPMA), enzyme-linked immunosorbent assay (ELISA) and serum-virus neutralization (SVN) test on sera from pigs experimentally infected with PRRSV isolate VR-2402 to characterize antibody response to PRRSV. Virus-specific antibodies were first detected by IFA test, IPMA, ELISA and SVN test at 9-11, 5-9, 9-13 and 9-28 days post inoculation (dpi) respectively. Using a regression analysis, the antibodies detected by these tests were estimated to decline to undetectable levels by 158, 324, 137 and 356 dpi respectively. Molina et al (2008) investigated the diagnostic performance of a commercial ELISA (HerdChek® PRRS Antibody 2XR, IDEXX laboratories) in a longitudinal experimental study in which pigs were inoculated with PRRSV isolate VR-2332 and followed for 193 dpi. The IgG response to PRRSV started at 14 dpi, peaked at 70 dpi and no significant difference was observed between the peak response and 193 dpi.

While IgG response of pigs to PRRSV infection has been reported most frequently, a few studies have illustrated kinetics of anti-PRRSV antibody isotypes. Joo et al (1997) followed IgG and IgM responses of pigs to field isolates and a vaccine strain of PRRSV using IFA test. IgG response was detected between 9 and 14 dpi and remained at a high titer during study period (49 dpi) while the IgM response was only detected between 5 and 28 dpi. Labarque et al (2000) assessed isotypes of antibodies against PRRSV using IPMA in both BAL and serum samples collected from experimentally infected with type 1 PRRSV over time. PRRSV-specific IgM antibody in both sera and BAL was only detected between 9 and 14 dpi and then rapidly declined while IgA antibody was first detected in 14 dpi, peaked at 25 dpi and was last detected at 35 dpi. Mulupuri et al (2008) assessed IgM and IgG response to recombinant structural and non-structural PRRSV proteins (N, GP5 5', GP5 3' and nsp2) using peptide ELISAs. IgG response to

both nsp-2 and N protein started around 7 dpi and peaked at 35 dpi. IgG response to GP5 ectodomain and endodomain appeared at 21 dpi and peaked at 35 dpi. IgM response to nsp2 and N protein was increased at 7 dpi which decreased to undetectable titer by 21 dpi. No IgM response to GP5 endodomain was observed although IgM response to GP5 ectodomain peaked at 21-28 dpi and declined thereafter.

Despite previous studies, the complete isotype profile of PRRSV-specific serum antibody response over time has not been fully understood. Effective use of serology to determine the stage of PRRSV infection and immunological status is yet one of the major challenges in prevention and control of PRRS. The objective of this study was to characterize the kinetics of PRRSV-specific IgG, IgM and IgA in pigs experimentally infected with VR-2332, the protype North American PRRSV, over 200 dpi using a commercial PRRS ELISA platform in comparison to virus neutralizing antibody response.

Materials and Methods

1.1. Study design

Serum samples used in this study were obtained from a previously published study (Molina et al., 2008). The study was a longitudinal study in which pigs were challenged with VR-2332 and followed for 202 days post inoculation (dpi) with re-challenge at 193 dpi. In brief, 14-day-old, PRRSV-negative, large white landrace cross-bred piglets (n=180) were initially purchased from a PRRSV naïve commercial swine operation and transported to a BSL2 animal facility at the Iowa State University. Upon arrival, the pigs were tested with a commercial ELISA kit and reverse transcriptase-polymerase chain reaction (RT-PCR) test to confirm their negative PRRS status. Pigs were randomly assigned to one of the following 4 treatment groups:

Group 1 = Inoculated at day 0, re-inoculated at day 193

Group 2 = Inoculated at day 0, not re-inoculated at day 193

Group 3 = Not inoculated at day 0, inoculated at day 193

Group 4 = Not inoculated at both day 0 and day 193

Each group was housed in a separate room within the same building. All pigs were acclimated for 5 days in the facility before use for the study. At each inoculation day, the pigs were inoculated intramuscularly with 1 ml of PRRSV VR2332 strain (104.0 TCID50/ml) in minimum essential medium (MEM, Sigma Chemical Co., M4655, St. Louis, MO, USA). Negative controls were inoculated with MEM. Serum samples were collected on 0, 7, 14 and, thereafter, every 2 weeks until 202 dpi; hence, each pig was evaluated for specific antibody response at each of the 18 time points after inoculation. A total of 27 pigs remained until the end of the study (i.e., 202 dpi). Serum samples from those pigs were used for the present study. Ten, 9, 4 and 4 pigs represented group 1, 2, 3 and 4, respectively. Samples were assigned a random number and stored in a -80 freezer until being tested by ELISA for different antibody isotypes specific for PRRSV.

1.2. Protocol optimization

Since levels of antibody isotypes were expected to be significantly different from each other, the ELISA protocol for each isotype was modified and optimized using a set of 4 serum samples with known infection status prior to testing. Briefly, a checkerboard titration system was used on 3 time points (dpi 0, 7 and 28), 4 serum dilutions (1:5, 1:10, 1:20 and 1:40) and 6 secondary antibody dilutions (1:10000, 1:15000, 1:20000, 1:25000, 1:50000 and 1:100000). Based on the evaluation, a 1:5 dilution of sera and a 1:15000 dilution of the secondary antibody in 0.01 M phosphate-buffered saline (PBS), pH7.2, were considered as optimum.

1.3. Isotype specific ELISA

A commercial ELISA kit, Herdcheck® PRRS X3 (IDEXX laboratories, Inc., Westport, ME) was used for the study with modifications. Briefly, serum was diluted 1:5 in kit diluent, and 100 µl of each diluted serum sample was added to each well and incubated for 30 min at room temprature. After plates were washed 3 times, 100 µl of optimally diluted goat anti-swine IgG, IgM or IgA antibody labeled with HRP (Bethyl Laboratories, Montgomery, TX) were added to each well and incubated for another 30 min at room temprature. After a second wash step, TMB substrate (KPL, Inc., Gaithersburg, MD) was added and incubated for 15 min at room temprature. The colorimetric reaction was stopped by adding 100µl 1M sulfuric acid to each well. Optical density (OD) of each well was measured on a microtiter plate reader at 450 nm wave length. Each sample was run in duplicate. Three known plate controls and IDEXX PRRS ELISA kit positive and negative controls were included in each plate in order to assess plate-to-plate variation between runs.

1.4. Fluorescent focus neutralization assay

Neutralization assay was done as previously described (Wu et al., 2001). In brief, 2-fold serial dilutions of each test serum were made in 100 µl RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Atlas biologics Inc. Fort Collins, CO), 100IU/ml penicillin (Sigma-Aldrich) and 100µg/ml streptomycin (Sigma-Aldrich). Each diluted sample was mixed with an equal volume of PRRSV VR-2332 adjusted to 100 fluorescent focus forming unit (FFU) per 100 µl. The virus-serum mixture was incubated at 37º C in a humidified CO_2 incubator for an hour and was then inoculated onto fresh MARC-145 cell monolayers prepared in 96-well plates. Inoculated cells were incubated for another hour under the same conditions described above. The inoculum was replaced with fresh RPMI-1640

supplemented with FBS and antibiotics and the plates were incubated for another 20 hours at 37º C in 5% CO_2 atmosphere. The cell culture media was discarded and cells were fixed with 80% ice cold acetone. After a washing step with 0.01 M phosphate-buffered saline (PBS, pH 7.2) the cells were incubated for 1 hour with 100 µl of PRRSV-specific monoclonal antibody SDOW17 diluted 1:10000, washed 3 times with PBS and incubated for 30 min with fluorescein isothiocyanate (FITC)-conjugated goat anti-murine IgG (ɤ) antibody (1:300 dilution). Cells were washed 3 times with PBS and observed under a fluorescent microscope. The neutralizing titer was expressed as the reciprocal of the highest dilution in which FFU was reduced by 90% or more. Each serum sample was run in duplicate.

1.5. Statistical analysis

Data was analyzed with SAS 9.2 (SAS Institute Inc., Cary, NC) and Medcalc® (MedCalc Software, Mariakerke Belgium) statistical software. OD values were log transformed and analyzed using repeated measures ANOVA model. Pair-wise differences among 4 treatment groups were assessed using Tukeys T-test by dpi. Receiver Operator Characteristic (ROC) analysis was done according to the known status of pigs in each treatment group. ROC curves were plotted based on a logistic regression model and the Areas Under the Curves (AUC), a plot of sensitivity (True positives) against 1-specificity (False positives) for all possible cut-off points were estimated to determine the optimum cut-off OD for both best sensitivity and specificity on each isotype ELISA. Neutralizing antibody titers among groups were analyzed using one-way ANOVA since normality assumptions were satisfied for log-transformed FFN titers.

Results

2.1. Cut-off OD determination for each isotype-specific ELISA

The optimum cut-off OD to maximize both sensitivity and specificity was first calculated for each isotype assay (Table 2). For the IgG assay, the optimized cut-off was 1.59 with diagnostic sensitivity of 100% (95% confidence interval=97.5 % to 100%) and diagnostic specificity of 99.4% (95% confidence interval=96.9 % to 100%). For the IgM assay, the cut-off was set at 1.49 with the corresponding sensitivity of 79.0% (95% confidence interval=54.4% to 93.9%) and specificity of 94.1% (95% confidence interval=86.7% to 98.0%). The optimized cut-off OD of 0.32 resulted in an IgA assay with sensitivity and specificity of 74.3% (95% confidence interval=66.2 % to 81.3%) and 90.0% (95% confidence interval=84.7% to 94.0%) respectively.

2.2. Isotyping of PRRSV N protein-specific ELISA antibody response

Group 4 (negative control) remained seronegative (all isotypes) for PRRSV throughout the study. After inoculation, pigs developed virus-specific IgG by 7-14 dpi (Fig. 1). PRRSV N proteins-specific IgG lasted at a high level until 202 dpi when the study was ended and was not boosted up by re-inoculation. Based on pair-wise comparison of the groups, the difference in IgG response between inoculated and not-inoculated groups was statistically significant from dpi 14 to the end of the study ($p<0.0001$).

PRRSV N protein-specific IgM appeared by 7 dpi and then rapidly declined (Fig. 2). By 28 dpi, no IgM was detectable in sera. Re-inoculation did not elicit IgM response whereas group 3 developed IgM antibody sharply after inoculation for the first time at 193 dpi. IgM response showed a significant difference between inoculated and not-inoculated groups at 7 and 14 dpi ($p<0.0001$) and also at 202 dpi ($p= 0.0006$). At dpi 202, the difference between groups 1 and 3 and between groups 2 and 3 were significant ($p= 0.0008$ and 0.0018 respectively).

IgA antibody specific for PRRSV N protein appeared by 14 dpi, started to decline after 70 dpi to negative level by 182 dpi, and was boosted up by re-inoculation (Fig. 3). On the IgA assay, the mean OD difference between inoculated and non-inoculated groups was significant from 14 to 168 dpi. At 202 dpi, the difference between groups 1 (challenged and re-challenged) and 4 (strict negative control) was significant ($p=0.001$).

2.3. Neutralizing antibody response

Neutralizing antibody response as determined by FFN assay was first observed at 28 dpi in one pig (Fig 4). By dpi 42 all sera from inoculated groups 1 and 2 were positive for neutralizing antibody with FFN titer ranging from 1:4 to 1:32 (Fig 4 and 5). The maximum FFN titer during the whole study time was 1:32 (Fig 4). FFN titers started to gradually decline after 154 dpi and some of the inoculated pigs did not have measurable FFN antibody at 182 dpi. Homologous challenge at dpi 193 boosted up neutralizing antibody titer in group 1 as early as dpi 196, indicating an anamnestic response, whereas group 2 continued to have declining neutralizing antibody. As shown in Figure 6, the difference in neutralizing antibody titers between the group 1 (i.e., re-inoculated at dpi 193) and group 2 (i.e., not re-inoculated at dpi 193) was statistically significant ($p< 0.05$). No neutralizing antibody response was detected in group 3 and group 4 (strict negative control).

Figures 7, 8 and 9 illustrate comparative kinetics of IgG, IgM and IgA response versus neutralizing antibody response to PRRSV infection, respectively. IgG OD values and FFN titers followed the same ascending pattern starting at 7 and 14 dpi respectively. While IgG response reached its peak at 28 dpi, FFN antibody response continued to increase up to 154 dpi. The different nature of the two responses became evident when re-inoculation at 193 dpi did not increase the IgG OD value; yet a sharp increase in FFN titer was observed in re-inoculated group

1 (panel A) as opposed to not-inoculated group 2 (panel B) as show in Figure 7. IgM response pattern had no similarity with the FFN titer pattern (Fig. 8). No increase in IgM OD value was observed in group1 after re-inoculation whereas re-inoculation resulted in a sharp increase in FFN titer. Among 3 isotypes, the IgA response pattern appeared to align better with the neutralizing antibody response. Re-inoculated group 1 depicted a parallel increase in both FFN titer and IgA OD value, whereas non-inoculated group shows no increase in IgA OD (Fig.9).

Discussion

There have been a good number of previous studies illustrating general antibody response or isotype-specific response to PRRSV and/or specific viral proteins in pigs after experimental infection (Joo et al., 1997, Labarque et al., 2000, Mulpuri et al., 2008, Murtaugh et al., 2002, Kittawornrat et al., 2012). Yet, the data is sparse and scattered. Different serologic methods were used in different studies. The lack of uniformity between studies makes it hard to interpret and collate data generated by different studies together. Furthermore, many of previous studies on the serologic response of pigs after PRRSV infection were commonly done for acute stage or for a relatively short period of time after infection. In this regard, the present study is one of rare studies to characterize the ontogeny of humoral immune response of pigs to PRRSV for a long period of time (>200 dpi) after experimental infection and assess isotype profile of antibody response in parallel to neutralizing antibody response.

This study utilized a commercial ELISA kit (HerdCheck® PRRS X3, IDEXX) which is based on N protein of PRRSV to assess kinetics of PRRSV-specific antibody isotypes instead of in-house ELISAs for several reasons. First, the kit has been extensively used for detecting serum antibody specific for PRRSV at least in North American veterinary diagnostic laboratories if not worldwide and has proven to be a reliable serologic tool. Second, studies in which recombinant

protein or peptide based ELISAs were used have demonstrated that N protein based ELISA has the best diagnostic performance in terms of specificity, sensitivity and broadness of reactivity (Mulpuri et al., 2008, Molina et al., 2008). Third, use of a commercial ELISA kit takes advantage of manufacturer's quality control and assurance in antigen preparation, which, in turn, minimizes plate-to-plate variation and enhances test reproducibility when used the same set of reagents. With the same thought process, all secondary antibody conjugates were purchased from the same vendor to minimize inconsistency in quality and batch-to-batch variation. Overall test results were highly repeatable when a subset of randomly selected samples was re-tested for quality assurance purpose (data not shown).

It was anticipated that the amount of IgM or IgA in serum samples would be much less than IgG. To increase our chance to accurately detect IgM and IgA antibodies, the ELISA protocol, particularly regarding sample dilution before testing had to be deviated from manufacturer's recommendation. Instead of 1:40 recommended sample dilution, all samples were tested after 1:5 dilution based on prior checker board evaluation to determine an optimal sample dilution. Due to lower sample dilution, IgG response to PRRSV in our study showed a sort of saturation effect (Fig. 1) which would have been attributed to the fact that antibody amount in some samples may have exceeded the amount of antigens available in wells. Nonetheless, the results obtained from our modified IgG ELISA particularly on samples collected at early stage of infection was in agreement with the results from a previous study (Molina et al., 2008) in which the same set of serum samples were tested by HerdCheck® PRRS 2X ELISA (i.e., earlier version) as directed by the manufacturer.

Overall, observed kinetics of IgM and IgG responses during our study were within expected profiles and were also in general agreement with previous reports by other investigators showing

that IgM response can be detected quickly but lasts for a short period of time after infection while IgG antibody is developed to a high titer and lasts long. Kinetics of IgA response could not be compared to previous studies ((Joo et al., 1997, Labarque et al., 2000, Mulpuri et al., 2008) since duration of those studies was much shorter than ours and did not evaluate IgA response to re-inoculation. The only agreement with the previous studies was that initial IgA response was detected around the same time after exposure. Based on our observations and others, a positive IgG result on the ELISA should be a good indicator of PRRSV exposure and IgG assay be a good serological too for surveillance as it lasts long after exposure. In comparison, a positive IgM result would be indicative of first and/or recent exposure to PRRSV in naïve pigs since re-infection with the homologous strain did not elicit an IgM response under study conditions. It remains to be interesting and needs to be further studied if previously exposed pigs mount IgM response when they are exposed to a 'heterologous' PRRSV strain. If so, an IgM assay can be a serological tool to determine immunological relatedness between PRRS viruses, particularly concerning cross protection.

An unexpected observation from our study was getting higher background reactivity for IgM to a certain level on the ELISA kit over time (Fig 2). A similar observation was also made with IgA assay (Fig. 3). Since non-inoculated pigs remained negative for IgG throughout the study (Fig. 1) and were not viremic (Molina et al 2008), the possibility of PRRSV spreading between inoculated and non-inoculated groups can be ruled out. Since increased background reactivity was not observed with IgG assay, the purity of antigen in the ELISA kit should not be questioned although a varying degree of non-specific background reactivity has been reported depending upon antigen type or antigen preparation method for ELISA (Technical guide for ELISA KPL, Inc) Then, increased background reactivity for IgM or IgA should be a normal phenomenon

associated with aging of pigs probably due to immune maturation as the animals get exposed to various microbes from the environment. Regardless of reason(s), such an unexpected increase in the background reactivity affected negatively on the sensitivity of the assay for IgM and IgA since a higher cut-off OD had to be selected to maintain a reasonable specificity. This should be taken into consideration for future development of isotype-specific assays.

The duration of protective immunity against PRRSV in pigs once exposed is still remained to be further understood. A previous study reported clinical protection from PRRSV infection for almost 2 years after the initial outbreak of PRRS in sows (Lager et al 1997). However, measurable anti-PRRSV antibodies may not last for that long since the protective immunity for PRRSV would require both humoral and cell-mediated immune components. A study estimated the time to seronegative for PRRSV in experimentally infected pigs by various serologic assays using a regression analysis based on declining trend of antibody level measured by each assay (Yoon et al 1995). The longest estimate was about a year for neutralizing antibodies. The current study data from FFN test seem supports that estimate as many of the pigs tested remained positive for neutralizing antibody at 193 dpi. More importantly it appears that pigs still had a memory response at 193 dpi as they mounted an anamnestic production of neutralizing antibody to a higher titer. Interestingly PRRSV N protein-specific IgA response seemed to align well with FFN antibody response in our study (Fig. 9) although levels of IgA in sera was much lower than those of IgM and IgG based on OD values. This observation may be taken into consideration if one were to look for an ELISA-based assay as alternative for neutralizing antibody assay since ELISA offers high-throughput capability, excellent reproducibility and shorter turnaround time of results.

Taken all together, it appears that isotype-specific serologic assessment can be useful to determine infection/immune status of pigs for PRRSV, although the ELISA protocol might need further adjustments and optimization to improve diagnostic performance.

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Figures and Tables

Figure 1. IgG response to PRRSV N protein in pigs experimentally infected with VR2332 strain over time as measured on a commercial PRRS ELISA kit. Groups 1 and 2 were inoculated intramuscularly with 10^4 $TCID_{50}$/ml of the virus at day 0. Group 1 was inoculated again at 193 days post inoculation while group 2 received the sham inoculum. Group 3 was inoculated with the virus only at day 193. Group 4 served as strict negative control.

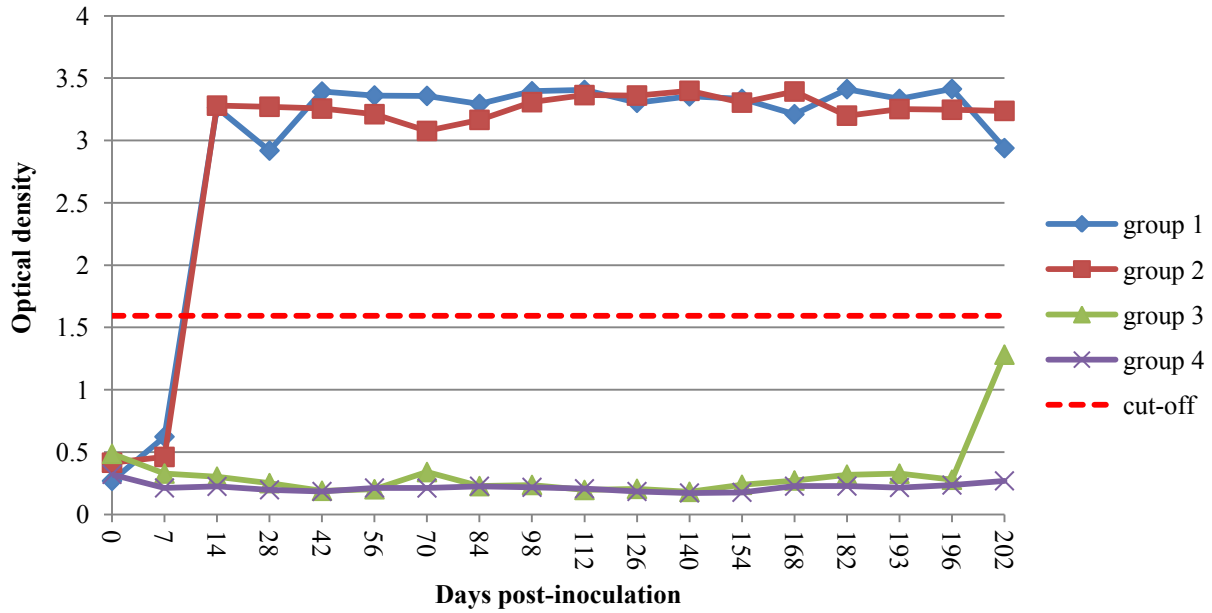

Figure 2. IgM response to PRRSV N protein in pigs experimentally infected with VR2332 strain over time as measured on a commercial PRRS ELISA kit. Groups 1 and 2 were inoculated intramuscularly with 10^4 $TCID_{50}$/ml of the virus at day 0. Group 1 was inoculated again at 193 days post inoculation while group 2 received the sham inoculum. Group 3 was inoculated with the virus only at day 193. Group 4 served as strict negative control. Group 4 data not shown because it closely followed group 3 kinetics.

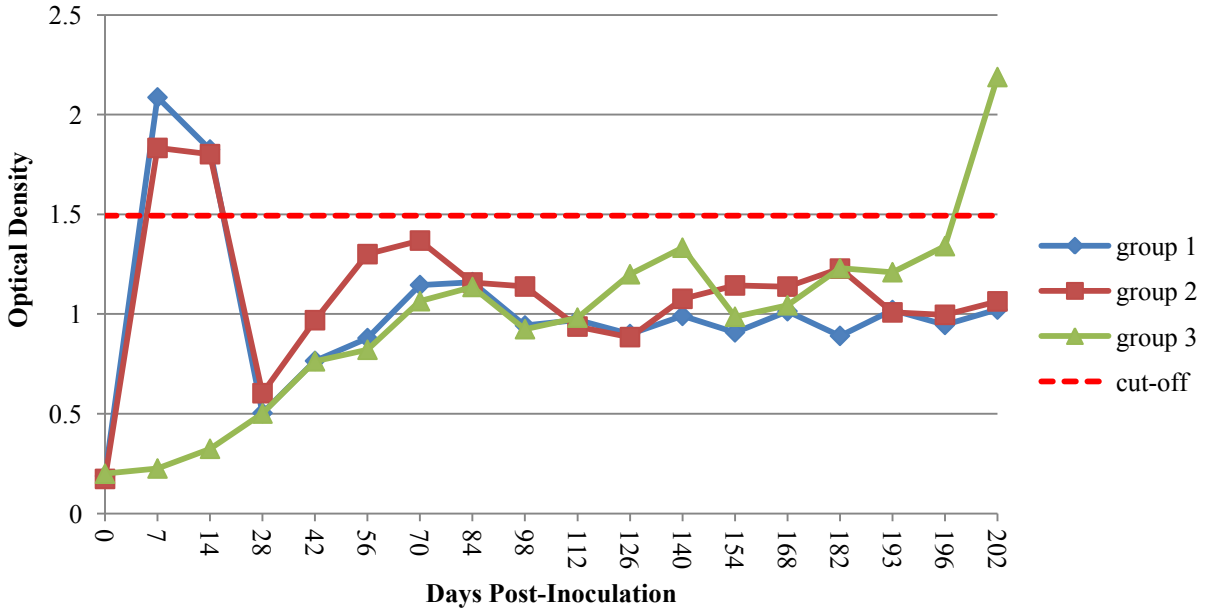

Figure 3. IgA response to PRRSV N protein in pigs experimentally infected with VR2332 strain over time as measured on a commercial PRRS ELISA kit. Groups 1 and 2 were inoculated intramuscularly with 10^4 $TCID_{50}$/ml of the virus at day 0. Group 1 was inoculated again at 193 days post inoculation while group 2 received the sham inoculum. Group 3 was inoculated with the virus only at day 193. Group 4 served as strict negative control.

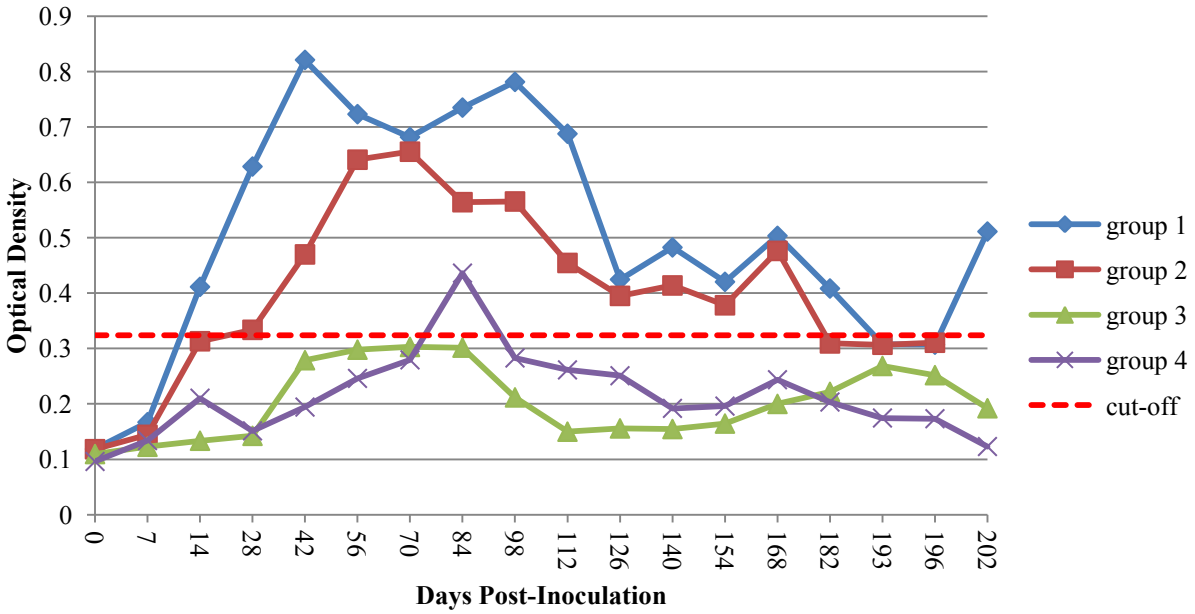

Figure 4. Neutralizing antibody response to PRRSV in pigs experimentally infected with VR2332 strain as measured by fluorescent focus neutralization (FFN) assay. Group 1 and 2 were inoculated intramuscularly with 10^4 $TCID_{50}$/ml of the virus at day 0 and group 1 was inoculated again with the virus at 193 days post inoculation while group 2 received the sham inoculum.

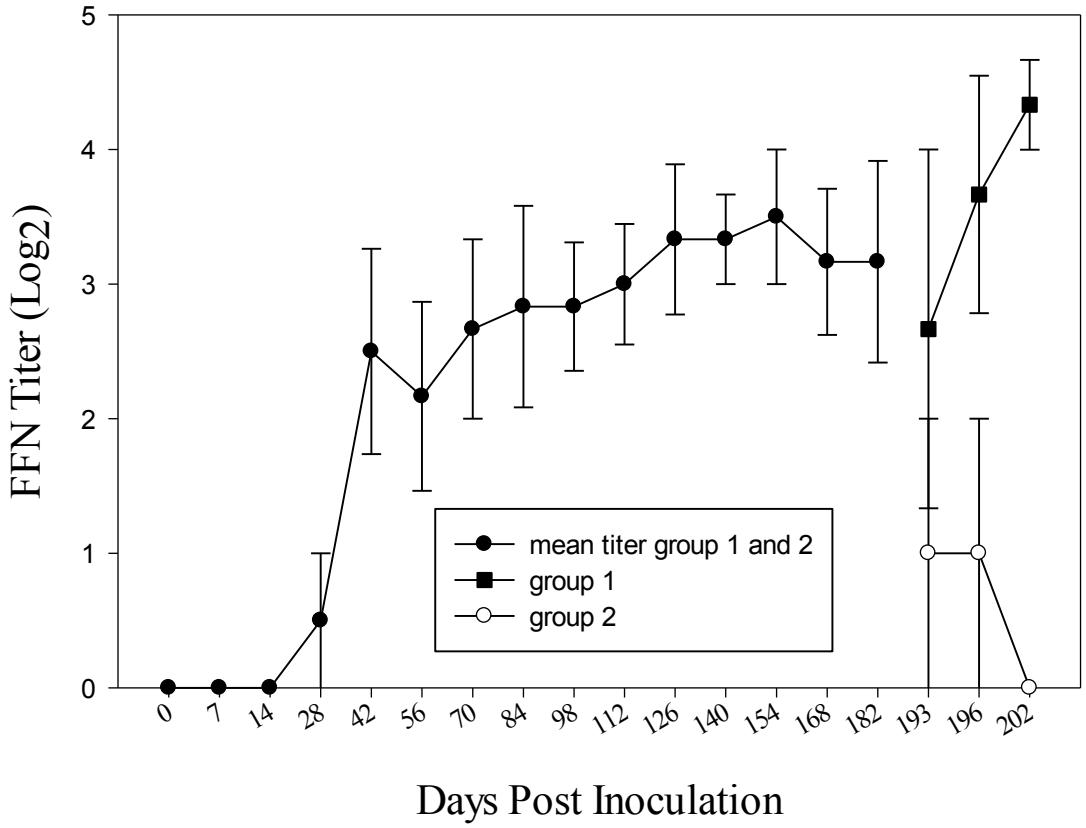

Figure 5. Distribution of neutralizing antibody titers to PRRSV in pigs experimentally infected with VR2332 strain. The animals were inoculated intramuscularly with 10^4 $TCID_{50}$/ml of the virus at day 0. Each box represents 75^{th} (upper) and 25^{th} (lower) percentile. The median and mean for each sampling day is represented by a solid line and dot respectively. Upper and lowers error bars represent 90^{th} and 10^{th} percentile, respectively.

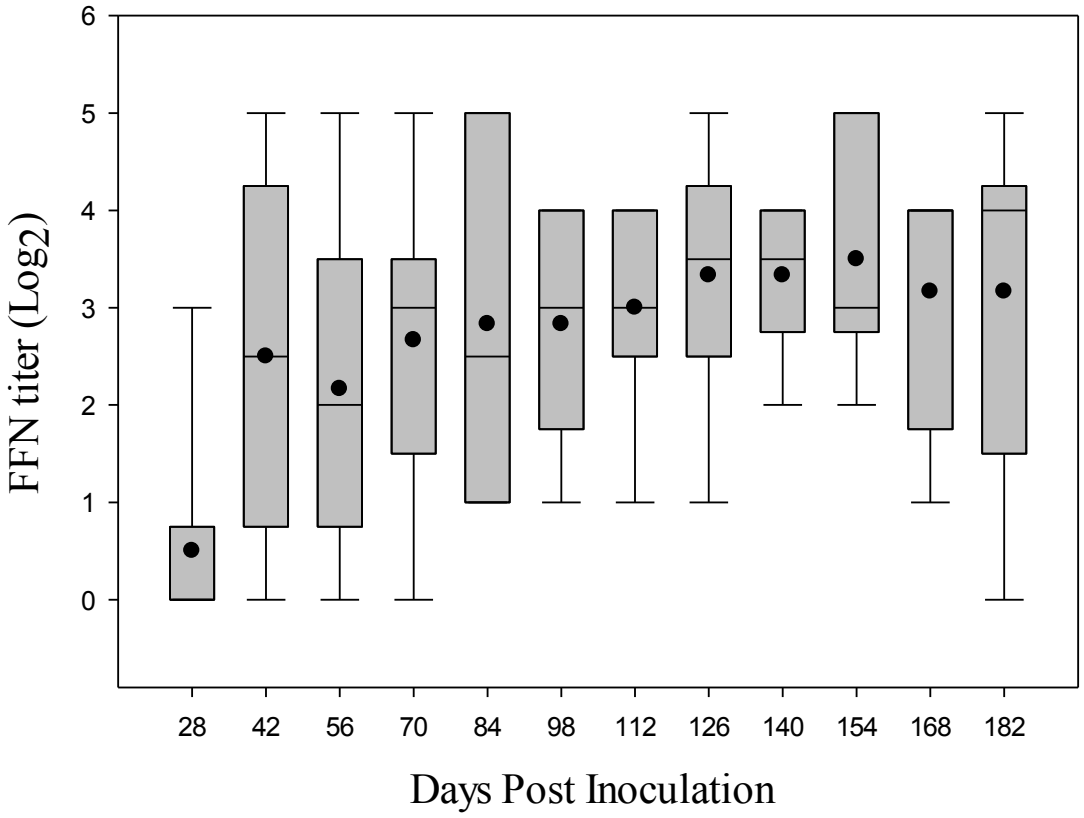

Figure 6. Anamnestic anti-PRRSV neutralizing antibody responses of pigs after re-challenge. Both group 1 and 2 were inoculated intramuscularly with VR2332 strain at the rate of 10^4 $TCID_{50}$/ml at day 0. Group 1 was re-inoculated with the virus at 193 days after the first inoculation while group 2 received the sham inoculum. Both groups were sampled at 193 (0), 196 (3) and 202 (9) days after the first inoculation (re-inoculation). Neutralizing antibody titers were measured by fluorescent focus neutralization (FFN) assay. Each box represents upper (75^{th}) and lower (25^{th}) percentile. The median and mean for each sampling day is represented by a solid line and dot respectively.

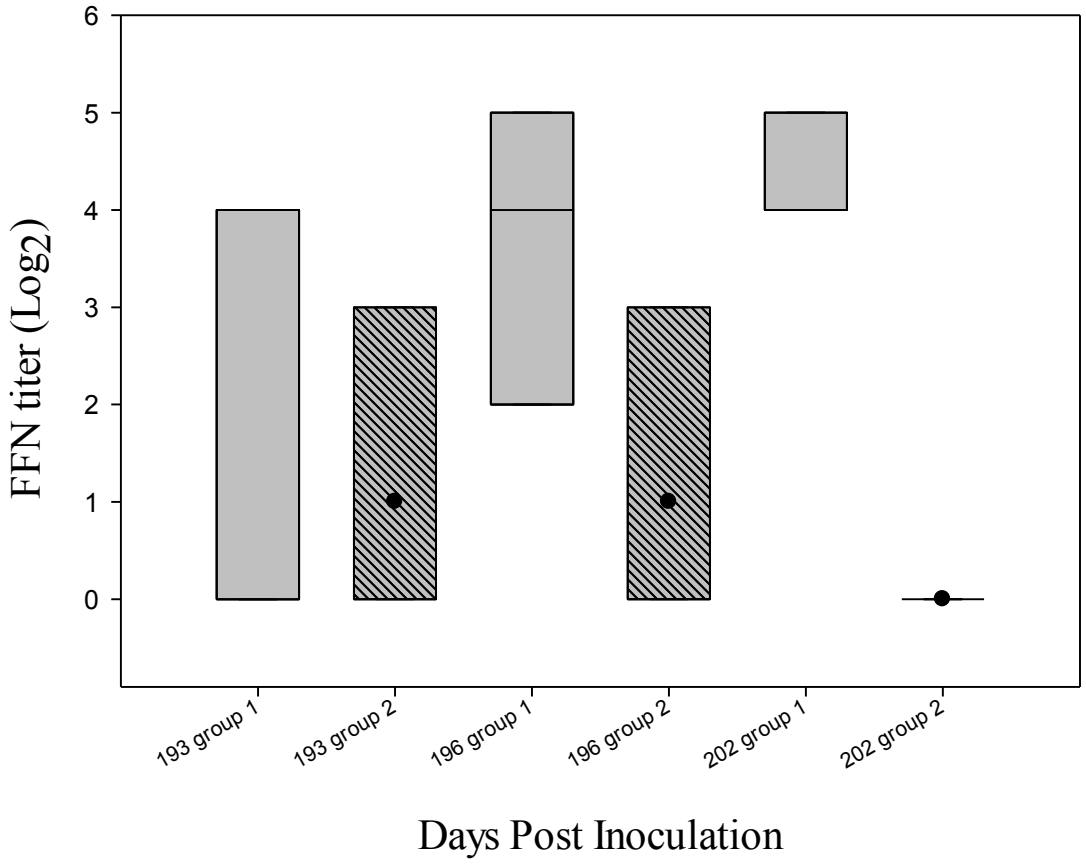

Figure 7. Comparative kinetics of virus-specific IgG antibody and neutralizing antibody response to PRRSV in pigs experimentally infected with VR2332 strain. Both group 1 (Panel A) and 2 (Panel B) were inoculated intramuscularly with 10^4 $TCID_{50}$/ml of the virus. Group 1 was re-inoculated with the virus at 193 days after the first inoculation while group 2 received the sham inoculum. PRRSV-specific IgG antibody was measured using a commercial ELISA kit while neutralizing antibody was measured by fluorescent focus neutralization (FFN) assay. Values denote mean ± S.E.

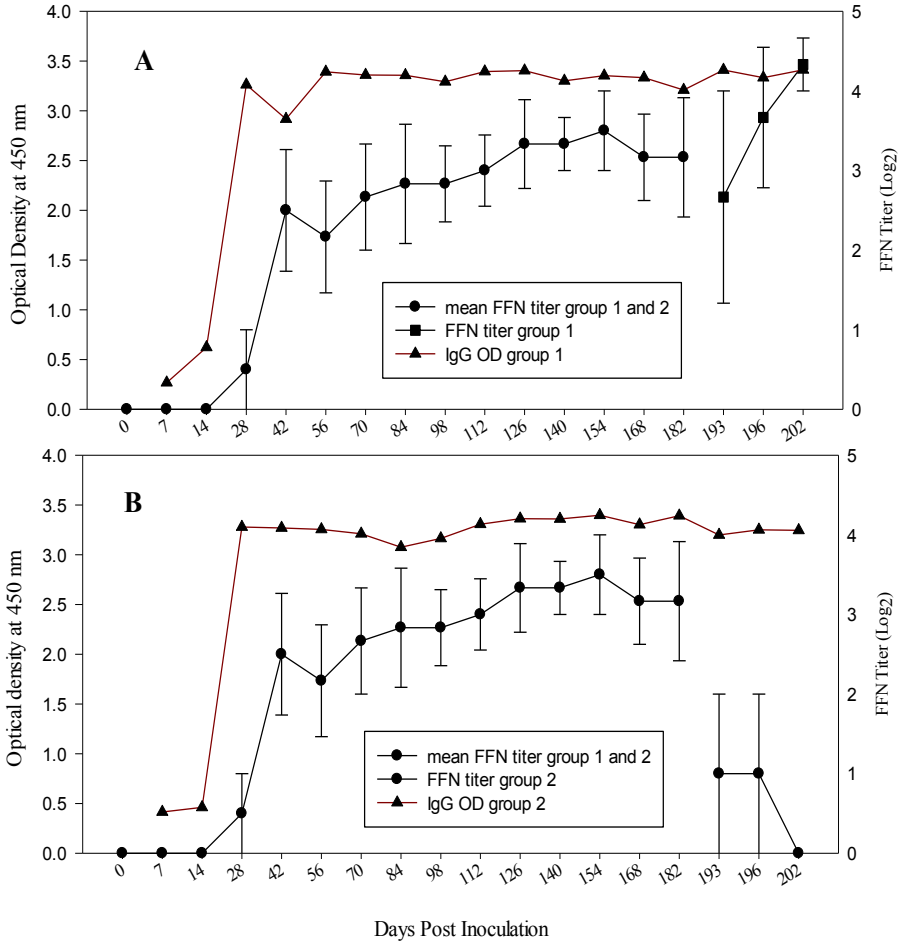

Figure 8. Comparative kinetics of virus-specific IgM antibody and neutralizing antibody response to PRRSV in pigs experimentally infected with VR2332 strain. Both group 1 (panel A) and 2 (panel B) were inoculated intramuscularly with 10^4 $TCID_{50}$/ml of the virus. Group 1 was re-inoculated with the virus at 193 days after the first inoculation while group 2 received the sham inoculum. PRRSV-specific IgM antibody was measured using a commercial ELISA kit while neutralizing antibody was measured by fluorescent focus neutralization (FFN) assay. Values denote mean ± S.E.

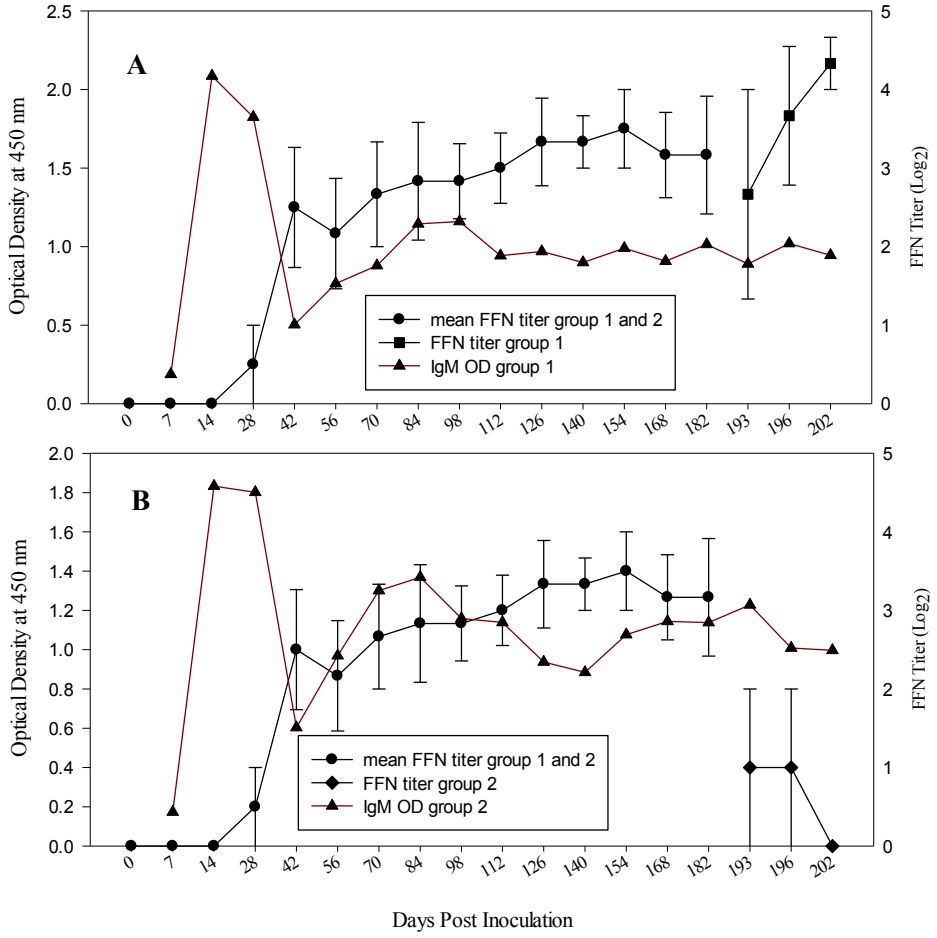

Figure 9. Comparative kinetics of virus-specific IgA antibody and neutralizing antibody response to PRRSV in pigs experimentally infected with VR2332 strain. Both group 1 (panel A) and 2 (panel B) were inoculated intramuscularly with 10^4 $TCID_{50}$/ml of the virus. Group 1 was re-inoculated with the virus at 193 days after the first inoculation while group 2 received the sham inoculum. PRRSV-specific IgA antibody was measured using a commercial ELISA kit while neutralizing antibody was measured by fluorescent focus neutralization (FFN) assay. Values denote mean ± S.E.

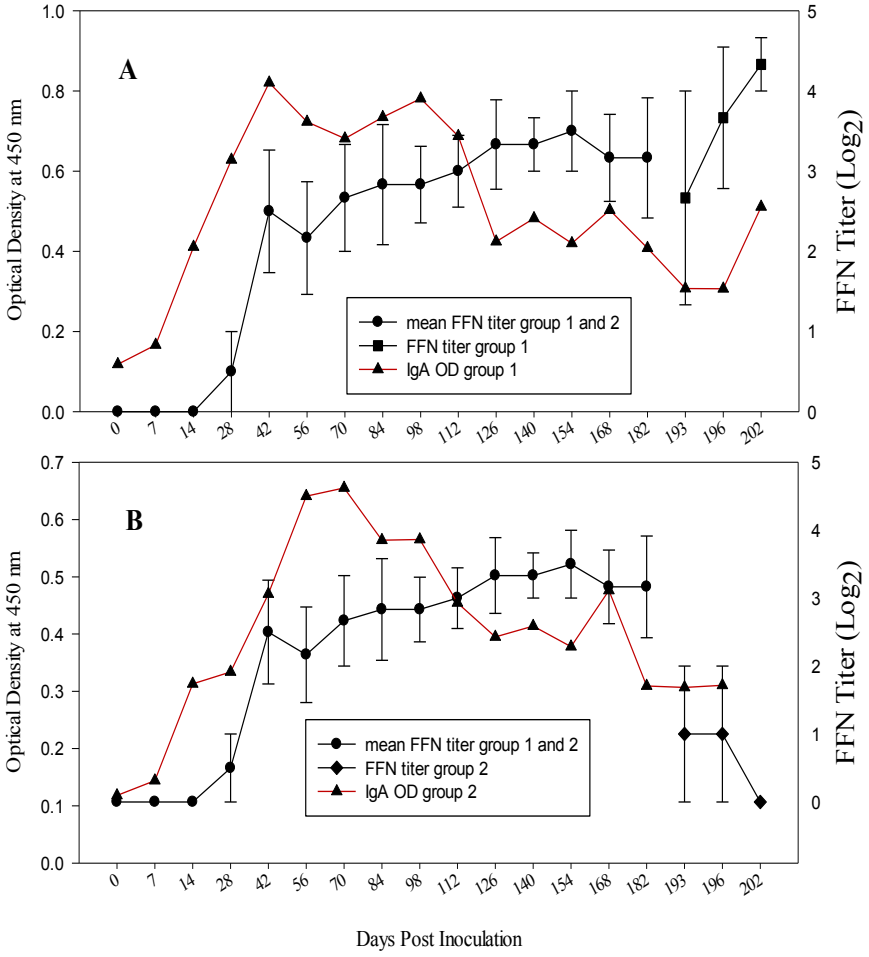

Table 1. Diagnostic performance of isotype-specific ELISA assays.

Elisa Assay	AUC[a]	SEM[b]	Sensitivity	Specificity	OD cut-off
IgG	1.00	0.0000548	100 (97.5 , 100)[c]	99.44 (96.9, 100)	1.59
IgM	0.905	0.0421	78.95 (54.4, 93.9)	94.05 (86.7, 98.0)	1.49
IgA	0.913	0.0155	74.29 (66.2, 81.3)	90.00 (84.7, 94.0)	0.32

[a]Area under the curve
[b]Standard error of mean
[c]95% confidence interval

CHAPTER 3. ISOTYPE-SPECIFIC AND NEUTRALIZING ANTIBODY RESPONSES OF PIGS TO AN EXPERIMENTAL PRRSV VACCINE

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Abstract

Porcine reproductive and respiratory syndrome (PRRS) continues to be one of the major challenges for the swine industry worldwide. Modified live virus vaccines against PRRS virus (PRRSV) have been used in North America since 1995 to aid in PRRS control. However vaccination has shown to generally induce low and delayed humoral and cell-mediated immune responses and has not always conferred optimal protection against heterologous challenge. Recently, a new modified live virus vaccine was introduced to the market in the United States with the label claim of aid in prevention of respiratory disease associated with PRRSV infection. The study objective was to evaluate isotype-specific and neutralizing antibody responses to this particular experimental vaccine. An isotype-specific ELISA test protocol based on a commercial PRRS ELISA kit (IDEXX) was used as was previously modified and optimized in our laboratory. The sample set was obtained from a vaccine safety study in pregnant sows. Ten PRRS naïve dams were given 1 dose of the vaccine at 90 days of gestation while 5 controls were given a sham inoculum. Serum samples were collected at 0, 3, 7, 10, 14, 21, 28/29 and 35/36 days post vaccination (dpv) and tested for isotypes by ELISA and for neutralizing antibody by fluorescent focus neutralization (FFN) assay. Neutralizing activity of the sera was evaluated against both homologous (the vaccine) and heterologous (VR2332) strains of PRRSV. Optical density (OD) values were recorded and analyzed with repeated measure ANOVA. FFN data were analyzed

using Kruskal-Wallis ANOVA on ranks and Wilcoxon rank sum test. The cut-off OD which was set by the previous study (Chapter 2) was used for each isotype. Virus-specific IgG was detected as early as at 10 dpv and continued to increase by 35 dpv. IgM was only detected between 10 and 28 dpv. The difference in IgA response between vaccinated group and non-vaccinated group was statistically significant at 10 and 14 dpv; however, when applied the previously established cut-off OD (0.3), none of the sera could be considered positive for IgA. Homologous neutralizing antibody response was first detected at 10 dpv and reached a FFN titer of 1:128 by 35 dpv. Heterologous neutralizing activity was detected in vaccinated pigs at 21 dpv and thereafter, although the maximum FFN titer never reached above 1:16. Overall, isotype-specific ELISA appears to be a useful tool in assessing the serologic status of vaccinated pigs. The vaccination did not induce detectable IgA antibody specific for PRRSV N protein under study conditions, which remains to be further studied.

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) belongs to *Arteriviridae* family genus *Arterivirus*. It is an enveloped virion with a positive-sense singled-stranded RNA genome of approximately 15 kb in length (Cavanagh, 1997, Allende et al., 1999, Wensvoort et al., 1991). Hallmarks of the virus are replication in host macrophages, high degree of virus evolution, and establishment of a persistent infection (Zimmerman et al., 1997, Wills et al 1997). European (type 1) and North American (type 2) genotypes of the virus have been identified and exist with high genetic variability between the genotypes and within each genotype (Kapur et al., 1996, Kim et al., 2007, Meng et al., 2000).

Clinical manifestations of PRRS have been characterized by respiratory disease in pigs of all ages and reproductive failure in breeding swine. The disease has cost the enormous economic

loss to the North American swine industry which has been estimated to be 664 million USD annually (Holtkamp et al., 2011). In response to the economic significance of PRRS, vaccination has been utilized to aid in prevention and control of PRRS. Both modified live virus and killed virus vaccines for PRRSV have been available for veterinary professionals and producers. In general, modified live virus (MLV) vaccines are perceived as more efficacious than killed virus (KV) vaccines, but their safety (i.e., reversion to virulence) has been occasionally in question (Torrison et al., 1996, Nielson et al., 2001, Shi et al., 2010). The effectiveness of the currently available commercial vaccines against heterologous strains is also of concern (Cano et al., 2007, Mengeling et al ., 2003, Meng et al., 2000, Murtagh et al., 2002, Huang et al 2010, Kimman et al 2009, Labarque et al., 2004).

Humoral immune response of pigs to PRRSV infection has been characterized by first appearance of a strong but non-neutralizing antibody response starting as early as 5-9 days post infection, while neutralizing antibody response does not appear until 4-5 weeks after infection (Murtaugh et al 2002, Yoon et al., 1995). Non-neutralizing antibody that appears in the early stage of infection was postulated to contribute to antibody-dependent enhancement of virus infection (Yoon et al 1996). Development of a weak and delayed active immune response to PRRSV has been proposed to be one of the reasons for vaccine failure (Murtaugh et al., 2002, Kimman et al 2009). Recently a new MLV vaccine (Zoetis Animal Health) was introduced to the market in the United States with the label claim of aid in prevention of respiratory disease associated with PRRS. Since there is no information available for the immunogenicity of this new vaccine, the following study was conducted to elucidate a humoral immune response profile of sows after vaccination. Specific objective was to evaluate isotype-specific and neutralizing

antibody response to this experimental vaccine. Cross neutralization activity against a heterologous strain was also assessed.

Materials and Methods

1.1. Serum samples

The serum sample set was obtained from a vaccine safety study in pregnant swine and used for the study. In brief, a total of 15pregnant sows at various parity numbers (P2 to P10) were purchased from a PRRSV naïve commercial farm and housed in the BSL2 Large Animal Infectious Disease Facility at Iowa State University. Ten randomly selected sows were vaccinated with one dose of the experimental modified live PRRSV vaccine (Fostera® PRRS, Zoetis) at 90 days of gestation as directed by the manufacturer and the remaining 5 sows were given with a sham inoculum (i.e., cell culture media). Blood samples were collected from vaccinated sows at 0, 3, 7, 10, 14, 21, 28 and 35 days post vaccination (dpv) and from unvaccinated sows at 0, 3, 7, 10, 14, 21, 29 and 36 dpv. All pigs were euthanized at 46 dpv. Bloods were processed for serum collection immediately after sampling. All serum samples were coded with random numbers and stored in -80° C until use.

A set of serum samples from piglets inoculated with VR-2332 strain from our previous study (Chapter 2) were also used in this study as reference when assessing cross-neutralization Fostera® PRRS vaccine virus and VR-2332, even though the antisera were generated in a different manner and the study platform (age of pigs and sample collection days) were different among the two studies. The sera were collected biweekly between 0 and 42 days post inoculation (dpi).

1.2.Viruses

Fostera® PRRS vaccine strain and VR-2332 virus strains were used. Open reading frame 5 sequence of Fostera® PRRS vaccine strain was compared to that of some selected well-characterized PRRSV strains (Lelystad, VR-2385, VR-2332, JA-142, ISU-P, SDSU73, MN-184) (Kim et al., 2007). As shown in Figure 1, strains MN184, VR-2332, VR-2385 and ISU-P were identified as heterologous strain to the vaccine virus since they shared less than 90% ORF5 sequence identity with the vaccine strain. Among the reference strains, the VR-2332 strain that shares 91.7% ORF5 identity with the vaccine virus was selected for the study since: a) VR-2332 strain is the parental strain of the most commonly used commercial MLV vaccine (Ingelvac® PRRS) in the US and b) a set of serum samples collected from pigs experimentally infected with VR-2332 was already in the possession.

1.3. Isotype-specific ELISA

A commercial ELISA kit, Herdcheck® PRRS X3 (IDEXX laboratories, Inc., Westport, ME) which is based on PRRSV N protein, was used for the study with modifications. Briefly, serum was diluted 1:5 in the diluent provide with ELISA kit, and 100 µl of each diluted serum sample was added to each well and incubated for 30 min. After plates were washed 3 times, 100 µl of optimally diluted HRP-labeled goat anti-swine IgG, IgM or IgA antibodies (Bethyl Laboratories, Montgomery, TX) were added to each well and incubated for another 30 min. After a second wash step, TMB substrate (KPL, Inc., Gaithersburg, MD) was added and incubated for 15 min. The colorimetric reaction was stopped by adding 1M sulfuric acid to each well. Optical density (OD) of each well was measured on a microtiter plate reader at 450 nm wave length. All reactions were conducted at ambient temperature. Each sample was run in duplicate. Three known plate controls and IDEXX's PRRS ELISA kit positive and negative controls were

included in each plate in order to assess plate-to-plate variation between runs. The corrected OD was calculated for each sample by subtracting OD of blank well from OD of well inoculated with serum sample. Samples with corrected OD ≥ 1.59, 1.49 and 0.32 were considered positive for IgG, IgM and IgA, respectively, on each isotype-specific ELISA.

1.4. Virus neutralization assay

Fluorescent focus neutralization (FFN) assay was performed to assess the presence/absence and level of neutralizing antibody against PRRSV in sera as previously described (Wu et al., 2001). In brief, 2-fold serial dilutions of each test serum were made in 100 µl RPMI-1640 (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Atlas biologics Inc. Fort Collins, CO), 100IU/ml penicillin and 100µg/ml streptomycin (Sigma-Aldrich). Each diluted sample was mixed with an equal volume of Fostera® PRRS vaccine strain or VR-2332 adjusted to 10^2 fluorescent focus forming unit (FFU) per 100 µl. The virus-serum mixture was incubated at 37º C in a humidified CO_2 incubator for an hour and was then inoculated onto fresh MARC-145 cell monolayers prepared in 96-well plates. Inoculated cells were incubated for another hour under the same conditions described above. The inoculum was replaced with fresh RPMI-1640 supplemented with FBS and the antibiotics, and the plates were incubated for another 20 hours at 37º C in 5% CO_2 atmosphere. The cell culture media was discarded and cells were fixed with 80% ice cold acetone. After a washing step with PBS, the cells were incubated for 1 hour with (100µl per well) of PRRSV-specific monoclonal antibody SDOW17 diluted 1:10000, washed 3 times with 0.01 M phosphate-buffered saline (PBS, pH 7.2) and incubated for 30 min with fluorescein isothiocyanate-conjugated goat anti-murine IgG (ɤ) antibody (1:300 dilution). Cells were washed 3 times with PBS and observed under a fluorescent microscope.

The neutralizing titer was expressed as the reciprocal of the highest dilution in which FFU was reduced by 90% or more. Each serum sample was run in duplicate.

1.5. Statistical analysis

Statistical analysis was performed with SAS 9.3 (SAS Institute Inc. Cary, NC). Repeated measure ANOVA test was done on log transformed ELISA data using Proc glimmix. Pair-wise comparison was performed with Student's *t* test adjusted by Tukey method. Neutralizing antibody titers among groups were analyzed using Kruskal-Wallis analysis of variance on ranks and Wilcoxon rank sum test. The significance level was set at 0.05.

Results

2.1. Isotyping of antibody response

Throughout the study period, the sham-inoculated group remained seronegative for PRRSV. In the vaccinated group, PRRSV N protein-specific IgG was first detectable at 10 dpv with mean OD of 1.79 ± 0.24, although a significant difference in IgG response between vaccinated and sham-inoculated groups was apparent starting at 3 dpv ($p=0.0273$) as summarized in Table 1. The increasing trend was evident until 35 dpv (last serum collection time point in this study). From 10 dpv to the end of the study, the overall difference in IgG response between vaccinated and sham-inoculated groups (Fig. 2) was highly significant ($p<0.0001$).

As for PRRSV N protein-specific IgM antibody, the difference between vaccinated and sham-inoculated groups was significant from 10 up to 28 dpv (Table 1). There was a sharp increase in the level of IgM antibody between 7 and 10 dpv (Figure 3). After 10 dpv, the level of IgM antibody started to gradually decline. Mean OD (± SE) values of vaccinated group for IgM from 10 to 28 dpv were above 1.5, while values of the sham-inoculated group at the same time

points were less than 0.8. The difference in IgM antibody response between the two groups at 35 dpv was no longer significant (p=0.0686).

On the IgA ELISA, the difference between the vaccinated and sham-inoculated groups was only statistically significant at 10 and 14 dpv with p=0.0028 and p=0.0002, respectively (Figure 4). However, when applied the previously established cut-off OD of 0.3 for PRRSV N protein-specific IgA (see Chapter 2), none of the vaccinated animals were considered positive for IgA.

2.2. Neutralizing antibody response

After vaccination, neutralizing antibody against the vaccine virus (i.e., homologous virus) was detected by 10 dpv with mean titer of 1:8 (ranging from 1:8 to 1:16). Then FFN titer continued to increase to 1:128 over time (Figure 5). No neutralizing antibody was detected in the sham-inoculated group at any of the time points. Accordingly, the overall difference in the median FFN titers between the vaccinated and sham-inoculated groups at all days post vaccination was statistically significant ($p< 0.001$). Pair-wise comparison of median FFN titers is presented in Table 2.

IgG response and FFN response showed a similarity: an increase in both responses starting 7 dpv and continuing to increase until 35 dpv (Figure 6). In contrast, kinetics of IgM or IgA response and showed a different pattern from FFN response. While IgM and FFN responses started to increase around 7 dpv, IgM rapidly declined by 10 dpv but FFN titer continued to increase until 28 dpv (Figure 7). In case of IgA, From 14 dpv IgA anrtibody started to decline from 14 dpv while FFN titer continued to raise (Figure 8).

When the same set of serum samples from vaccinated sows were tested against VR-2332 strain (i.e., heterologous virus) in FFN assay, cross-neutralizing antibody was observed at 21, 28

and 35 dpv with mean neutralizing titer (log_2) of 1.0 (±0.53), 1.8 (±0.4) and 1.4 (±0.4), respectively, which were overall significantly ($p<0.05$) lower than FFN titers against the homologous virus (Figure 9). The highest heterologous titer obtained was 1:16 in one pig at 28 dpv. When the serum set collected from pigs inoculated with VR-2332 was tested against Fostera® PRRS vaccine strain (i.e., heterologous virus) in FFN assay, cross-neutralizing antibody was observed at 28 and 42 dpi with mean FFN titers (log_2) of 2.2 (±0.74) and 2.5 (±0.56), respectively (Figure 10). It appears that no significant difference was detected in the ability of VR-2332 antisera to neutralize homologous and heterologous virus at both 28 and 42 dpi ($p= 0.09$).

Discussion

The objective of this study was to characterize isotype profile of antibody response to a novel experimental vaccine in pigs as well as neutralizing antibody response and to evaluate if each isotype-specific response is capable of depicting the immune status of vaccinated pigs. Vaccinated sows developed IgG antibody by 10 dpv although a statistically significant difference in IgG level between vaccinated and unvaccinated groups was evident as early as 3 dpv (p-value=0.02) and remained seropositive with continuous increase in the titer until the end of the study (Fig. 2). On the other hand, IgM antibody was also detectable in vaccinated pigs by 10 dpv and then started to quickly decline below the positive cut-off by 28 dpv (Fig. 3). The timeline for initial detection of virus-specific IgG and IgM responses was in agreement with previous reports illustrating detection of positive IgG and IgM antibody in pigs by a commercial ELISA at 10 and 7 days after vaccination with Ingelvac® PRRS MLV respectively (Charerntantanakul et al., 2006, Kittawornrat et al., 2013). A statistical evidence of earlier

seroconversion to the vaccine virus on IgG assay in our study may have been attributed to the fact that a lower serum dilution (1:5 instead of 1:40) was used for isotype testing.

An interesting observation from the current study was the lack of IgA response in vaccinated sows when using pre-established cut-off OD for positives. Statistically, however, there was a significant difference in IgA response between vaccinated and unvaccinated groups at 10 through 21 dpv (p-value < 0.05) which could be considered positive if lowering the cut-off OD. Even so, the duration of IgA response was a lot shorter than what was demonstrated in a previous study (Chapter 2) with VR-2332 in young swine. Those young pigs had detectable IgA specific for PRRSV N protein between 14 and 168 days post inoculation. Although exact attributes to different IgA profile between vaccinated sow and infected piglets remain to be further investigated, one could speculate that differences in age, virus strain, and/or antigen presentation (adjuvanted versus wild type) may be accounted for it.

Age can be a factor influencing the duration of viremia as younger pigs tend to be viremic quickly and have prolonged viremia as compared to sows (Klinge et al., 2009) Yet, adaptive immune response (i.e., antibody level) measured by a commercial ELISA and Nsp-2 specific ELISA appeared to be age-independent (Klinge et al., 2009). When pigs of 3 different age groups (i.e., piglets, finishers and sows) were challenged by two different strains and followed for 63 dpi, kinetics of IgG response measured by a commercial ELISA was the same in all 3 groups with the peak titer at 21 dpi. The only difference was that piglets had a higher average antibody titer throughout the study than the other groups (Klinge et al., 2009). The effect of age on kinetics of IgM and IgA response remains to be further investigated.

The Fostera® PRRS vaccine seemed to be able to induce neutralizing antibody earlier (10 dpv) and quickly to a higher titer (1:128 by 21 dpv) than other MLV vaccines. The highest neutralizing titer induced by VR-2332 strain, the parental strain of the most commonly used commercial MLV vaccine in the US (Inglvac® PRRS MLV) has been reported to be 1:32. In a previous study, a low level (≤1:8) of neutralizing antibody was first detected at 28 dpv (Li et al., 2014). In another study, the Inglvac® PRRS MLV vaccine did not produce detectable neutralizing antibody prior to challenge although vaccinated pigs developed neutralizing antibody (1:4-1:32) after challenge with field strains (Charerntantanakul., 2006).

There are several factors influencing immune response of pigs after vaccination. First, vaccine type has an effect on the response. As studied elsewhere, MLV and KV vaccines generally elicit immune response in a very different manner. Animals immunized with a MLV vaccine tend to rapidly seroconvert starting 14 dpv. Homologous challenge of those animals at 28 dpv does not result in an increase in antibody response. On the other hand, animals vaccinated with a KV product do not seroconvert until after challenge when a sharp increase in antibody response is observed. In one study, neither MLV nor KV vaccine induced neutralizing antibody response until after challenge where only the KV vaccine group developed a sharp neutralizing antibody increase. Interestingly, the MLV vaccinated groups cleared viremia after challenge whereas KV vccinatd group could not (Zuckermann et al., 2007). Second, adjuvant itself can influence immune response. Different adjuvants, including bacterial endotoxin, ORF5 peptides, IL-12, Poly ICLC and IFNα, have been tested with MLV vaccines. The choice of adjuvant did not influence antibody response measured by ELISA as none of these adjuvants were able to elicit neutralizing antibody higher than MLV alone. Yet MLV vaccine adjuvanted with IL-12 and ORF5 peptides induced a higher IFNγ response compared to MLV alone (Charerntantanakul

et al 2006, Foss et al 2002). Third, vaccine delivery platform and route can also influence the immune response in conjunction with adjuvant. For instance, a novel intranasal delivery of a MLV vaccine adjuvanted with *Mycobacterium tuberculosis* whole cell lysate was able to mount high levels of neutralizing antibody and IFNγ. These effects were attributable to the specific adjuvant used since the results were significantly different when MLV vaccine was used alone (Dwivedi et al., 2011).

Neutralizing antibody titer has been reported to be a strong correlate of protective immunity and vaccine effectiveness (Lopez et al., 2007). In a series of experiments where piglets were intraperitoneally injected with immunoglobulins with various neutralizing titers and subsequently challenged with PRRSV, pigs receiving a 1:8 neutralizing antibody titer did not develop viremia while the virus still replicated in the bronchial lymph nodes. In contrast, pigs received neutralizing antibody titers lower than 1:8 became viremic. It was also shown that a neutralizing antibody of 1:32 would be needed in order to obtain full protection defined by block of viremia, no proliferation in peripheral organs and no animal to animal transmission (Lopez et al., 2007). It was previously shown that passive transfer of anti-PRRSV hyperimmune serum to pregnant gilts at day 90 of gestation provides sterilizing immunity against reproductive loss b PRRSV (Osorio et al., 2002).

Our study showed that antibody produced by Fostera® PRRS vaccine cross-neutralized a heterologous strain (VR-2332) although cross neutralizing antibody titer was low (≥ 1:16) and was only detected at 21, 28 and 35 dpv. It appears that antibodies induced by VR-2332 and Fostera® PRRS vaccine strain are able to cross-neutralize each other to some extent. Interestingly, cross neutralizing antibody titers of anti VR-2332 sera against Forstera® PRRS vaccine virus were not statistically different from homologous FFN titers, whereas anti-VR2332

sera were able to cross-neutralize Fostera® PRRS vaccine virus; yet those titers were not statistically different from homologous FFN titers.

Vaccination elicited isotype kinetics of antibody response similar to that by a wild type strain except for the IgA response which did not align with neutralizing antibody response. IgM response might be able to detect first exposure to vaccine strain but IgG response was the only isotype-specific response that appeared at the earliest dpv (3 dpv) and followed the same pattern as neutralizing antibody response; therefore in the current study IgM and IgA-specific antibody response might not be a useful tool to assess humoral response to this particular vaccine. However, high levels of neutralizing antibody response produced by the novel experimental vaccine compared to data available from other MLV vaccines currently in use offers a promising area for future vaccine research. Other areas in which additional research is needed are effect of antigen basis on isotype profiling (i.e., nucleocapsid protein vs other proteins) and field application of isotype-specific serology.

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Figures and Tables

Figure 1. Phylogenetic relationship between Fostera® PRRS vaccine strain and other reference field isolates of PRRSV based on ORF5 sequence.

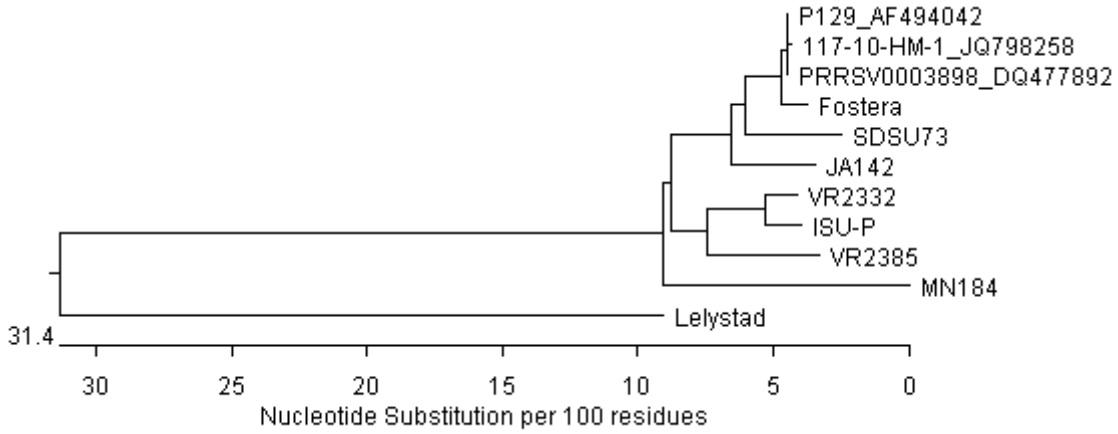

Figure 2. PRRSV N protein specific IgG response to Fostera® PRRS in vaccinated sows as measured using a commercial PRRS ELISA kit (IDEXX). Values at each time point are expressed as mean ± S.E.

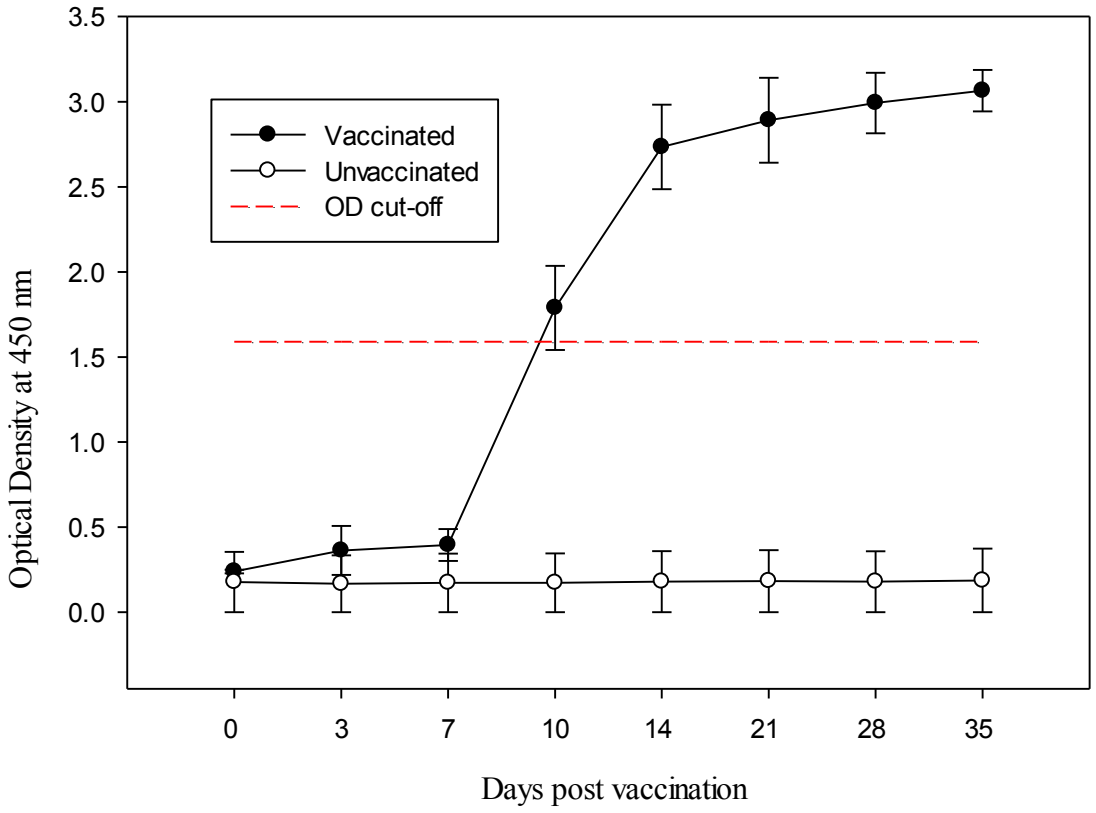

Figure 3. PRRSV N protein specific IgM response to Fostera® PRRS vaccine in vaccinated sows as measured using a commercial PRRS ELISA kit (IDEXX). Values at each time point are expressed as mean ± S.E.

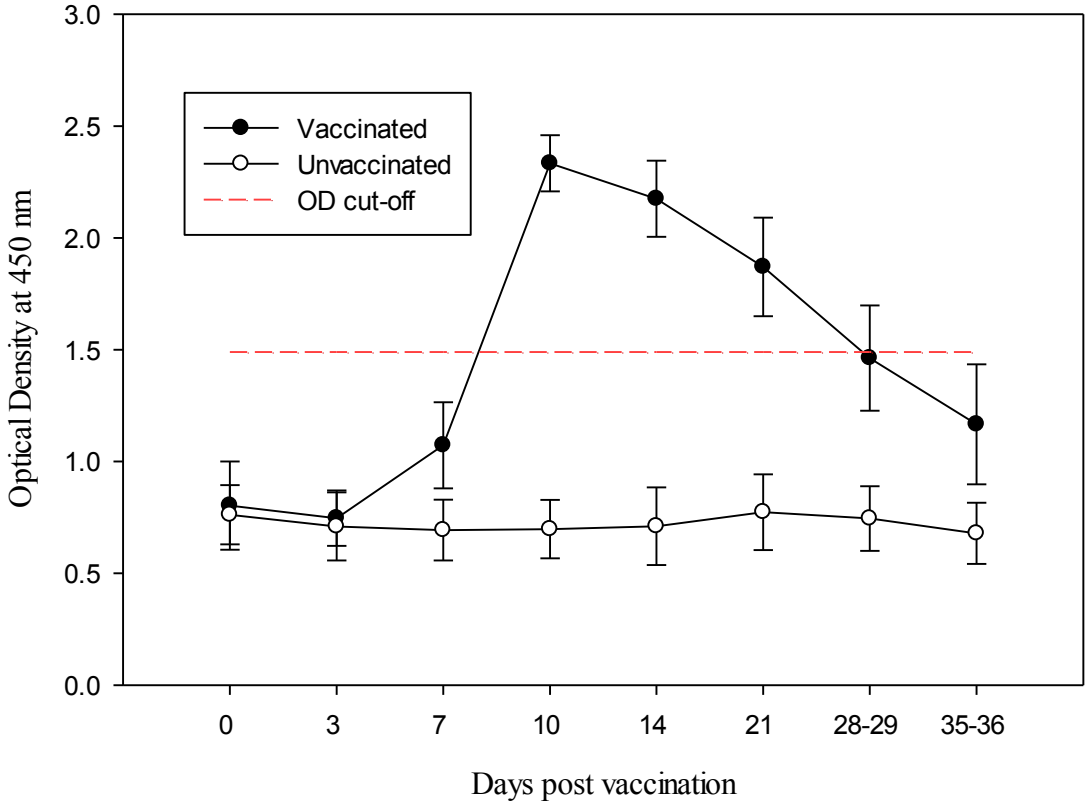

Figure 4. PRRSV N protein specific IgA response to Fostera® PRRS vaccine in vaccinated sows as measured using a commercial PRRS ELISA kit (IDEXX). Values at each time point are expressed as mean ± S.E.

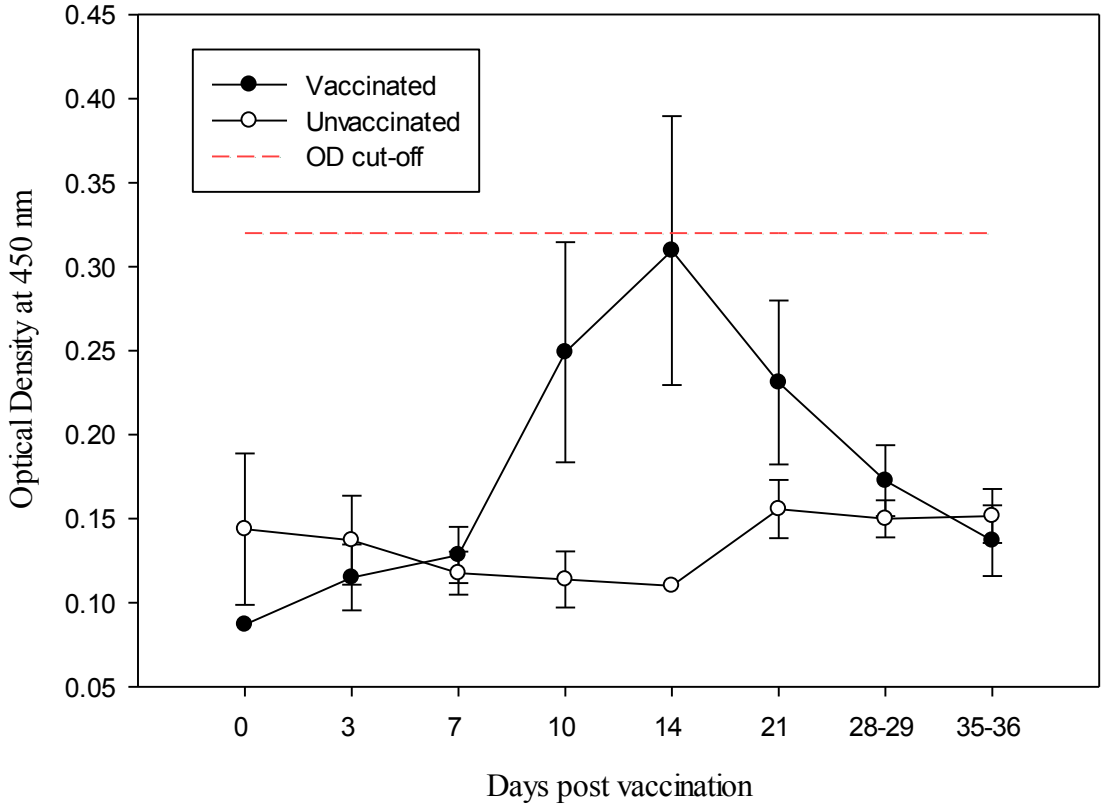

Figure 5. Homologous serum-virus neutralizing activity of sows vaccinated with Forstera® PRRS vaccine as measured by fluorescent focus neutralization (FFN) assay. FFN titers are expressed as geometric mean ± standard error in log_2 basis.

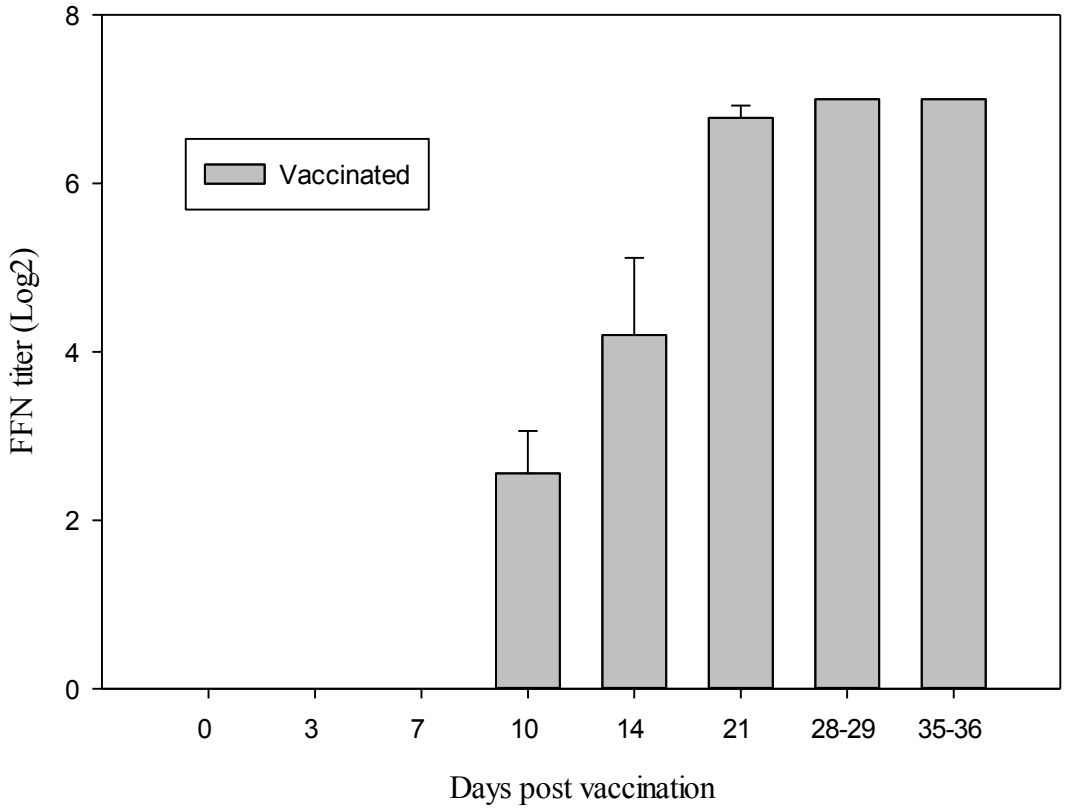

Figure 6. Comparative kinetics of IgG antibody and neutralizing antibody response to PRRSV in sows vaccinated with Fostera® PRRS vaccine at 90 days of gestation. Pigs were inoculated with one dose of the vaccine as directed by the manufacturer. PRRSV-specific IgG antibody was measured on a commercial PRRS ELISA kit (IDEXX) while neutralizing antibody was measured by fluorescent focus neutralization (FFN) assay. Values denote mean ± S.E.

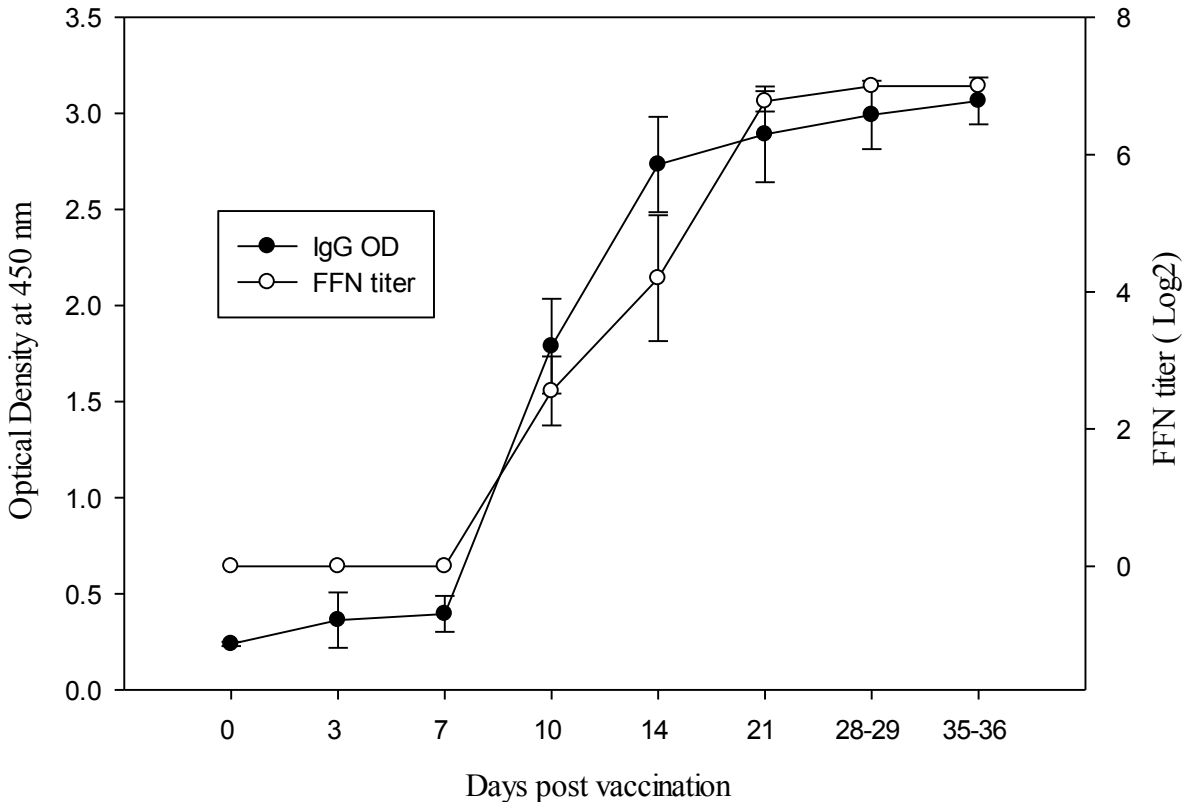

Figure 7. Comparative kinetics of IgM antibody and neutralizing antibody response to PRRSV in sows vaccinated with Fostera® PRRS vaccine at 90 days of gestation. Pigs were inoculated with one dose of the vaccine as directed by the manufacturer. PRRSV-specific IgM antibody was measured on a commercial PRRS ELISA kit (IDEXX) while neutralizing antibody was measured by fluorescent focus neutralization (FFN) assay. Values denote mean ± S.E.

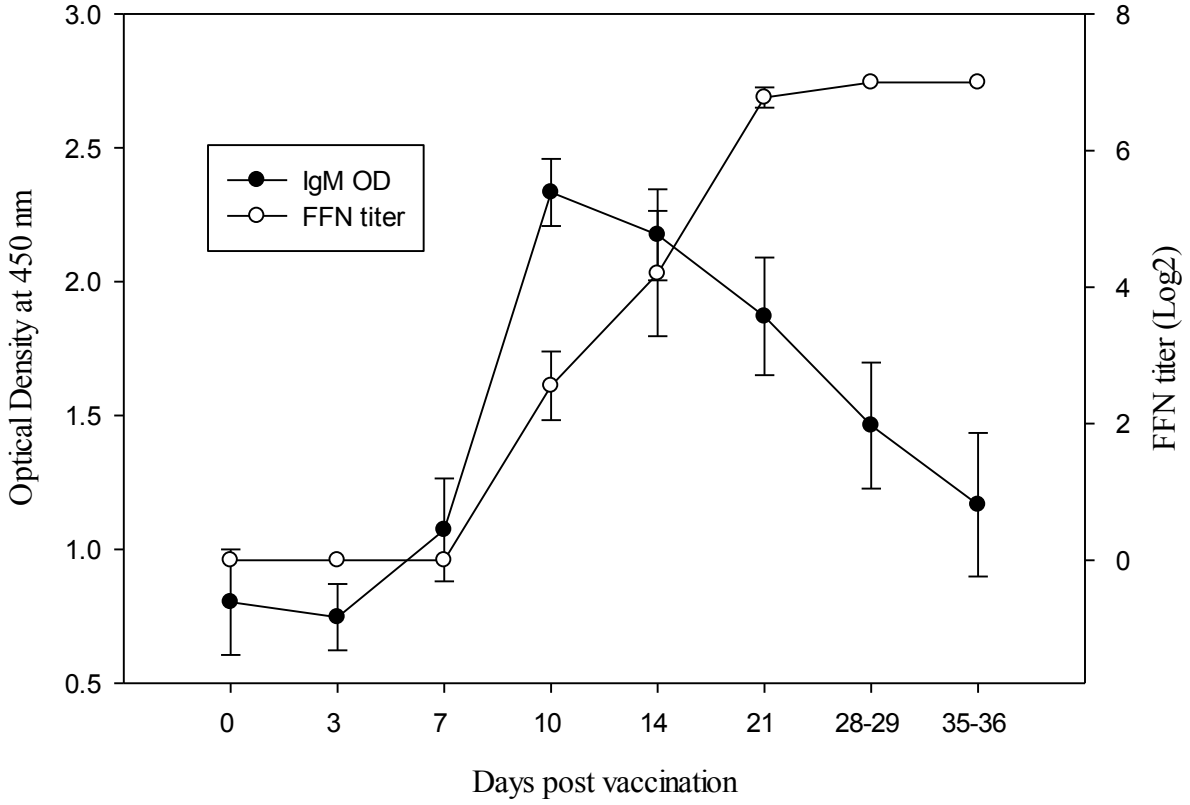

Figure 8. Comparative kinetics of IgA antibody and neutralizing antibody response to PRRSV in sows vaccinated with Fostera® PRRS vaccine at 90 days of gestation. Pigs were inoculated with one dose of the vaccine as directed by the manufacturer. PRRSV-specific IgA antibody was measured on a commercial PRRS ELISA kit (IDEXX) while neutralizing antibody was measured by fluorescent focus neutralization (FFN) assay. Values denote mean ± S.E.

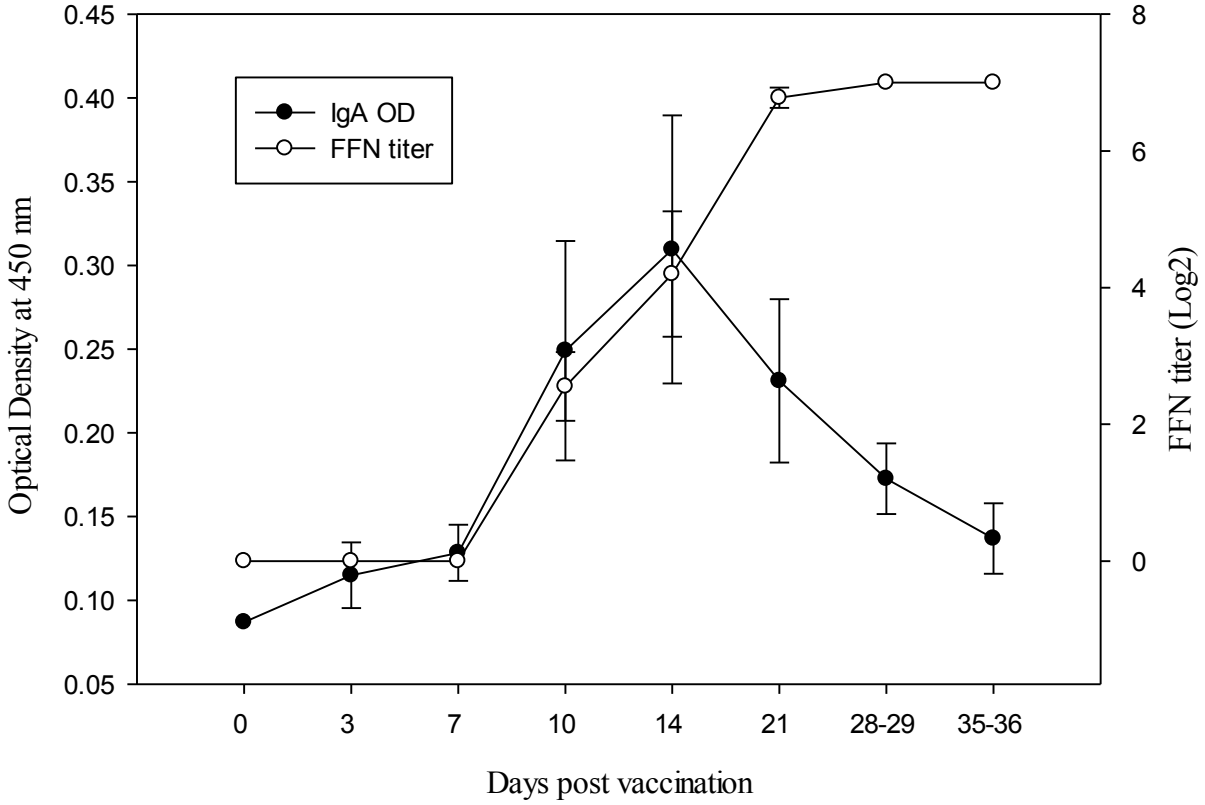

Figure 9. Comparison of anti-PRRSV neutralizing activity against homologous (black bars) and heterologous (grey bars) strains in sera from sows vaccinated with Fostera® PRRS vaccine

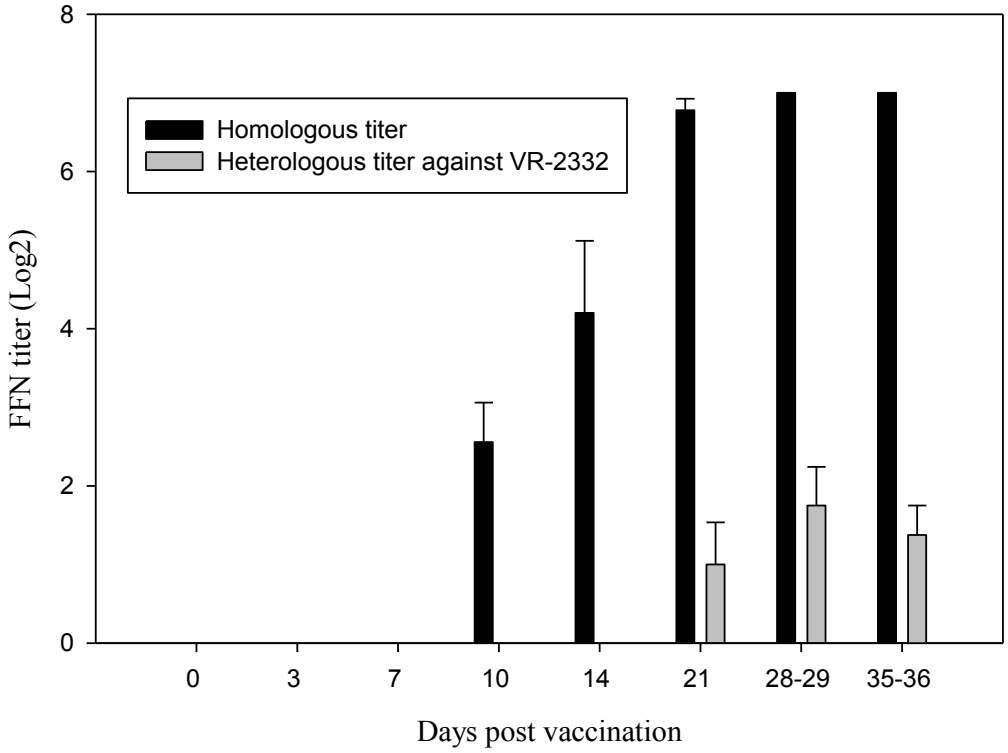

Figure 10. Comparison of anti-PRRSV neutralizing activity against homologous (black bars) and heterologous (grey bars) strains in sera from pigs inoculated intramuscularly with VR-2332 at the rate of 10^4 $TCID_{50}$/ml

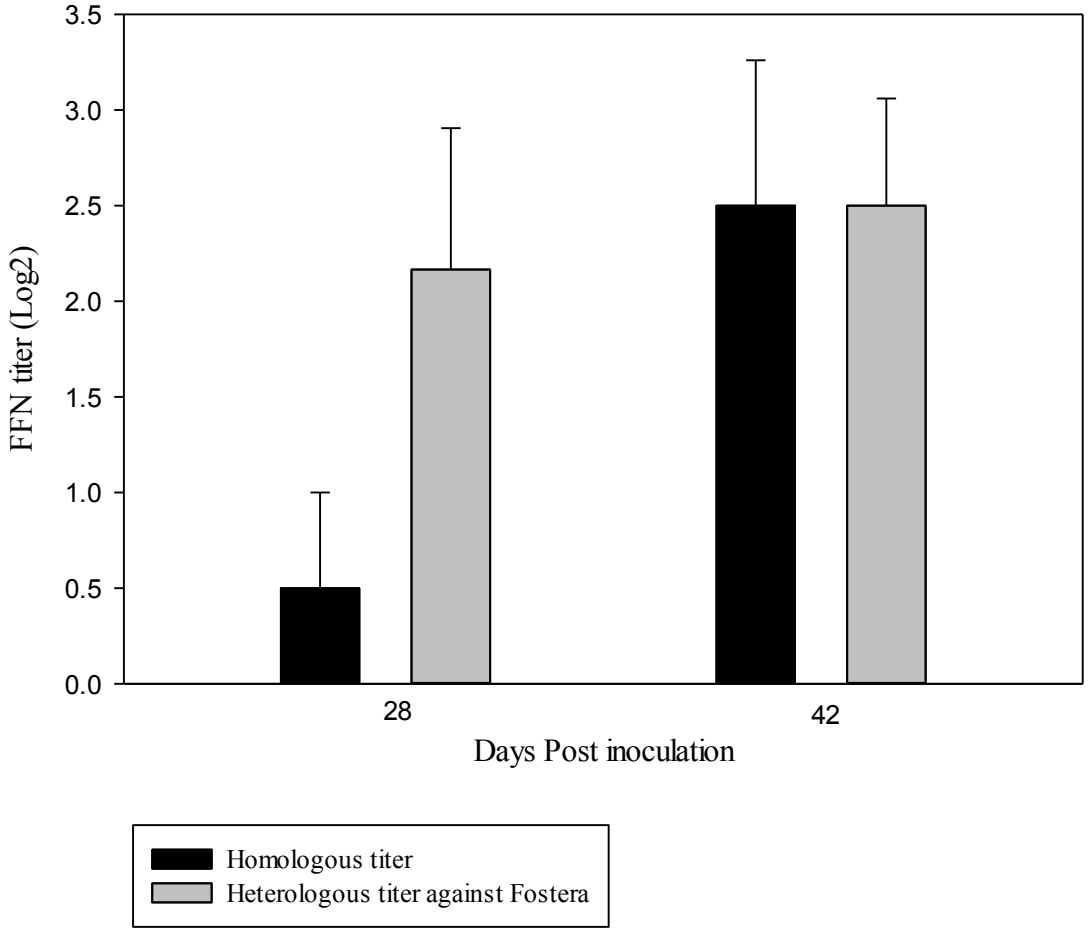

Table 1: Comparisons of PRRS vaccinated and unvaccinated groups at each time point. Adjustment for Multiple Comparisons was done by Holm Tukey Method. P value < 0.05 was considered significant.

dpv	Adjusted P-value for difference between vaccinated and unvaccinated groups		
	IgG	IgM	IgA
0	0.2324	0.9631	0.1360
3	0.0273	0.8218	0.3697
7	0.0020	0.1143	0.8108
10	<.0001	<.0001	0.0028
14	<.0001	<.0001	0.0002
21	<.0001	0.0006	0.1606
28	<.0001	0.0133	0.5359
35	<.0001	0.0686	0.5055

Table 2. Pair-wise comparison of median neutralizing antibody titers (log_2) against homologous Fostera® PRRS vaccine virus in vaccinated pigs at different days post vaccination.

Comparison	Difference of Ranks	P<0.05
dpv 28 vs dpv 10	25.833	Yes
dpv 28 vs dpv 14	20.700	Yes
dpv 28 vs dpv 21	3.667	No
dpv 28 vs dpv 35	0.000	-
dpv 35 vs dpv 10	25.833	Yes
dpv 35 vs dpv 14	20.700	Yes
dpv 35 vs dpv 21	3.667	-
dpv 21 vs dpv 10	22.167	Yes
dpv 21 vs dpv 14	17.033	Yes
dpv 14 vs dpv 10	5.133	No

CHAPTER 4. GENERAL CONCLUSIONS

Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) continues to be one of the most challenging diseases in the swine industry. Given the limitations associated with PRRSV diagnostic assays including serological assays, insufficient data available on isotype-specific antibody response, and the short duration of most challenge/vaccine studies. It was recognized the need to fully elucidate the ontogeny and isotype kinetics of antibody response to PRRSV infection for a long period of time after experimental infection. The other objective of the study was to evaluate whether or not the virus type i.e., attenuated vs. wild type can influence isotype profile of antibody response.

In two research papers presented in this thesis, isotype-specific response to VR-2332 strain (wild type) and a novel modified live virus vaccine (Fostera® PRRS) strain was measured and compared with neutralizing antibody response. Interestingly, in case of the wild-type inoculated pigs, all isotype-specific assays (IgG, IgM and IgA) provided valuable information: a) IgG response is a reliable indicator of exposure; b) IgM response can be used to detect first exposure to PRRSV; and c) IgA response pattern is very similar to neutralizing antibody response. However, diagnostic performance of these assays as measured by sensitivity and specificity need to be yet improved to be able to compete with commercial assays. Further optimization of these isotype-specific assays might be needed to increase diagnostic performance.

In case of vaccinated animals the isotype-specific response pattern was different from that of wild-type inoculated animal, specifically for IgA antibody response. Therefore future studies are needed to determine which factors affect IgA response. Other areas of future research could focus on the effect of antigen basis on isotype profile since the assays we used were nucleocapsid

protein-based, even though N protein based ELISA has been reported to have the best diagnostic performance as compared to other viral protein or peptide based ELISAs. Field application of isotype-specific humoral immune-response also needs to be addressed.

In conclusion, our study was able to broaden the knowledge on PRRSV humoral immune response and demonstrated that isotype-specific serology can be utilized as a useful diagnostic tool to determine infection/immune status of pigs for PRRSV.

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