Mechanisms of immune protection against porcine reproductive and respiratory syndrome virus (PRRSV)

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Dedication

This dissertation is dedicated to

my incredible daughters

Sophie Grace Robinson and Catherine Maisy Robinson,

whose boundless energy, enthusiasm and determination

inspire me each and every day.

Abstract

Porcine reproductive and respiratory virus (PRRSV) is a rapidly evolving and diverse RNA virus that causes significant disease to swine populations globally. There are no specific treatments for PRRSV; biosecurity measures and immunization are the mainstays of PRRSV prevention and control. Although immunization plays a critical role in reducing disease and stabilizing virus-endemic herds, vaccines provide incomplete and unpredictable cross-protection against the diversity of PRRSV strains. A limitation for improving immunization strategies is that mechanisms responsible for immunity against PRRSV and correlates of cross-protection are poorly understood. The objective of this dissertation was to re-evaluate aspects of PRRSV immunity in light of contemporary information for novel clues to identify mechanisms of protection. The approach was to investigate the role of recently discovered PRRSV protein ORF5a in immunity, and to re-evaluate the role of neutralizing antibodies in PRRSV cross-protection. The findings herein demonstrate that ORF5a protein is not important for immune protection against PRRSV but the maintenance of ORF5a in fine evolutionary balance with the GP5 variable glycosylation region suggests it plays a critical role in the virus life cycle. High levels of broadly-neutralizing antibodies to PRRSV were identified from sows in typical commercial settings. Neutralizing antibodies isolated from sows provided cross-protection to naïve animals against diverse strains of PRRSV. This information provides a basis for renewed investigation into the mechanisms of neutralizing antibody-mediated protection against PRRSV and strategies for induction of broadly-neutralizing antibodies. Improved understanding of immune mechanisms to PRRSV will enable more effective control, prevention and ultimately, elimination of the virus.

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List of Abbreviations

Ab	antibody
ADE	antibody dependent enhancement
CAP	caprylic acid
cDNA	complementary deoxyribonucleic acid
dN	rate of nonsynonomous substitution
dS	rate of synonomous substitution
EAV	equine arteritis virus
ELISA	enzyme-linked immunosorbant assay
FACS	fluorescent assisted cell sorting / flow cytometry
FBS	fetal bovine serum
FFN	fluorescent focus neutralization
GP	glycoprotein
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
Ig	immunoglobulin
IM	intramuscular
IP	intraperitoneal
MLV	modified live vaccine
MOI	multiplicity of infection
OD	optical density
ORF	open reading frame
PAM	porcine alveolar macrophage
PBS	phosphate buffered saline

PCR	polymerase chain reaction
PRRSV	porcine reproductive and respiratory virus
RNA	ribonucleic acid
RT-qPCR	reverse transcriptase quantitative polymerase chain reaction
SAS	saturated ammonium sulfate
SDS_PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SN assay	serum neutralizing assay
S/P	signal to positive ratio
$TCID_{50}$	tissue culture infectious dose 50

Chapter 1 Immunity to PRRSV

1.1 Introduction

Since the emergence and proliferation of *porcine reproductive and respiratory syndrome virus* (PRRSV) to become endemic in swine producing regions across the world, there has been intense investigation of the biology, evolution, epidemiology and immune response to the virus due to the immense impact it has had on animal health and economics of swine production. PRRSV is a highly successful pathogen; it has continued to expand and circulate, evolve and diversify, despite immense efforts to reduce its impact through prevention, control, and elimination of the virus by herd management strategies, improved biosecurity and immunologic approaches. The capacity of PRRSV to spread in the air and with movement of animals and people facilitates its persistence, as do other features, such as the propensity of PRRSV to have a prolonged course of infection and therefore transmission, and to re-infect populations owing to the rapid evolution and diversity of the virus. Furthermore, characteristics of swine production, such as high population turnover with constant influx of animals via births and replacement breeding animals, and incomplete immunity to PRRSV following recovery from infection provides a continued supply of susceptible hosts. Whilst immunity tends to be robust to homologous strains, rapid viral evolution leads to sufficient antigenic diversity between strains such that individuals may have limited or incomplete cross-protection. PRRSV is enigmatic; despite all that is known, understanding of immunity to PRRSV is superficial in terms of mechanisms that underlie protective immunity, prolonged infection dynamics and eventual clearance from an animal. The *objective of this dissertation* was to re-evaluate aspects of PRRSV immunity in light of contemporary information to identify novel clues that might inform mechanisms of protection. The *approach* was to investigate the role of the recently discovered PRRSV protein ORF5a in immunity, and to re-evaluate the role of

neutralizing antibodies in PRRSV cross-protection in light of new information emerging from other rapidly evolving RNA viruses.

1.2 Literature Review

Challenges associated with rapidly evolving RNA viruses

For any viral disease, it is desirable to have a protective vaccine, to prevent transmission and spread, achieve herd or population level immunity, eliminate, and ultimately, eradicate the virus. However, achieving these steps is one of the biggest challenges facing infectious disease researchers. Viruses have, and continue to evolve a seemingly infinite array of strategies to evade or subvert their hosts' defenses. Furthermore, other factors such as social, political, economic, and management factors can significantly influence outcomes or dynamics of infectious disease. Despite success stories such as eradication of smallpox and rinderpest, some current examples highlight the role of non-biologic factors hindering such efforts; poliovirus eradication efforts in unstable regions of the world, and the discrepancy between developed and developing world with respect to prevention and control of rabies in domestic animal reservoirs for human zoonotic transmission (Khan and Qazi, 2013; Vallat, 2011). Effective vaccines exist in the case of poliovirus and rabies, but remain elusive for many viral diseases. Vaccine development has proven exceptionally challenging for a number of RNA viral diseases such as human immunodeficiency virus/acquired immune deficiency syndrome, influenza, dengue and other viral hemorrhagic fevers, foot and mouth disease, and bovine viral diarrhea. Common features of these problematic and difficult to control viruses are their rapid evolution, antigenic diversity, immune evasion, and poor immunologic control (Burton et al., 2012; Steinhauer and Holland, 1987).

Despite 25 years of investigation since PRRSV emerged as a swine pathogen, the rapidly evolving RNA virus has proven challenging to control. It belongs to the order *Nidovirales*, family *Arteriviridae*, and genus *Arterivirus*, along with other similarly host-restricted family members; equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV) of mice, and simian hemorrhagic fever virus (SHFV) (Faaberg et al., 2011). Porcine reproductive and respiratory syndrome appeared around the same time in commercial swine herds in Europe and North America, the respective viral isolates having >60% difference at the genomic level, and restricted serologic cross-reactivity, establishing two distinct genotypes (Allende et al., 1999; Collins et al., 1992; Magar et al., 1995; Murtaugh et al., 1995; Nelsen et al., 1999; Nelson et al., 1993; Wensvoort et al., 1991). The two genotypes have remained genotypically distinct but have spread to co-circulate in swine producing regions around the world (Murtaugh et al., 2010; Nelsen et al., 1999). Irrespective of genotypic differences, the impact of PRRSV infection on swine herds results from morbidity and mortality due to reproductive failure in sows (including abortions, stillbirths and weak born piglets), and respiratory disease in growing pigs, as detailed in (Zimmerman et al., 2012). The estimated economic impact on swine producers in 2012 was $664 million annually for the United States (Holtkamp et al., 2012). Hence, from an economic and animal welfare standpoint, there is a substantial need to find improved solutions for PRRSV prevention and control. Characteristics of the virus and its interaction with the porcine immune system are discussed in the context of challenges to achieving protective immunity.

Characteristics of PRRSV relevant for host cell infection and immunity

PRRSV has a compact linear, capped and polyadenylated RNA genome of about 15 kb in size, and uses mechanisms such as ribosomal frameshifting and overlapping open reading frames (ORF) to maximize coding capacity (Fang et al., 2012; Maclachlan and Edward, 2011). The

genome contains 10 ORFs, with the 5' region being made up of ORF1a/b that is translated into two polyproteins in the cytoplasm by the cellular machinery upon uncoating. These polyproteins are then processed by virally encoded proteases into 14 nonstructural proteins comprising the PRRSV replicase, including an RNA-dependent RNA polymerase for viral genomic replication (Fang and Snijder, 2010). In addition to viral replication, PRRSV nonstructural proteins modulate host innate immune defenses in a variety of ways, which have been reviewed elsewhere (Fang and Snijder, 2010; Sun et al., 2012; Yoo et al., 2010).

PRRSV structural proteins glycoprotein (GP)2, GP3, GP4, GP5, M (membrane) and N (nucleocapsid) arise from ORFs 2-7 respectively, following discontinuous transcription to form a 3' co-terminal nested set of subgenomic mRNAs in which the 5' proximal ORF is translated (detailed in Dokland, 2010; Veit et al., 2014). Subgenomic mRNA 2 and 5 are bicistronic, whereby along with ORF2a and ORF5 coding for GP2 and GP5 respectively, contain small overlapping ORFs, leading to production of an additional protein product; E (from downstream ORF2b) and recently discovered ORF5a protein (from upstream ORF5a) (Firth et al., 2011; Johnson et al., 2011; Snijder et al., 1999; Wu et al., 2001). The viral nucleocapsid protein encases the viral genome and the remaining structural proteins are incorporated in the viral envelope derived from internal cell membranes (Figure 1.1). PRRSV virions have a relatively smooth surface profile, with few protruding features, consistent with predicted small ectodomains of GP5 and M (30 residues for GP5 and 16 for M) (Dokland, 2010; Spilman et al., 2009).

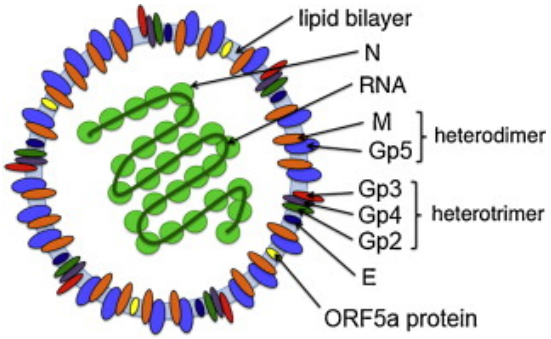

Figure 1.1 PRRSV virion structure. From (Veit et al., 2014).

PRRSV has a limited cell tropism. In the host, PRRSV primarily infects macrophages, but infection of dendritic cells has also been demonstrated (Baumann et al., 2013; Silva-Campa et al., 2010; Wang et al., 2007; Wensvoort et al., 1991; Yoon et al., 1992). Growth on continuous cell lines has been limited to simian renal epithelial CL2621/MA104 and the derivative MARC 145 (Collins et al., 1992; Kim et al., 1993). It was previously thought that interaction between major envelope protein dimers comprising GP5 and M with sialoadhesin on the surface of macrophages was responsible for PRRSV infectivity (Delputte and Nauwynck, 2004; Van Breedam et al., 2010; Van Gorp et al., 2008). Subsequently, it was demonstrated that the CD163 receptor (scavenger receptor cysteine rich family) was sufficient for PRRSV infection, and explained permissivity of simian MARC 145 cells which express CD163, but not sialoadhesin (Calvert et al., 2007; Duan et al., 1998; Van Gorp et al., 2008; Wissink et al., 2003b). The role of GP5 sialic acids binding sialoadhesin (CD169) on macrophages remained controversial, with conflicting evidence regarding its role in infection, with some studies showing higher titer of infection in the presence of CD169 and CD163 compared to CD163 alone, with others showing no difference (Van Gorp et al., 2008; Welch and Calvert, 2010). Recent studies have demonstrated that sialoadhesin is not necessary for infection in CD169 knockout pigs, and removal of viral N-

glycans does not reduce infectivity (Li and Murtaugh, 2015; Prather et al., 2013). Furthermore, minor envelope proteins GP2, GP3, GP4 and E associate to form a multimeric complex in the viral envelope, and are the major determinant of viral tropism in cell culture (Das et al., 2010; Tian et al., 2012; Wissink et al., 2005). GP2 and GP4 directly interact with host cell CD163 receptor (Das et al., 2010). The minor envelope proteins are required for infectivity, whereas GP5-M and N are necessary for virus particle formation (Wissink et al., 2005). However, the precise structure, specific interactions, and functions of each of these proteins are not known at this time.

PRRSV virions are coated with an array of N-linked glycosylation sites on envelope glycoproteins 2 through 5 (reviewed in Dokland, 2010; Veit et al., 2014). There is variability in the number and position of glycosylation sites between and within genotypes, with a few highly conserved sites. In addition to interacting with host cell sialoadhesin, glycans function in protein maturation and transport during virion formation (Wei et al., 2012a; Wissink et al., 2005). Briefly, GP2 has two conserved glycosylation sites that are not required for infectivity (Wissink et al., 2004). GP3 protein is the most heavily glycosylated envelope protein of PRRSV, with six sites adding as much as 16 kDa to its mass (Gonin et al., 1998). The glycan close to the GP3 signal peptide has been proposed to mask the cleavage site, allowing it to act as a signal anchor (Matczuk et al., 2013). Studies on the role of individual GP3 sites in replication and antigenicity are conflicting (reviewed in Veit et al., 2014). Gp4 proteins of PRRSV have four conserved glycosylation sites, and mutation of individual sites does not affect virus replication, but double, triple or quadruple mutants of the glycosylation sites are lethal for virus production, suggesting a functional requirement for glycans during viral assembly (Das et al., 2011).

GP5 contains multiple N-glycosylation sites, with conserved positons at N44 and N51 in type 2 viruses and N46 and N53 in type 1. Lack of the oligosaccharides linked to N44/N46 in GP5 impairs the production of infectious progeny virus and significantly reduces viral infectivity (Ansari et al., 2006; Wissink et al., 2004). In a study that evaluated 1301 genotype 2 GP5 sequences, 1 to 6 N-linked glycosylation sites were observed at 8 different positions (Delisle et al., 2012). Glycosylation at positions 44 and 51 were essentially fixed, whereas other positions were variable and located in or near previously described hypervariable regions that flank a proposed neutralizing epitope (Ostrowski et al., 2002). Thirty-eight unique glycotype patterns were observed, with no evidence of phylogenetic clustering indicative of selection (Delisle et al., 2012). In contrast, another study found the extent of GP5 glycosylation was correlated with the degree of neutralization, and minimally glycosylated GP5-M dimers that induce cross-neutralizing antibodies have been patented for vaccine development (Harris and Erdman, 2014; Vander Veen et al., 2009). Still, bacterially expressed GP5-M ectodomain fragments lacking glycosylation did not induce neutralizing antibodies *in vivo* (Li and Murtaugh, 2012).

GP2 or GP4 glycosylation mutants were not more sensitive to antibody neutralization, nor elicited higher titers of neutralizing antibodies after infection of piglets, whereas a glycan shielding effect of antibody epitopes has been proposed for GP3 and GP5 (Das et al., 2011; Faaberg et al., 2006; Vu et al., 2011; Wei et al., 2012b; Wissink et al., 2004). An immunological benefit of glycans has been demonstrated in other viruses such as HIV by shielding critical epitopes, making them inaccessible to the host (Wei et al., 2003). On the flip side, glycans themselves are a target of broadly neutralizing anti-HIV antibodies (Moore et al., 2012; Mouquet et al., 2012). However, reports for PRRSV are conflicting in this regard and there is no consensus regarding the specific role of glycosylation in immunological protection.

It is perhaps not surprising that GP3 and GP5 glycosylation site number and position is most variable, given they are the most variable PRRSV proteins by amino acid sequence (Meng et al., 1995; Murtaugh et al., 1995). Whether variability in glycosylation is a mechanism for immune evasion as has been proposed, or simply variation in the face of a rapidly mutating virus or other selective forces to maintain glycan residues that are needed for other functions is unknown (Murtaugh et al., 2010; Vu et al., 2011).

Genetic diversity of PRRSV continues to expand, giving rise to a heterogeneous viral population with concomitant antigenic diversity (Brar et al., 2015; Meng, 2000; Murtaugh et al., 2010). Diversity is generated via mutations arising from polymerase infidelity during genome replication, recombination between strains or variants within a cell, and potentially, modulation by host antiviral enzymes (Murtaugh et al., 2010). High mutation rates lead to rapid evolution as variants undergo selection in response to the environment, whether more fit due to replication or transmission efficiency or evasion of the host immune response. Traditionally, the ORF5 coding sequence for GP5 has been used for phylogenetic comparison of PRRSV isolates due to its variability and perceived immunologic importance. ORF5 sequence analysis has been valuable for determining relatedness of strains globally, change over time in a particular population, whether an outbreak is due to persistence of an endemic strain versus reintroduction of a similar strain or an unrelated isolate, and evaluating effectiveness of control measures (Brar et al., 2015; Murtaugh et al., 2010). Next generation sequencing of PRRSV genomes is rapidly becoming more accessible and cost effective, and the database of complete genomes is quickly expanding. Deep sequencing analysis will illuminate minor sequence variants and polymorphisms, providing opportunities to discover novel viral features and mechanisms of host-pathogen interactions (Lu

et al., 2014). Comparative whole genome analyses may facilitate understanding of viral heterogeneity, antigenic diversity and their implications for cross-protective immunity.

Features of the porcine immune system and response to PRRSV

Early observations of PRRSV infection suggested that PRRSV was immunosuppressive, leading to persistent or prolonged infection of an animal in the face of humoral and cell-mediated responses (Benfield et al., 1998; Drew, 2000; Horter et al., 2001; Molitor et al., 1997). Other members of the *Arteriviridae* are also characterized by prolonged or persistent infections (Maclachlan and Edward, 2011). It was subsequently determined that despite prolonged viremia and presence in lymphoid tissues leading to an extended duration of transmission in absence of clinical disease, individuals eventually clear the virus to achieve sterilizing immunity (Benfield et al., 1998; Torremorell et al., 2002; Wills et al., 2003). Whilst this information is useful for strategies to eliminate PRRSV from a herd, the immunologic mechanisms for eventual clearance of PRRSV are still unknown. PRRSV modulates the immune system in a number of ways, which can vary substantially depending on the virus strain. Furthermore, other factors likely influence the response, such as age, genetics, stress, and co-infections with other pathogens. Some relevant features of the immune response to PRRSV are discussed.

Innate immune cells of the myeloid lineage are the primary target cell of PRRSV. Macrophages typically serve to protect the host against pathogens and other insults as part of the innate immune system by surveillance, scavenging, phagocytosis, and chemotaxis. Macrophages also have a significant role in priming the adaptive immune system via antigen presentation. PRRSV infects and replicates in macrophages, co-opting the host cell machinery and modulating defense mechanisms. PRRSV may also infect dendritic cells, modulating release of IFNα to influence

antigen presentation (Baumann et al., 2013; Wang et al., 2007). Studies investigating the effect of PRRSV on innate immunity have demonstrated alterations in interferon responses, immunomodulatory cytokine profiles, natural killer cell cytotoxicity, induction of autophagy and apoptosis, and antigen presenting capacity (Amadori and Razzuoli, 2014; Darwich et al., 2010; Kimman et al., 2009; Murtaugh and Genzow, 2011; Sang et al., 2011; Sun et al., 2012; Yoo et al., 2010). Detailed examination of innate immunity to PRRSV is outside the scope of this review; suffice to say many complexities remain to be unraveled.

The role of cell-mediated immunity in PRRSV infection is largely unknown, owing mostly from lack of tools to study antigen specific T cells. The cell-mediated response to PRRSV has primarily been evaluated by IFNγ ELISPOT, cytokine assays and phenotyping of cells by flow cytometry. T cell responses to PRRSV have been characterized as weak, delayed, variable, and there is no convincing evidence that they are correlated with level of viral loads or protection. In-depth evaluation of cell-mediated immunity to PRRSV is outside the scope of this review, and has been discussed in detail elsewhere (Amadori and Razzuoli, 2014; Batista et al., 2004; Bautista and Molitor, 1997; Darwich et al., 2010; Kimman et al., 2009; Molina et al., 2008; Murtaugh and Genzow, 2011; Xiao et al., 2004).

The main focus for this review is humoral immunity to PRRSV. A rapid and robust humoral immune response is generated to PRRSV, and antibodies are detected as soon as 5 days post infection, with a typical IgM response followed by a class-switched IgG response. Antibodies are generated against PRRSV structural and nonstructural proteins to varying degrees by protein and by individual animal. The antibody response peaks around 4 weeks post infection and gradually declines over time, reaching very low levels by 300 days post infection. The early non-neutralizing serologic response is not related to protection or clearance of viremia. Detailed review of the antibody kinetics from PRRSV infection are provided elsewhere (Amadori and

Razzuoli, 2014; Darwich et al., 2010; Kimman et al., 2009; Lopez and Osorio, 2004; Murtaugh and Genzow, 2011). Seroconversion, as demonstrated by immunofluorescence (IFA), enzyme-linked immunosorbant assay (ELISA), or immunoperoxidase monolayer assay (IPMA) is frequently used diagnostically to demonstrate evidence of infection (Ludemann and Lager, 2010).

PRRSV-specific memory B lymphocytes are present in lymphoid tissues (Mulupuri et al., 2008). However, typical anamnestic response to challenge of immune animals does not occur. One hypothesis proposed that neutralizing antibodies prevent productive infection such that sufficient antigen is not present to stimulate memory B cells (Murtaugh and Genzow, 2011). The role of neutralizing antibodies in protective immunity for PRRSV has long been debated. Following primary infection, neutralizing antibodies are slow to appear, and then only at low levels compared with the non-neutralizing antibody response (Lopez and Osorio, 2004). Neutralizing antibodies arising from primary infection tend to be relatively strain-specific (Martinez-Lobo et al., 2011; Trible et al., 2015; Yoon et al., 1997). The value of neutralizing antibodies has been questioned because in some instances it is not necessary to have a measurable neutralizing antibody response to resolve or prevent infection (Diaz et al., 2006; Karniychuk et al., 2012; Loemba et al., 1996; Vézina et al., 1996). However, this has no bearing on whether they are beneficial for protection against a secondary infection. Neutralizing antibodies have been associated with clearance or clinical protection more frequently than any other type of immunologic response, and are therefore perhaps the best correlate of protection available for PRRSV, even if not currently understood well enough to utilize effectively (Batista et al., 2004; Dwivedi et al., 2011; Geldhof et al., 2013; Labarque et al., 2000; Li et al., 2014; Lopez et al., 2007; Lopez and Osorio, 2004; Molina et al., 2008; Osorio et al., 2002; Thacker, 2004; Vanhee et al., 2009; Yaeger, 2000; Yoon et al., 1996). Based on this alone, there is a critical need for better

mechanistic understanding of neutralizing antibodies to PRRSV to determine their potential value as a correlate of protection or a strategy for PRRSV prevention.

Neutralizing antibodies provide homologous protection when administered to PRRSV-naïve animals (Lopez et al., 2007; Osorio et al., 2002; Yoon et al., 1996). In each of these studies, ammonium sulfate precipitated immunoglobulins from PRRSV hyperimmune sera with high neutralizing activity was administered intraperitoneally to PRRSV-naïve pigs prior to virulent viral challenge. Osorio et al.(2002) demonstrated complete protection of sows and prevention of transplacental infection to their offspring. In a follow-up using the same stock of immunoglobulins, the degree of protection afforded by neutralizing antibodies to weaned pigs was dose-dependent, however a higher titer was required for sterilizing immunity in the young pigs (Lopez et al., 2007). For these two studies, neutralizing antibodies were induced in the donor sows by an intense regimen of immunization with virulent viruses and Freund's complete adjuvant over a 7 to 14 month time-period.

The third passive transfer study also showed dose-dependent prevention of viremia, however, at subneutralizing concentrations, antibody dependent enhancement (ADE) of infection was observed (Yoon et al., 1996). Viremia was of longer duration and greater magnitude where a low dose of neutralizing immunoglobulin was administered compared with control (PRRSV-negative) immunoglobulin. The enhancing effect of low concentrations of neutralizing antibodies on virus yield *in vitro* was reduced with increasing concentration of protein A, suggesting the effect was mediated by the Fc portion of immunoglobulins, as has been observed for Dengue virus where weakly neutralizing antibodies bring infectious virus to macrophages via Fc receptor binding, increasing uptake (Halstead, 2003). ADE to other viruses typically results where an initial immune response sensitizes the host followed by sequential infection by another antigenic type or from rapid evolution of antigenic diversity during a chronic infection (Halstead et al., 2010). In

the instance of PRRSV described by Yoon et al. (1996), enhancement was to the homologous strain that the neutralizing antibodies were raised to. However, the immunoglobulin preparation injected in the animals was pooled from serum of nine donor animals, so it is possible that complex interactions between the virus and neutralizing and non-neutralizing antibodies whose dynamics are altered by dose of immunoglobulins caused the phenomenon (Klasse, 2014; McGuire et al., 2014).

ADE has potential consequences with regard to waning maternally-derived neutralizing antibodies. PRRSV-neutralizing antibodies are passed from sows to piglets in colostrum and milk, with half-life estimates of 8 days and 3 weeks, detectable to 10 weeks (Liu et al., 2008; Senn et al., 1998). This window coincides with the period where most severe respiratory disease is observed in young pigs. In contrast to this, one study demonstrated a nadir of PRRSV serum neutralizing activity between 9 and 11 weeks of age, with a coincident reduction in proportion of viremic animals during this time, however the magnitude of viremia and neutralizing antibodies were not evaluated for correlation from individual animals, so this finding may be misleading (Liu et al., 2008). Whilst important to consider when studying the role of neutralizing antibodies in cross-protection against PRRSV, ADE has not been shown to enhance disease in the field or in numerous vaccine challenge studies (Murtaugh and Genzow, 2011).

Many studies have examined PRRS viral targets of neutralizing antibodies. Initially, GP5 was the focus of such studies, owing to identification of neutralizing antibodies targeting the equivalent protein in other arteriviruses EAV and LDV (Balasuriya et al., 1997; Lopez and Osorio, 2004; Plagemann, 2001). Epitopes of GP2, GP3, GP4, and M protein have also since been described, and findings of the numerous studies evaluating neutralizing epitopes are summarized in Trible et al. (2015). There are inconsistencies and contradictions between studies of neutralizing epitopes and it was recently suggested that “one explanation for the absence of agreement in

characterization of PRRSV neutralizing epitopes is a lack of understanding regarding the homologous versus heterologous nature of the different antibody reagents used in experiments" (Trible et al., 2015). This is likely to be true, as few of those studies evaluated neutralization of heterologous strains.

Thus, the literature regarding PRRSV neutralizing antibodies contains a lot of 'noise', owing to individual study differences, particularly with respect to viral strains and neutralizing assay methodology. Most critical though, is evaluation of homologous versus heterologous neutralizing activity, possibly explaining conflicting results on their role in protection and epitope targets of cross-neutralization. In spite of these differences, the weight of evidence strongly suggests an association of neutralizing antibody titers with better outcome or recovery from infection, clearance of viremia, or protection (Batista et al., 2004; Dwivedi et al., 2011; Geldhof et al., 2013; Labarque et al., 2000; Li et al., 2014; Lopez et al., 2007; Lopez and Osorio, 2004; Molina et al., 2008; Osorio et al., 2002; Thacker, 2004; Vanhee et al., 2009; Yaeger, 2000; Yoon et al., 1996).

Neutralizing antibodies are crucial for vaccine-mediated protection against viral diseases (Burton, 2002). However, infections with rapidly evolving RNA viruses are often characterized by ineffective neutralizing antibody responses, resulting either from antigenic diversity or immune evasion (Burton et al., 2012). A number of breakthroughs in recent years have allowed huge strides forward for understanding mechanisms for production of broad and potent neutralizing antibodies to rapidly evolving viruses such as HIV, influenza, and dengue (Corti and Lanzavecchia, 2013). These findings were facilitated by development of high throughput neutralizing assays, and culture and sorting techniques to identify memory B cell or plasma cells that produce broadly neutralizing antibodies, and next-generation sequencing methods to analyze immunoglobulins (Burton et al., 2012). Such advances have led to improved understanding of

mechanisms involving viral targets and structure of broadly neutralizing antibodies, and co-evolution of viruses and neutralizing antibodies (Corti and Lanzavecchia, 2013). New information elucidated from other RNA viruses has been enlightening in the context of re-evaluation of neutralizing antibodies to PRRSV, with the realization that is takes time (2 to 4 years) for neutralizing breadth to develop, and only occurs in a small proportion of individuals (around 1% of HIV-infected individuals are categorized as elite neutralizers) (Gray et al., 2011; Simek et al., 2009). Given it takes time for breadth to develop owing to affinity maturation and mutation over time in presence of the antigen, that PRRSV can be present in the host for prolonged periods, and that commercial animals are commonly exposed to multiple virulent and vaccine viruses over time, re-evaluation of neutralization to PRRSV is indicated (Liao et al., 2013). In addition, other lines of evidence that support a possibility for high levels of neutralizing antibody production are the ability to induce high levels of neutralizing activity with hyperimmunization regimens, that breadth increased when viremia was prolonged, and titers or breadth are increased when exposed to multiple or different strains (Islam et al., 2014; Li et al., 2014; Osorio et al., 2002; Trible et al., 2015; Yaeger, 2000).

In reference to evidence that neutralizing antibodies are capable of conferring protection against PRRSV, and the comparatively inefficient neutralizing assays in use for PRRSV, (Butler et al., 2014) suggests “it would be a shame if the current belief in poor PRRSV virus neutralizing activity is a consequence of selected and limited sampling”. Recent development of a high-throughput ELISA-based PRRSV neutralizing assay, and improved understanding of porcine immunoglobulin loci and repertoire, provide new possibilities for investigation of PRRSV neutralizing antibodies (Dawson et al., 2013; Li and Murtaugh, 2012; Schwartz et al., 2012a; Schwartz et al., 2012b; Schwartz and Murtaugh, 2014). It is therefore critical to investigate the

role and characteristics of PRRSV-neutralizing antibodies to evaluate the potential for harnessing neutralizing antibody-mediated protection.

Limitations for PRRSV prevention, control and elimination

PRRSV is a successful pathogen, evidenced by its prevalence in, and negative impact on swine herds worldwide. Currently, there are no specific treatments to ameliorate disease resulting from PRRSV infection. Immunity resulting from vaccination with attenuated PRRSV strains reduces severity of disease to varying degrees, but does not reliably prevent infection. Furthermore, attenuated vaccine strains replicate and may cause disease in the host, maintain infection for duration equivalent to natural infection, can be transmitted to contact animals, and have the potential to revert to virulence and cause disease (Murtaugh and Genzow, 2011; Murtaugh et al., 2003). Features that facilitate continued dissemination and persistence of PRRSV in the swine population at large will briefly be described.

PRRSV is transmitted horizontally through direct contact with infected animals, fomites, semen, and by airborne spread, and also vertically by in utero transmission or via colostrum and milk (Zimmerman et al., 2012). Primary means of transmission between herds are introduction of infected animals or semen, inadvertent carriage on personnel or transport vehicles, as well as dissemination through the air (Zimmerman et al., 2012). PRRSV infection in individual animals may be prolonged, with virus detected in lymphoid tissues for as long as 8 months in absence of clinical signs of disease (Wills et al., 2003). Eventually though, the virus is cleared resulting in sterilizing immunity (Corzo et al., 2010; Torremorell et al., 2002). Mechanisms of clearance are unknown, and different theories have been proposed but not tested (e.g. Amadori and Razzuoli, 2014; Murtaugh and Genzow, 2011; Xiao et al., 2004).

PRRSV may persist for prolonged periods or indefinitely in swine herds owing to population turnover providing a continued supply of susceptible individuals due to births, introduction or co-mingling of weaned pigs and replacement breeding animals, as well as re-infection of convalescent animals with incomplete protective immunity (Nodelijk et al., 2000). Nursery pigs may or may not have maternally-derived protection, and have a period of vulnerability as maternal protection wanes during a time when they are exposed to high viral loads from other young pigs. Young pigs are particularly susceptible to PRRSV infection, with longer duration and level of viremia compared with adults despite similar magnitude and timing of antibody response (Klinge et al., 2009). Young pigs are just as efficient, if not more so, in their ability to make neutralizing antibodies upon primary infection (Robinson et al., 2015b). The relative susceptibility of young pigs is more likely a consequence of age-dependent permissivity of porcine alveolar macrophages (PAM) to infection. We and others have observed increased frequency of infected cells and viral titers grown in PAMs from pigs less than 8 weeks of age (Ludemann and Lager, 2010; Mengeling et al., 1995; Robinson et al., 2015b; Thanawongnuwech et al., 1998). We demonstrated the difference is not mediated by differential expression of CD163 or CD169 receptor expression and are currently investigating the possible influence of host restriction factors on age-dependent resistance of PAMs to PRRSV (Robinson et al., 2015b). In any case, nursery and weaned pigs serve as a reservoir for PRRSV in a herd.

PRRSV control strategies aim to limit adverse effects in endemic herds, and are broadly classified as management or immunologic. Management includes measures such as improving biosecurity to prevent new introductions to a herd and separating batches of pigs of similar age and immune status in all-in-all management, rather than continuous flow which maintains pathogens in the population (Zimmerman et al., 2012). The mainstay of immunologic control of PRRSV is vaccination. Detailed review of available vaccines and results of experimental trials with novel

immunization approaches has been provided elsewhere (Johnson, 2009; Murtaugh and Genzow, 2011). Currently, the most effective and therefore widely used are commercial modified live vaccines made from a single attenuated strain, and live virus inoculation with circulating virulent farm isolates. The primary goals of vaccination are to achieve uniform immunity and herd stability to reduce clinical disease. Vaccination strategies are therefore dependent on generating cross-protective immunity if they are to provide benefit against infection with PRRSV strains other than that used in the vaccine.

Modified live vaccines tend to provide effective protection to homologous strains, although immunity may not be complete or life-long (reviewed in Murtaugh and Genzow, 2011). Despite the undoubted clinical benefits of vaccines in swine populations, protection afforded against heterologous strains is variable and frequently incomplete (reviewed in Murtaugh and Genzow, 2011). So although vaccines are crucial for reduction and control of disease, protection is unpredictable and this fact hampers efforts to eliminate PRRSV from herds or regions. Therefore, improved understanding of correlates of cross-protection is a critical need in PRRSV research.

1.3 Specific Aims

Despite substantial knowledge regarding the complex interactions between the rapidly evolving virus and the porcine immune system, PRRSV continues to pose significant problems. Substantial gaps exist in our understanding of PRRSV immune mechanisms, particularly with respect to factors responsible for cross-protective immunity. From an immunological standpoint, the fundamental difficulty in achieving PRRSV prevention is a poor understanding of what constitutes a protective immune response to PRRSV. Correlates of protection that predict immunity or assess response to interventions are lacking, and there are insufficient immune strategies and tools available. These deficiencies limit the ability to reliably induce protective immunity to prevent infection and transmission. To address this need, we propose to investigate two interesting observations that have potential to uncover new information important for understanding PRRSV immunity. The goal of this dissertation was to re-evaluate aspects of PRRSV immunity in light of contemporary information for novel clues to identify mechanisms of protection. The approach was to investigate the role of recently discovered PRRSV protein ORF5a in immunity, and to re-evaluate the role of neutralizing antibodies in PRRSV cross-protection in light of new information emerging from other rapidly evolving RNA viruses.

Specific objectives were to:

- Evaluate the role of novel PRRSV ORF5a protein in immune protection against PRRSV
- Examine influence of overlapping ORF5a on major envelope glycoprotein GP5
- Characterize PRRSV neutralizing characteristics of serum from commercial sows
- Determine breadth and protective role of anti-PRRSV cross-neutralizing antibodies
- Investigate PRRS viral targets of cross-neutralizing antibodies

Chapter 2 Role of novel ORF5a protein in immune protection against PRRSV

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Immune response to ORF5a protein immunization is not protective against porcine reproductive and respiratory syndrome virus

Sally R Robinson, Marina C Figueiredo, Juan E Abrahante, Michael P Murtaugh

2.1 Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is an RNA virus responsible for PRRS in swine; a disease with globally significant animal welfare and economic concerns. There is no specific curative treatment, and variably effective immune protection from previous exposure or vaccination (Benson et al., 2000; Cano et al., 2007a; Cano et al., 2007b; Opriessnig et al., 2007). Despite widespread use of vaccines and live virus inoculation with circulating field strains as a means to promote immune protection against PRRSV, outbreaks of PRRS continue to occur in naïve and PRRSV-exposed populations, resulting in significant losses to producers and the swine industry (Holtkamp et al., 2012). While viral genetic and antigenic diversity likely contribute to variable immune protection, precise molecular mechanisms responsible for protective immunity also are poorly understood.

Recently, a novel ORF5a protein, encoded in an alternative open reading frame (ORF) of subgenomic mRNA 5, was discovered in all Arterivirus family members (Firth et al., 2011; Johnson et al., 2011). ORF5a protein is a structural protein in PRRSV virions, is present in PRRSV-infected cells, and elicits antibody production in infected pigs (Johnson et al., 2011). This, along with its evolutionary conservation indicates an important biological role. However the

function of ORF5a protein and its contribution to host immunity to PRRSV is unknown. Here, we examined the role of ORF5a protein in the immune response to PRRSV by immunization of pigs with ORF5a protein and evaluation of the response to virulent viral challenge, with the hypothesis that ORF5a immunization would reduce viral infection.

2.2 Materials and Methods

Animals

Twenty-two, four week old male and female cross-bred pigs sourced from a commercial PRRSV-negative swine herd were randomly assigned to four groups (Table 2.1). Pigs had been weaned one week prior and were acclimated for 5 days in groups of 5 or 6 pigs, housed in separate rooms of the University of Minnesota College of Veterinary Medicine animal isolation facility. Pigs were tested for viremia and antibodies (assays described below) to PRRSV to ensure they were negative at the beginning of the study. Pigs were immunized on days 0, 10, and 20, following a standard protocol (Li and Murtaugh, 2012). Viral challenge was performed by intramuscular inoculation on day 28 with 1 ml cell culture media containing 3×10^5 $TCID_{50}$ of PRRSV strain VR2332 (Genbank accession number U87392) (Yoon et al., 1999).

Blood was collected on days 0, 10, 20, 28, 35, 42, and 49 in serum separator tubes and serum was stored frozen at -20 °C for determination of antibody levels and viremia. Clinical response to challenge was evaluated by daily measurement of rectal temperature for 7 days from day of challenge, and observation for disease symptoms such as coughing, sneezing or lethargy. On day 49, pigs were anesthetized with an intramuscular combination of tiletamine, zolazepam and xylazine for blood collection and euthanized by intravenous barbiturate overdose. Necropsies were conducted to evaluate gross lung pathology.

The study was approved by and conducted under the guidelines of the University of Minnesota Institutional Animal Care and Use Committee.

Immunogens

Synthetic full length PRRSV VR2332 ORF5a protein (51 amino acids) was provided by the University of Minnesota Biomedical Genomics Core Peptide Synthesis Facility (Minneapolis, MN) for use as the primary immunogen. Immediately prior to use, lyophilized ORF5a protein was suspended in phosphate buffered saline (PBS) and emulsified in an equal volume of two different adjuvant preparations; (a) incomplete Freund's adjuvant (IFA) (Sigma-Aldrich, St. Louis, MO), and (b) equal volumes of IFA and a liposome preparation containing monophosphoryl lipid A (MPL) (Ernst et al., 2006) (Molecular Express Inc., Rancho Dominguez, CA) using the double-hubbed needle method (Berlin and McKinney, 1958; Freund and Thomson, 1945). MPL is a low-toxicity derivative of lipopolysaccharide, a TLR4 agonist, shown in other systems to aid robust adaptive immune responses without the proinflammatory activity of LPS (Ernst et al., 2006; Mato-Haro et al., 2007; Olson et al., 2010). The dose of synthetic ORF5a in 1 ml preparations for each of the three immunizations at day 0, 10 and 20 of the study was 50 ug, 20 ug and 200 ug respectively per pig.

PRRSV nonstructural protein 7 (nsp7) ELISA

Nsp7 was expressed and purified for antibody capture ELISA as described (Brown et al., 2009; Johnson et al., 2007). Plates were coated with 100 ng nsp7 per well, and serum samples were diluted 1:50 in 5 % non-fat dry milk (NFDM) in phosphate-buffered saline containing 0.05 % Tween 20 (PBST). Detection antibody, horseradish peroxidase-conjugated goat anti-pig IgG

(Bethyl Laboratories Inc. Montgomery TX), was used at a 1:100,000 dilution in 5 % NFDM in PBST. Immune complexes were revealed by oxidation with TMB peroxidase substrate (KPL, Gaithersburg MD) for 15 min and stopped with 1 M phosphoric acid. Absorbance was read at 450 nm in a ThermoMax Microplate Reader (Molecular Devices, Sunnyvale CA). Controls for the assay included known PRRSV-positive and negative serum.

ORF5a protein ELISA

ORF5a protein containing myc- and 6xhis- tags was expressed and purified as described (Johnson et al., 2011) for antibody capture ELISA. ELISA was performed as described and plates were coated with 100 ng ORF5a per well (Johnson et al., 2007). Serum samples were diluted 1:50 in 5 % non-fat dry milk (NFDM) in phosphate-buffered saline containing 0.05 % Tween 20 (PBST). Detection antibody, horseradish peroxidase-conjugated goat anti-pig IgG (Bethyl Laboratories Inc. Montgomery TX), was used at a 1:25,000 dilution in 5 % NFDM in PBST. Controls included buffer only, known ORF5a-positive serum and known PRRSV-negative serum. Absorbance values are represented as the mean of the duplicate wells or the group average ± standard error. Cutoffs were assigned as twice the value of the background level absorbance, determined as the mean absorbance of the negative control wells.

Viral RNA isolation and reverse transcription-quantitative PCR (RT-qPCR)

Viral RNA was isolated from serum using a QIAmp Viral RNA Mini kit (Qiagen, Valencia CA) eluted into 50 ul of RNase-free water and stored at -80 °C. Complementary cDNA was synthesized with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad CA) with random hexamer primers. Primer sequences GATAACCACGCATTTGTCGTC

(forward) and TGCCGTTGTTATTTGGCATA (reverse) amplified a 149 bp PRRSV ORF6 fragment. Primer specificity for Type II PRRS viruses was confirmed by BLAST analysis against the NCBI nonredundant nucleotide database. PCR reactions were performed in a total volume of 20 ul, containing 5 ul of cDNA, 10 ul of SYBR Green PCR Master Mix (PerfeCTa SYBR Green FastMix, Quanta Biosciences, Gaithersburg MD) and 200 ng of each primer. Reactions were run in a Stratagene Mx3000P thermal cycler (Agilent Technologies, Inc. Santa Clara CA), with activation at 95 °C for 1 minute, 35 cycles of denaturation at 95 °C for 3 seconds and annealing/extension at 60 °C for 25 seconds, followed by a dissociation step. All samples were run in duplicate.

PRRSV was quantified using the standard curve method similar to (Wasilk et al., 2004). A standard curve was generated from gel purified ORF6 PCR product from the same stock of PRRSV VR2332 used to infect the pigs using the same method of viral RNA isolation, cDNA synthesis and primers for PCR described above. After purification, cDNA concentration was estimated by spectrophotometry and number of copies of the template calculated (Staroscik, 2004). Serial 10-fold dilutions were used to construct a standard curve, ranging from $>10^5$ to <1 copy per reaction. Identification of positive samples was determined by the presence of a quantification cycle value and analysis of the dissociation product. Negative controls without cDNA were used in all PCR plates.

Serum neutralization assay

MARC145 cells were plated at 5000 per well in ninety six-well Nunc™ tissue culture plates (Thermo Scientific, Rochester NY) in Minimal Essential Medium (MEM) (Gibco, Grand Island NY) supplemented with sodium bicarbonate, non-essential amino acids, HEPES buffer (Sigma,

St. Louis MO) and gentamycin sulfate (Cellgro Mediatech, Manassas VA) with 10% fetal bovine serum (FBS) (Sigma, St. Louis MO) and cultured for 48 hours at 37 °C in 5% CO_2 before use. Porcine pulmonary alveolar macrophages were similarly plated at 80,000 per well in RPMI 1640 (Cellgro Mediatech, Manassas VA) with sodium pyruvate (Cellgro Mediatech, Manassas VA) in place of sodium bicarbonate, supplemented with 5 % FBS and cultured for 1 day at 37 °C in 5 % CO_2 before use.

Serum samples were heat-inactivated at 56 °C for 30 min, two-fold serially diluted in MEM or RPMI without FBS, and mixed with an equal volume of 3 x 10^5 $TCID_{50}$/ml VR-2332 for 1 hour at 37 °C. After incubation, 50 ul of the serum-virus mixture was added to MARC145 and PAM cells and incubated for 1 hour at 37 °C. Inoculates were then removed and cells were cultured with MEM or RPMI containing FBS for 1 day. Controls included serum with previously determined PRRSV neutralizing activity, virus alone with no serum, and uninfected cells.

After culture, cells were fixed with 3.7 % formaldehyde (Sigma, St. Louis MO) in PBS for 30 min at room temperature, permeabilized with 0.1 % Triton X-100 (Sigma, St. Louis MO) in PBS for 5 min, and blocked with NFDM in PBS, pH 9.6, for 1 hour at 37 °C. After washing with PBS, 1:10,000 diluted anti-nucleocapsid mAb SR30-A (Rural Technologies, Brookings SD) was added to cells for 1 h at 37 °C. After washing, the secondary antibody HRP-conjugated goat-anti-mouse IgG (H+L) (KPL, Gaithersburg MD) was diluted 1:500 and then added to cells for 1 h at 37 °C. Finally color was developed with TMB peroxidase substrate (KPL, Gaithersburg MD) for 15 min and stopped with 1 M phosphoric acid. Absorbance was read at 450 nm in a ThermoMax Microplate Reader (Molecular Devices, Sunnyvale CA). Results were calculated as the percent inhibition of viral infection at each dilution compared with virus only controls (with background absorbance from uninfected cells subtracted).

Statistics

Pairwise comparison of viremia between groups was conducted using the nonparametric Wilcoxon rank sum test. Fisher's exact test was used to examine pairwise comparisons of proportion of viremic pigs per group. As no statistical or biological differences in viremia or antibody titers were observed between the immunized groups regardless of adjuvant preparation (group 2 and 3 in Table 2.1), data from these two groups were combined for graphical presentation in Figure 2.1 and Figure 2.2.

The difference in distribution of virus neutralizing capacity between the paired pre-immune and immune sera was investigated for each serum dilution using a paired two-tailed t test. Correlation coefficients were calculated between serum neutralizing capacity and ORF5a antibody level, and tested by a two tailed t test with n-2 degrees of freedom with the hypothesis that they were correlated.

2.3 Results and Discussion

Pigs were determined to be naïve to PRRSV at the beginning of the study as antibody responses to PRRSV ORF5a (Figure 2.1) and nsp7 (data not shown) were negative indicating absence of prior exposure, as well as by absence of viremia in the RT-qPCR assay. All pigs immunized with ORF5a protein had seroconverted by the day of challenge (day 28) (Figure 2.1). At this time, the antibody response (as evidenced by a plateau in the antibody response curve for each individual pig) was fully developed. Following challenge, all pigs developed viremia, with no quantitative differences between immunized and non-immunized groups (Figure 2.2, $p>0.05$). There was no significant difference between the proportion of viremia-positive pigs per group in challenged

groups irrespective of immunization as determined by Fisher's exact test, showing that the results were not skewed by individual pig responses.

Despite viremia in all challenged animals the response of pigs to the PRRSV VR2332 challenge of 3 x 10^5 $TCID_{50}$ was subclinical. No coughing or sneezing was observed in the challenged animals, nor were there differences in body temperature between groups or evidence of fever in individual pigs (data not shown). Similarly, gross lung lesions were minimal or absent.

To determine whether ORF5a immune sera has virus neutralizing capacity, an ELISA-based assay was used to quantify PRRSV infection of MARC 145 cells and porcine alveolar macrophages after pre-incubation with sera (Li and Murtaugh, 2012). Sera from a sow with PRRSV neutralizing activity of greater than 50 % at a 1:16 dilution was used as a positive control for the assay. Neutralizing capacity of immune sera at challenge on day 28 was compared to day 0 baseline for all pigs. There was less than 50 % neutralizing activity of ORF5a immune sera at a 1:2 dilution and no difference in neutralizing capacity between paired pre-immune and immune sera across all animals ($p = 0.38$) (Table 2.). Furthermore, there was no correlation between the level of anti-ORF5a antibodies in serum and virus neutralizing capacity. Similarly, serum level of ORF5a antibody was not correlated with the level of viremia.

ORF5a protein is a predicted membrane-spanning structural protein in the virion (Johnson et al., 2011). Therefore, we examined its potential role in anti-PRRSV immunity, particularly with respect to induction of a neutralizing antibody response. It was observed that in response to PRRSV infection the antibody response to ORF5a is relatively late in onset (4 weeks after PRRSV infection) and low level (Johnson et al., 2011), consistent with that described for PRRSV-neutralizing antibodies (reviewed in (Lopez and Osorio, 2004)). Pigs had a robust

antibody response to ORF5a protein immunization, showing that it is highly antigenic in swine. However, ORF5a protein seroconversion did not translate to protection against PRRSV viremia. Immunized pigs had equivalent levels of viremia which was resolved at the same rate as in the non-immunized group. As PRRSV infection was subclinical in this study, it remains unknown whether ORF5a immunization would alter the course or severity of clinical disease. Immune serum did not significantly neutralize PRRSV, and the level of ORF5a protein antibodies was not correlated with the virus neutralizing capacity of sera. No effects of MPL-liposome inclusion in the adjuvant emulsion with IFA were observed. Collectively, the data show that ORF5a protein immunization elicits antibodies that do not have virus neutralizing activity, and the immune response is not protective to PRRSV challenge.

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*Juan Abrahante designed and optimized the PRRSV PCR assay used in this study, and Marina Figueiredo performed the qPCR reactions.

Table 2.1 Treatment groups

Group	Treatment	N
1a	Untreated negative control	3
1b	ORF5a protein-IFA immunization, non-challenged	3
2	ORF5a protein-IFA immunization, PRRSV challenged	6
3	ORF5a protein-liposome-IFA immunization, PRRSV challenged	5
4	Non-immunized, PRRSV challenged	5

Table 2.2 Comparison of pre- and post-ORF5a immunization 50% serum neutralization titers

Group	pigs with 50% SN titer ≥ 1:2 / total pigs	
	pre-immunization	**post-immunization**
1a	0/3	0/3
1b	0/3	0/3
2	0/6	0/6
3	0/5	0/5
4	0/5	0/5

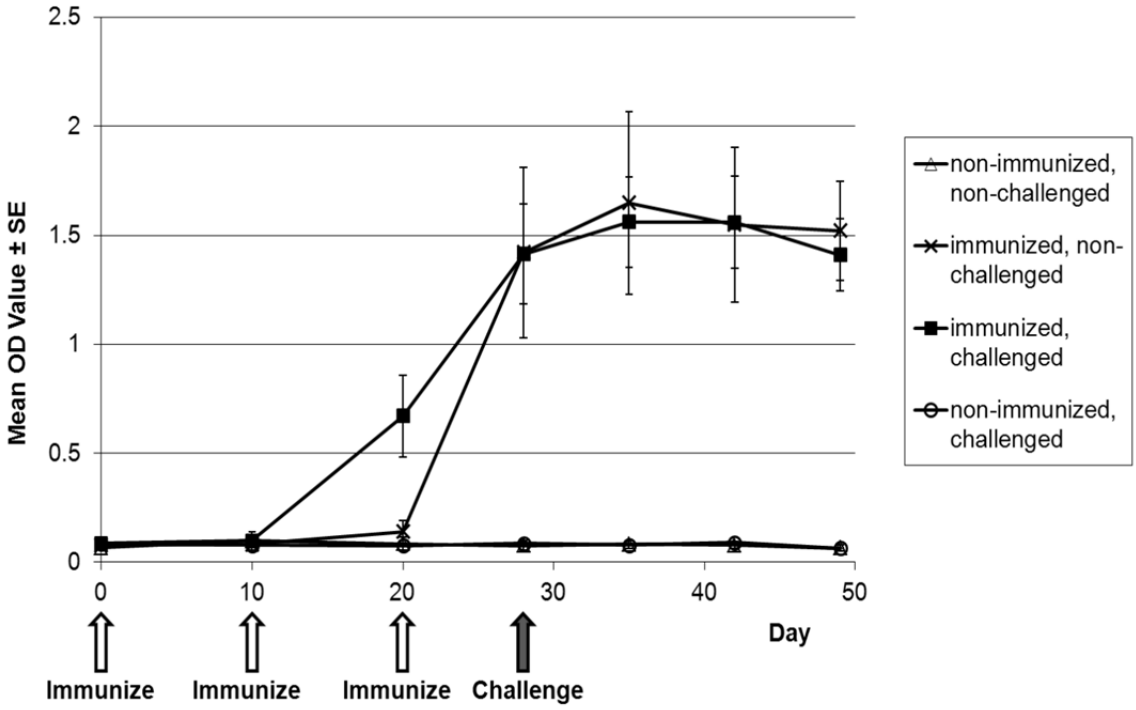

Figure 2.1 Antibody response to ORF5a protein immunization.

Serum samples were stored and tested on the same day by ELISA as described in Materials and Methods.

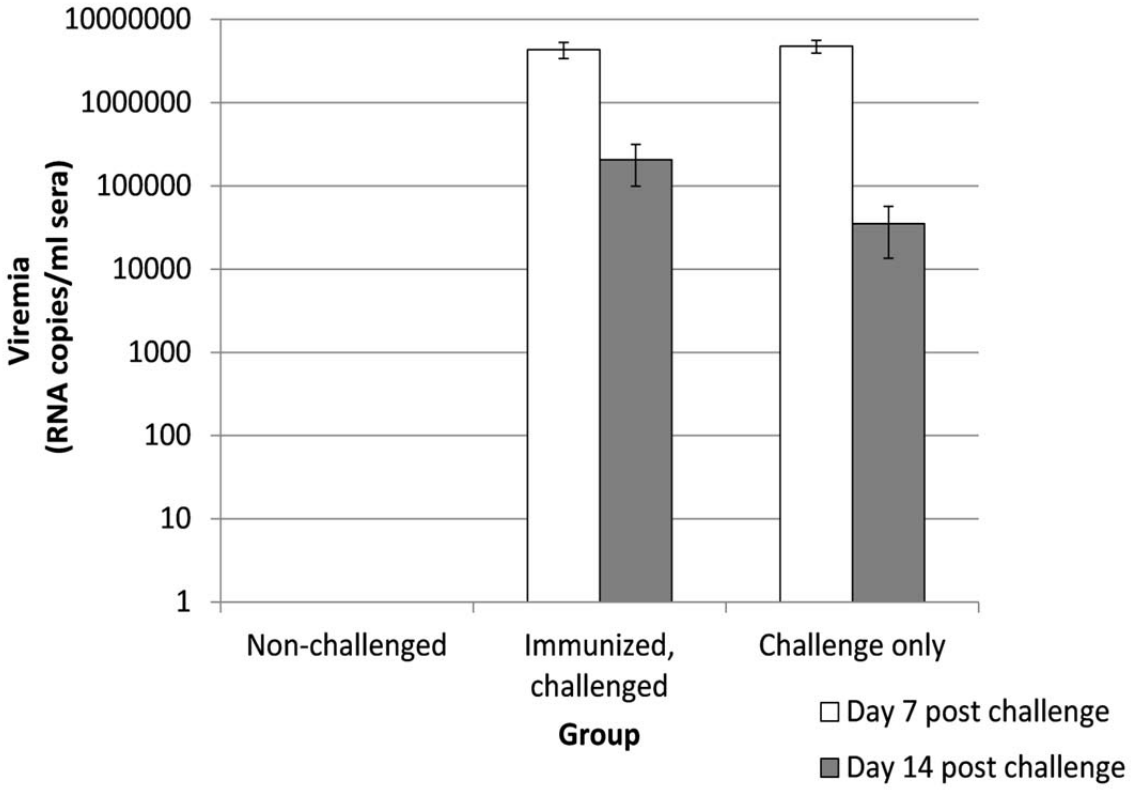

Figure 2.2 Effect of immunization on PRRSV viremia.

Quantitative RT-PCR was performed as described in Materials and Methods using the standard curve method with a gel-purified PCR product.

Chapter 3 Co-evolution of PRRSV ORF5a protein and glycoprotein 5

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Purifying selection in Porcine reproductive and respiratory syndrome virus ORF5a protein influences variation in envelope glycoprotein 5 glycosylation

Sally R. Robinson, Juan E. Abrahante, Craig R. Johnson, Michael P. Murtaugh

3.1 Introduction

Porcine Reproductive and Respiratory Syndrome (PRRS) virus is an Arterivirus in the Order Nidovirales. It causes PRRS in swine, a devastating disease with significant animal welfare and economic implications for which there is no specific treatment and incomplete protection by vaccination. Despite extensive research yielding substantial knowledge of PRRSV biology, epidemiology and genome structure since its emergence in the late 1980s, the precise mechanisms responsible for virulence, pathogenesis and protective immune response to PRRSV remain poorly understood. These factors limit progress toward development of effective measures for prevention and treatment of this disease.

The ~15kb PRRSV RNA genome is extremely compact, with 98% coding capacity. There are conserved untranslated regions (UTRs) at both the 5' and 3' end of 150 to 220 nt, and a single non-coding nucleotide between open reading frames (ORFs) 1b and 2, at the transition between coding sequences for the non-structural and structural proteins. Coding potential of PRRSV is maximized through a number of mechanisms, including ribosomal frameshifting and proteolytic cleavage of translated polypeptides to yield multiple non-structural proteins, and overlapping open reading frames and nested subgenomic (sg) mRNA in the structural protein coding sequences (Fang et al., 2012; Maclachlan and Edward, 2011).

The structural proteins of PRRSV are translated from a 3' co-terminally nested set of sgmRNAs resulting in expression of each protein from separate sgmRNA fragments. Two additional small envelope proteins, E and ORF5a protein, are expressed from overlapping bicistronic sgmRNA 2 and sgmRNA 5, respectively (Firth et al., 2011; Johnson et al., 2011; Snijder et al., 1999; Wu et al., 2001). In each case, overlapping GP2 and E proteins, and ORF5a protein and GP5 are translated from alternate reading frames. The novel PRRSV ORF5a protein is initiated 10 bp upstream of, and encoded in the +2 reading frame from, the major envelope glycoprotein GP5 in Type 2 PRRS viruses (Firth et al., 2011; Johnson et al., 2011). The presence of ORF5a in sgmRNA5 is evolutionarily conserved throughout the Arteriviridae (Firth et al., 2011; Johnson et al., 2011).

ORF5a protein is a small (46 – 51 amino acid) type 1 membrane protein with a short ectodomain, a single transmembrane region and an endodomain tail containing a highly conserved 8 aa arginine-glutamine (RQ) motif (Johnson et al., 2011). Paradoxically, the conserved RQ-motif is encoded in the same nucleotide sequence that encodes a hypervariable glycosylation region of the GP5 ectodomain, first described in 1995 (Kapur et al., 1996; Meng et al., 1995; Pirzadeh et al., 1998). GP5 is predicted by protein structure modeling software to contain an N-terminal signal peptide (Meulenberg et al., 1995). It directs GP5 to the secretory pathway of the endoplasmic reticulum/Golgi complex, where it can undergo maturation, including folding and N-linked glycosylation prior to virion assembly (Faaberg et al., 2011; Mardassi et al., 1996). The N-terminal portion of mature GP5 protein is proposed to contain a neutralizing epitope flanked by two hypervariable glycosylation regions that provide a glycan shield for immune evasion and that are under intense immunological selection (Ansari et al., 2006; Faaberg et al., 2006; Lopez and Osorio, 2004; Meng et al., 1995; Ostrowski et al., 2002; Vu et al., 2011). However, the significance of GP5 glycosylation and neutralization in PRRSV immunity remains controversial

(Li and Murtaugh, 2012; Murtaugh and Genzow, 2011). Furthermore, the implications of differential selective forces on the overlapping reading frames of this potentially important region for PRRSV immunity have not been evaluated. To investigate the mechanism by which the sgmRNA5 nucleotide sequence simultaneously accomplished conservation of the RQ-motif in ORF5a protein and hypervariability in GP5 glycosylation, we analyzed 3466 unique and diverse type 2 PRRSV ORF5 sequences.

3.2 Material and methods

PRRSV ORF5 nucleotide sequences of 603 bp in length (the coding sequence for GP5 from amino acid position 1 to 201) were obtained from the University of Minnesota Veterinary Diagnostic Laboratory PRRSV database, containing more than 8,000 ORF5 sequences, including field isolates and vaccine strains, and representing the full diversity of type 2 PRRSV (Shi et al., 2010). Sequences were manually edited in CLC sequence viewer (CLC Bio, Cambridge MA), aligned using MUSCLE (Edgar, 2004), and translated in both ORF5a protein and GP5 reading frames. ORF5 sequences begin at the GP5 translation start codon, so are devoid of the first 10 nucleotides of ORF5a. Hence, translated ORF5a protein sequences start at amino acid 5. Sequences were clustered at 100% similarity using CD-HIT to remove identical sequences (Li and Godzik, 2006).

ORF5a and GP5 nucleotide and amino acid frequency logos were generated with WebLogo (Crooks et al., 2004). GP5 N-linked glycosylation motifs were identified with the N-GlycoSite tool (Zhang et al., 2004). The relative frequency of ORF5a and GP5 synonymous and non-synonymous nucleotide substitutions was determined using Molecular Evolutionary Genetics Analysis (MEGA) 5 (Tamura et al., 2011).

For N-terminal sequencing of GP5, PRRSV strain VR2332 (Genbank accession U87392.3) was grown in MARC145 cells and purified from cell culture supernatant as described, except that sucrose pellets were banded in 1.25 g/ml CsCl (Johnson et al., 2011). Purified virions were electrophoresed on a 10% SDS-polyacrylamide gel. Coomassie blue-stained bands in the molecular weight regions of 18, 20, 25 and 28 kDa were excised and submitted to the Mayo Clinic Proteomics Core (Rochester, MN) for automated Edman degradation protein sequencing on an ABI Procise system (Applied Biosystems, Carlsbad, CA).

Codon usage was analyzed using MEGA (Tamura et al., 2011) for ORF5a R and Q residues and GP5 N and S residues in the overlapping region. In addition these were compared with codon usage in the complete PRRSV VR2332 genome (Genbank accession U87392.3) and expressed swine genes obtained from the codon usage database (http://www.kazusa.or.jp/codon/) (Nakamura et al., 2000). Statistical comparisons were made between observed codon usage frequencies in the 3466 ORF5 sequences and frequencies expected from random codon usage using the Chi-squared test for residues with 6 possible codons (R and S) and Fisher's exact test for those with 2 codons each (Q and N). A p value of 0.05 was considered significant.

Theory

Coincident nucleotide sequence coding for a highly conserved motif in one protein and a highly variable motif in another is paradoxical. We hypothesized that selective codon usage driven by purifying selection to maintain the conserved ORF5a protein RQ-motif simultaneously facilitates nonsynonymous mutations increasing glycosylation site variability in GP5. Glycosylation site diversity as an indirect consequence of RQ-motif conservation provides an alternative to immunological selection, for which little direct evidence has been marshaled.

3.3 Results

Genetic selection pressure on 3466 Type 2 PRRSV ORF5 sequences is purifying across the majority of the 201 aa GP5 sequence, with the exception of the first 35 N-terminal aa. Selection pressure here is neutral or diversifying, and a hypervariable glycosylation region is present in aa 32 to 35 (Figure 3.1). The difference in selection pressure was previously utilized to predict the dual-coding nature of ORF5 (Firth et al., 2011). Of the 3466 ORF5a protein sequences, seventy six percent were 46 aa long; the remaining 24% were 51 aa in length, consistent with previous findings (Firth et al., 2011).

Ninety-six percent of 3466 ORF5a proteins exclusively contained R and Q residues in the RQ-motif (amino acid position 32-39) (Figure 3.2A). In overlapping amino acid positions 29-36 of the GP5 protein, N-linked glycosylation motifs (defined by N-X-S/T, where N = asparagine, X = any amino acid, S/T = serine or threonine) were identified; the majority of full-length GP5 sequences (94%) were found to contain 2 to 4 N-linked glycosylation motifs (Figure 3.3A). Along with the essentially fixed motifs at GP5 positions 44 and 51, additional N-linked glycosylation motif variation occurs in the ORF5a overlapping region at positions 30, 32, 33, 34 and 35 (Figure 3.3B), and extensive use of N and S residues is evident (Figure 3.2A).

The discordance between R/Q conservation in ORF5a protein and glycosylation diversity in GP5 is associated with differences in the reading frame of specific nucleotide changes that are shared between the two ORFs. The nucleotide variation in this region of ORF5a is largely restricted to the third codon position, with mean frequency of sequence variation 21%, compared to codon position 1 (1%) or 2 (6%). Third codon position variation frequently results in synonymous substitutions due to the degenerate nature of the amino acid code, preserving the R/Q motif (Figure 3.2A). By contrast, the same mutation occurs in the second codon position of ORF5,

causing a non-synonymous substitution that disrupts glycosylation motifs with high frequency (Figure 3.2A).

The overall selective pressure in this region, assessed by the relative frequency of synonymous and non-synonymous nucleotide substitutions, was neutral (dN=dS) or diversifying (dN>dS) for GP5, whereas it was strongly purifying (dN<dS) or neutral in ORF5a, with some diversification evident at residues 37 and 38 (Figure 3.2B). ORF5a codons in which C was followed by A/G variation in the second position (positions 33, 35, 36, 37) resulted in a change from R to Q or Q to R. Thus, simple analysis of dN and dS to infer selective forces underestimates conservation of the RQ-motif as the R/Q substitution is a non-synonymous change.

Strongly purifying selection is observed at ORF5a protein residues 32, 33, and 34, which overlap the GP5 signal peptide. The signal peptide is cleaved between amino acids 31 and 32 to form mature GP5, as predicted by SignalP (www.cbs.dtu.dk/services/SignalP/) and confirmed by N-terminal amino acid sequencing. Electrophoresis of purified PRRSV VR2332 virions revealed two visible bands were present in the molecular weight regions of 25 and 28 kDa, consistent with molecular weight heterogeneity observed for GP5 due to a combination of N-linked high-mannose and complex oligosaccharide types (Figure 3.4A) (Mardassi et al., 1996). The first 10 residues of each band yielded the sequence S-DSSSHLQL, which corresponds to VR2332 GP5 residues 32-41 (Figure 3.4B). The second cycle did not yield a product, consistent with the presence of a glycosylated asparagine. Thus, amino acid 32 defines the amino terminus of mature GP5. Consequently, the predicted N-linked glycosylation motif at amino acid position 30 in 32% of 3466 ORF5 sequences (Figure 3.3B) is not present in PRRSV virions.

A notable feature of the hypervariable glycosylation region is the absence of U nucleotides in GP5 sequences that code for amino acid residues 30 to 36 (Figure 3.5A). To analyze the

mechanism for U residue depletion, the theoretical usage of U nucleotides in the possible codons for N, S, R and Q were compared to the actual observed codon usage in this region. U nucleotides are present in 6 of the 8 codons for N and S, accounting for 7 of the 24 possible codon nucleotide positions, or 29%, in these key glycosylation residues. By contrast, U occurs only once in the 8 possible codons for R and Q, at only 1 of 24, or 4%, of potential coding nucleotides, in the overlapping ORF5a coding sequence. However, the observed frequency of U nucleotides in 3466 sequences was substantially lower, at 0.1%. The low observed frequency of U in this region is due to codon usage bias in N and S residues imposed by ORF5a protein (Figure 3.5B), since U is absent in Q and is only in 1 of 18 possible R codon positions. The observed codon usage bias differs from random utilization of N and S codons significantly (χ^2 test, p ~0) and also differs from the frequency of use in other regions of the PRRSV genome and from codon usage in expressed swine genes (Figure 3.5B). Strikingly, of the 8 possible S and N codons, AGC (coding for serine) and AAC (coding for asparagine) are utilized almost exclusively in this region (Figure 3.5B). These are the only codons for S and N that do not contain U and are the only codons that allow dual coding of S/N in the GP5 reading frame and R/Q in the ORF5a protein +2 reading frame. A striking codon bias is also observed for R residues than would be expected from random codon usage (χ^2 test, p ~0) (Figure 3.5C).

3.4 Discussion

The paradox of a highly conserved RQ-motif coexisting with a hypervariable glycosylation region existing in a uracil desert is the mechanistic result of ORF5a codon wobble whose concomitant position two change in the overlapping GP5 codon alters the pattern of N-linked glycosylation motifs. Previously, immunological selection to escape neutralizing antibodies was

proposed to account for variable glycosylation pattern evolution in GP5. However, direct functional evidence for immunological selection is lacking, and the significance of neutralizing antibody responses to PRRSV immunity is not firmly established (reviewed in (Murtaugh and Genzow, 2011)). Disruption of N-linked glycosylation motifs is reported to result in increased susceptibility to virus neutralization *in vitro* (Ansari et al., 2006; Faaberg et al., 2006; Vu et al., 2011). Reversion of mutant virus to the glycosylation phenotype has been observed *in vivo*, with corresponding increased resistance to *in vitro* neutralization (Vu et al., 2011). However, neutralizing monoclonal antibodies have been shown to bind both glycosylated and non-glycosylated forms of GP5 (Pirzadeh and Dea, 1997), and immunization with non-glycosylated GP5-M ectodomains did not elicit neutralizing antibodies even though partial protection was observed (Li and Murtaugh, 2012). Our analysis revealed that GP5 S and N codon usage is highly restricted to accommodate ORF5a protein R and Q residues in the alternate reading frame. Selective codon usage in ORF5a R, in particular, serves to maintain hypervariable glycosylation sites in GP5. Thus, in addition to purifying selection to maintain the conserved ORF5a protein motif, selective pressure also exists to maintain glycosylation sites in GP5. Our results, combined with lack of compelling evidence for an immunological benefit to GP5 glycosylation or for phylogenetic clustering of GP5 glycotypes indicative of selection (Delisle et al., 2012), indicate that glycosylation motif position variation in GP5 is a mechanism to maintain a functional requirement for glycan addition in the GP5 ectodomain, such as for correct folding (Wei et al., 2012a), whilst preserving an undetermined function of the RQ-motif in ORF5a protein.

The strong, purifying selection to maintain Type 2 PRRSV ORF5a protein RQ-motif, combined with genetic ablation studies (Sun et al., 2013) indicate that ORF5a is necessary for production of viable progeny virions. While an RQ-motif is present in Type 1 PRRSV and other arteriviruses, its conservation appears to be less pronounced (Johnson et al., 2011); Firth et al., 2011). A

relative paucity of ORF5 and ORF5a sequences for other arteriviruses at this time precludes a detailed comparative analysis of selection pressures. However, genetic ablation of ORF5a in equine arteritis virus (EAV) substantially reduced, but did not eliminate, virion production in infected cells (Firth et al., 2011; Sun et al., 2013), suggesting that ORF5a is not essential in EAV. Also, translation of ORF5a of Type 1 PRRSV, lactate dehydrogenase-elevating virus (LDV) and simian hemorrhagic fever virus (SHFV) is initiated downstream of ORF5, rather than upstream as occurs in Type 2 PRRSV and EAV (Firth et al., 2011). The significance of this difference is not known. The specific ORF5a amino acid residue patterns in the RQ-rich region vary among the arteriviruses, as does the overlapping GP5 sequences (Firth et al., 2011; Johnson et al., 2011). The exact position within this region of N-linked glycosylation motifs, and proposed neutralizing epitopes also differs between Type 1 and 2 PRRSV (Ostrowski et al., 2002; Plagemann et al., 2002; Wissink et al., 2003a). It is possible that the high degree of RQ-motif conservation and overlap with the hypervariable glycosylation motif of GP5 contribute to the higher virulence of Type 2 PRRSV.

3.5 Conclusions

PRRSV has evolved a mechanism of highly selective codon usage, utilizing the ORF5a wobble position to maintain ORF5a protein RQ-motif conservation in the face of positive selective pressure for GP5 N-linked glycosylation motifs. The extreme bias for codon usage resulting in a uracil desert indicates evolution of a fine-tuned mechanism to accommodate the dual coding nature of the nucleotide sequence region and suggests co-evolution of the two proteins to accommodate the functional needs of both a GP5 N-linked glycosylation motif and an ORF5a protein RQ-motif. Since GP5 glycosylation variability is assumed to result from immunological selection against neutralizing antibodies, these findings show that an alternative possibility

unrelated to immunological selection not only exists, but provides a foundation for investigating previously unsuspected aspects of PRRSV biology.

ORF5a protein appears to play a key role in the Arteriviridae since its genetic ablation in equine arteritis virus severely impacts growth in permissive cells (Firth et al., 2011), and abolishes growth in PRRSV (Sun et al., 2013). The high level of ORF5a protein RQ-motif conservation throughout Type 2 PRRS viruses indicates that this region, in particular, is critical. Hence, subtle modifications of nucleotide sequence in this region based on rare, viable field isolates may provide an avenue to rational development of new vaccines.

Acknowledgements

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*Juan Abrahante compiled and manually edited sequence data for use in this study, performed alignments and initial nucleotide selection analysis. Craig Johnson performed N-terminal sequencing experiments.

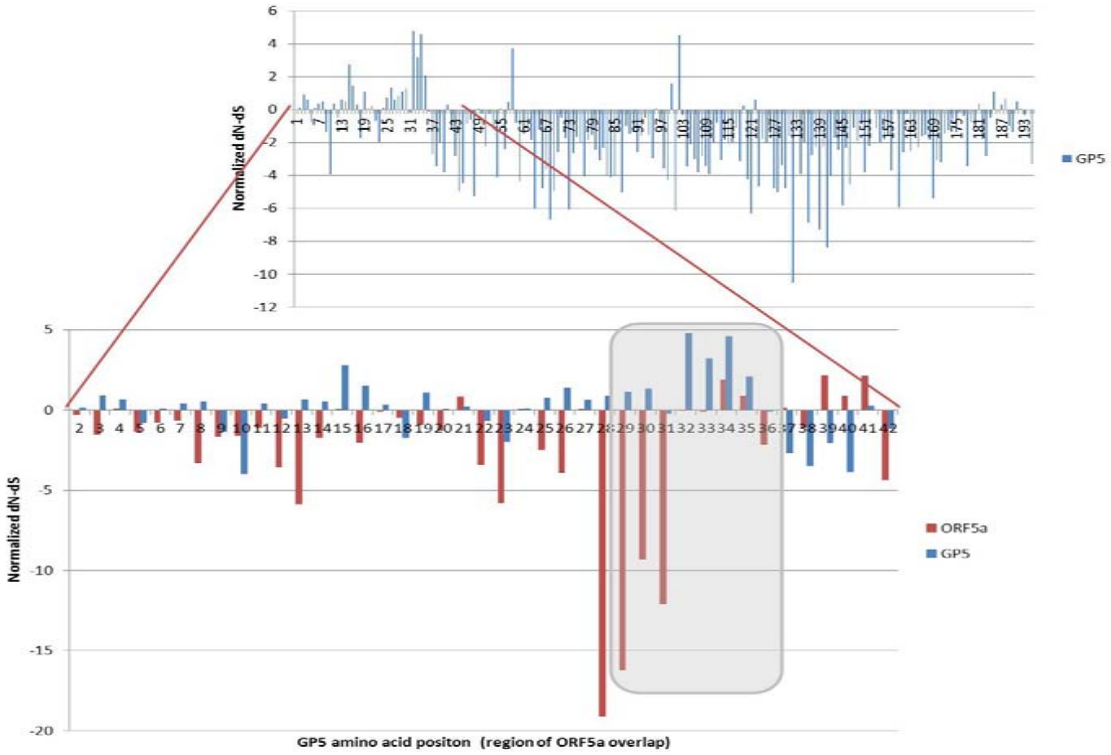
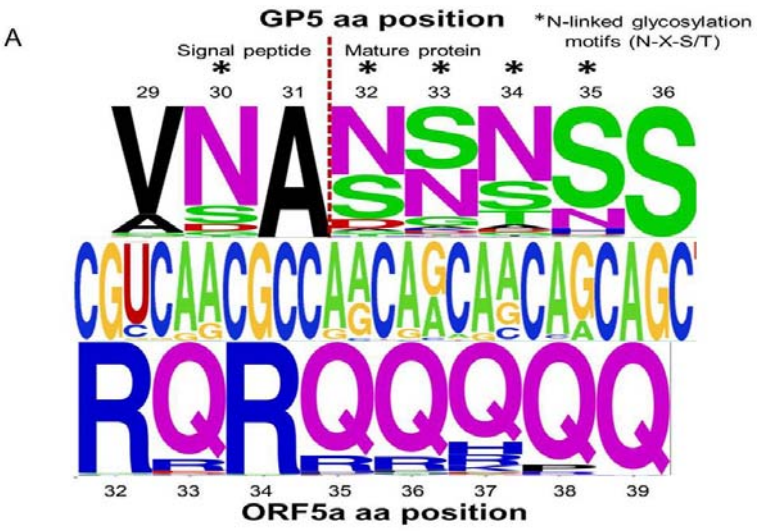
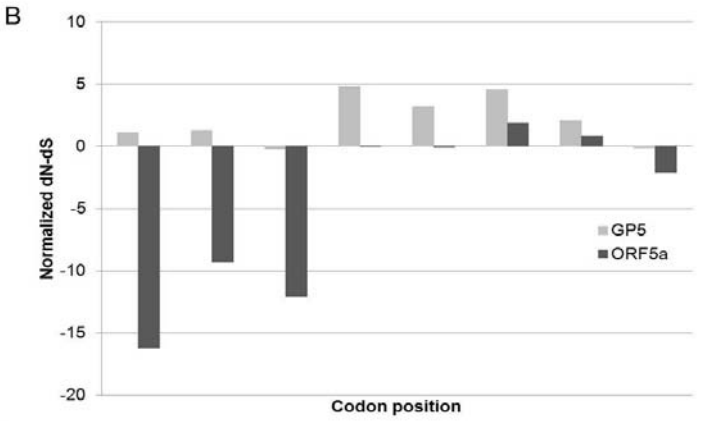


Figure 3.1 Analysis of 3466 PRRSV glycoprotein 5 (GP5) coding sequences.

Comparison of rate of nonsynonymous (dN) and synonymous (dS) changes per site as a measure of selective pressure across the GP5 coding sequence (top figure, and shown in blue), and in the region of ORF5a protein overlap (bottom figure, and shown in red). Shaded area represents the region of interest containing the GP5 hypervariable glycosylation region and the ORF5a protein RQ-motif.





GP5	29	30	31	32	33	34	35	36
ORF5a	32	33	34	35	36	37	38	39

Figure 3.2 PRRSV sgmRNA5 dual coding sequence at the GP5 hypervariable region and ORF5a protein RQ-motif overlap.

A) Relative frequency of nucleotide residues in the 3466 sgmRNA5 nucleotide sequences (from nucleotides 84-104 of the GP5 reading frame), and amino acid sequences translated in the GP5 and ORF5a protein reading frames. Amino acid positions are shown for GP5 and ORF5a protein. GP5 N-linked glycosylation motif positions are marked by asterisks and signal peptide cleavage site is shown by a dashed line. B) Comparison of synonymous and nonsynonymous changes in GP5 and ORF5a protein coding sequences, calculated in MEGA5 (Tamura et al., 2011).

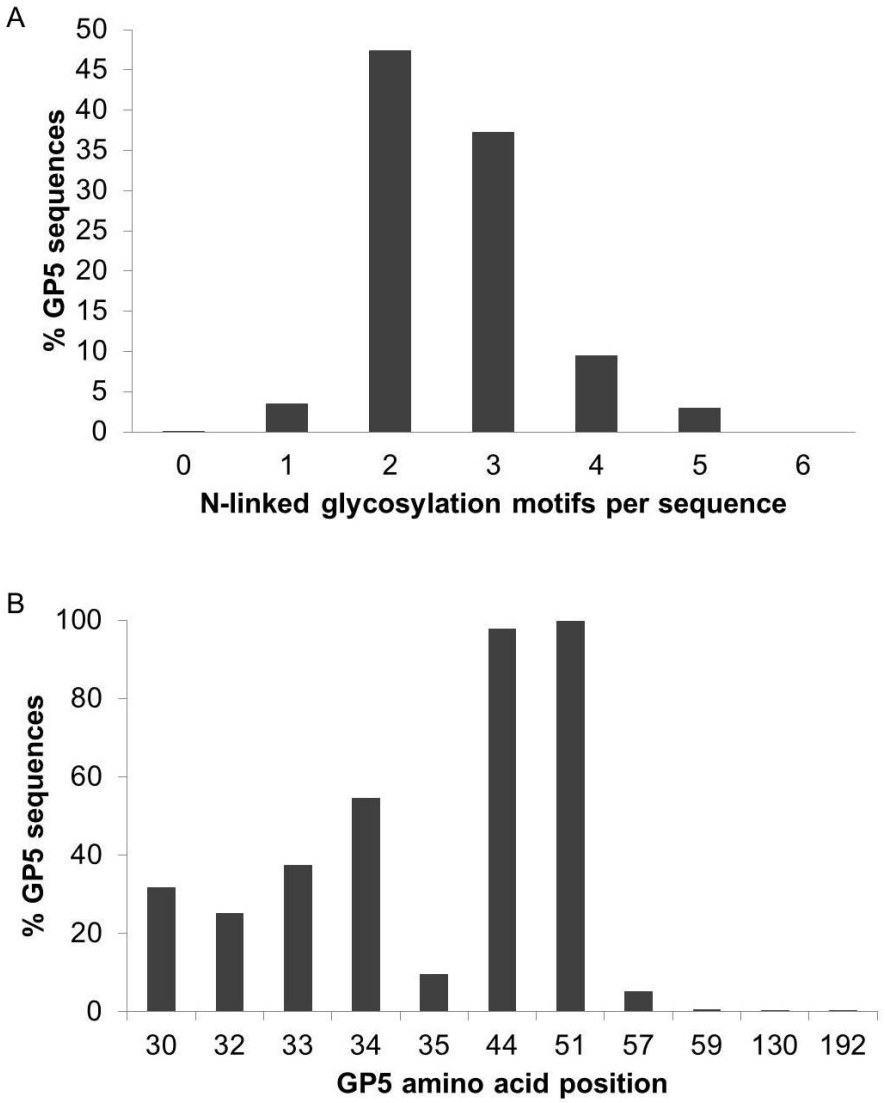


Figure 3.3 Predicted GP5 N-linked glycosylation patterns.

A) Variation in number of predicted N-linked glycosylation motifs across 3466 GP5 sequences.

B) Frequency of predicted N-linked glycosylation motifs at each position in GP5.

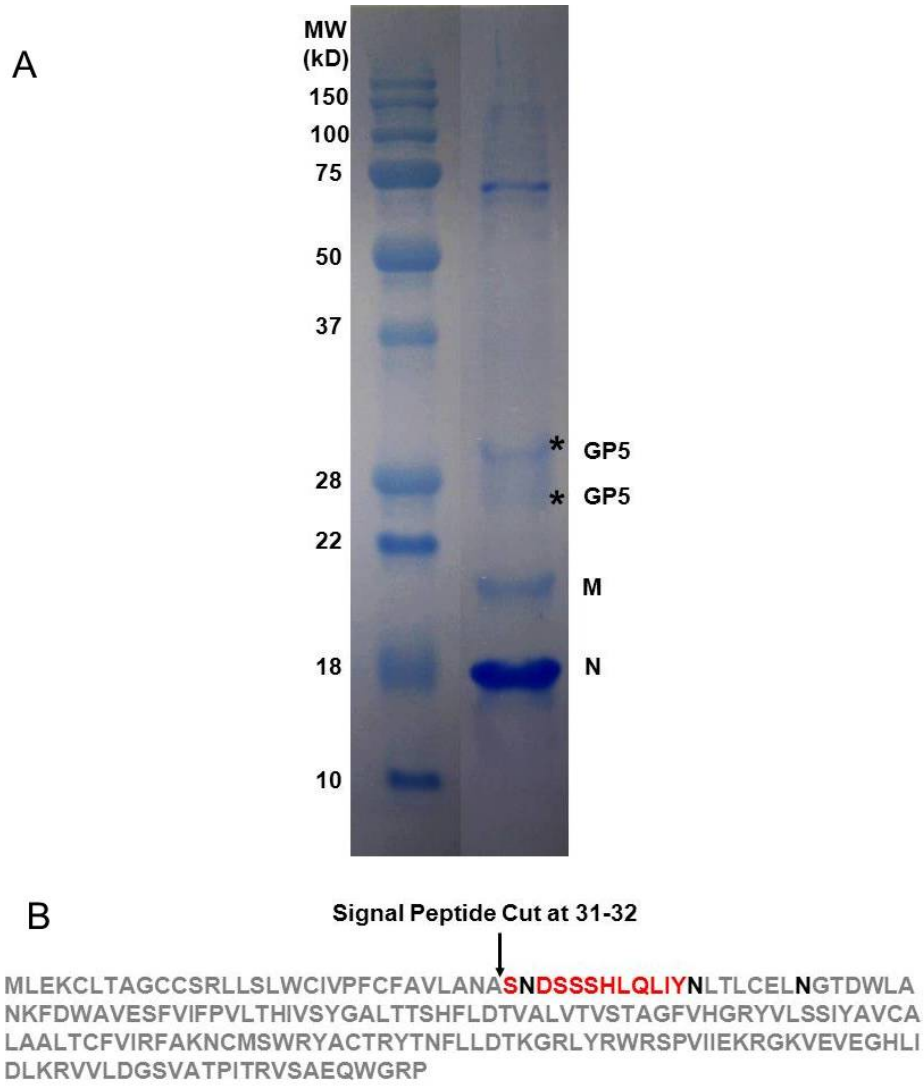


Figure 3.4 N-terminus of mature GP5.

A) Purified PRRSV VR2332 virions electrophoresed on a 10% SDS-polyacrylamide gel, and stained with Coomassie blue. Molecular weight marker is shown in lane 1. Bands identified as GP5, M, and N were excised from the gel and subjected to N-terminal amino acid sequencing by Edman degradation. B) PRRSV VR2332 GP5 amino acid sequence. N-terminal amino acid residues identified by protein sequencing are shown in red, and the signal peptide cleavage site is marked by an arrow between residues 31 and 32. Asparagine (N) residues in bold denote N-linked glycosylation motifs at positions 33, 34 and 51.

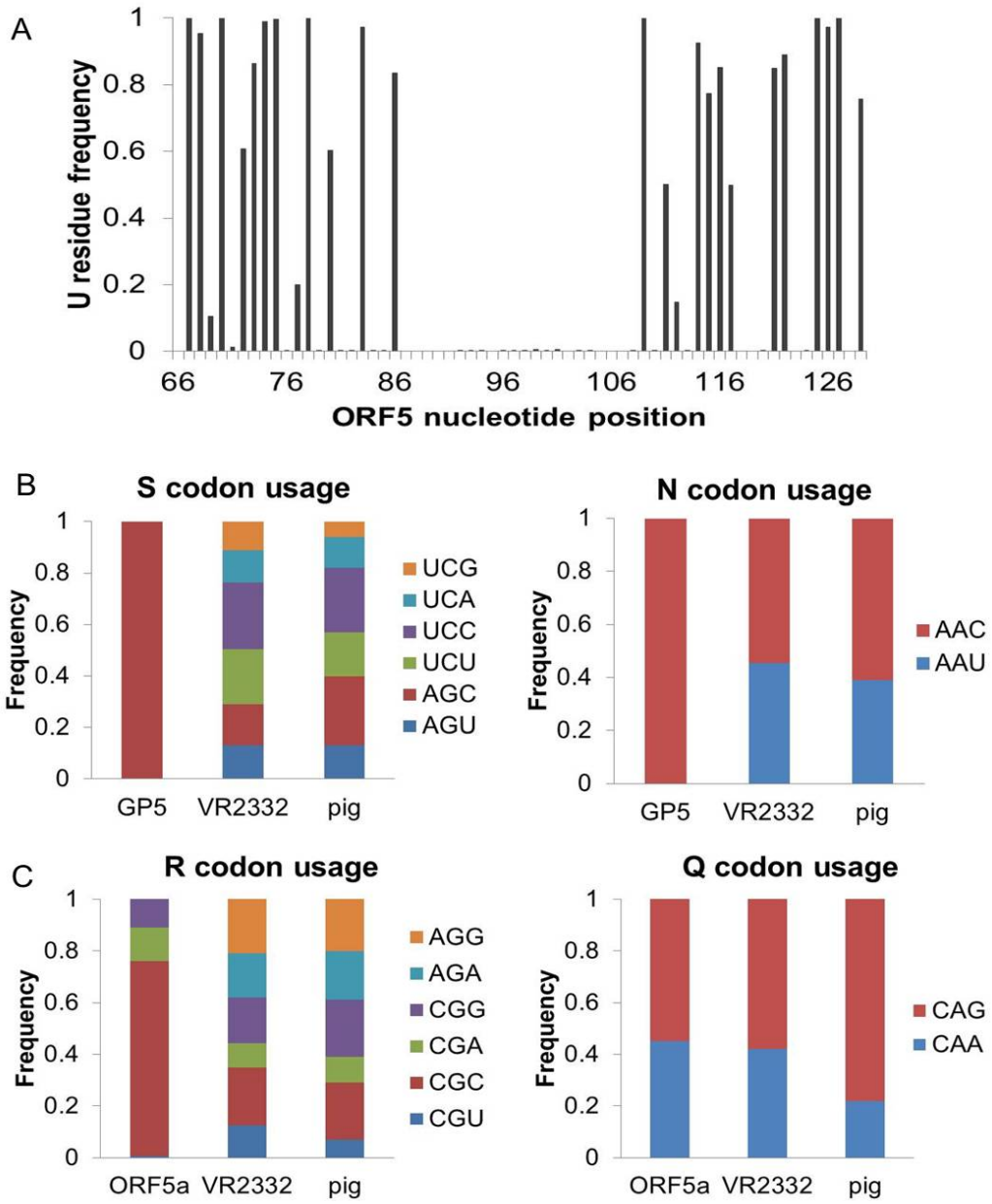


Figure 3.5 Uracil desert in the GP5/ORF5a protein overlap.

A) Frequency of uracil residues at ORF5 nucleotide positions 66-126 for 3466 sequences. B) Codon usage for GP5 N and S residues in the hypervariable glycosylation region lacking uracil. Relative codon usage for the entire coding sequence of PRRSV VR2332 and of 3000 pig coding sequences is shown for comparison. c) Codon usage for ORF5a protein R and Q residues in the uracil desert.

Chapter 4 Characterization of broadly neutralizing antibodies against PRRSV

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Broadly neutralizing antibodies against the rapidly evolving Porcine reproductive and respiratory syndrome virus.

Sally R Robinson, Juan Li, Eric A Nelson, Michael P Murtaugh

4.1 Introduction

Neutralizing antibodies are a critical part of the immune armory for defense against viruses, and the mechanism by which many effective vaccines work to protect against viral infections. However, infections by rapidly evolving and genetically diverse viruses are often characterized by ineffective neutralizing antibody responses. Immune responses to rapidly evolving RNA viruses, such as influenza, hepatitis C, and human immunodeficiency virus (HIV), may show neutralizing profiles that are easily evaded, or not cross-protective (Liao et al., 2013; von Hahn et al., 2007; Walker et al., 2009; Wei et al., 2003), and ineffective responses leading to chronic infection or superinfection are comparatively weakly neutralizing (Pestka et al., 2007; Smith et al., 2006). Additionally, antibody-dependent enhancement (ADE) of infection is related to ineffective cross-protection in some viral infections such as Dengue and influenza viruses (Halstead, 2003; Vincent et al., 2012). Development of effective vaccines against rapidly evolving and diverse RNA viruses, for which suboptimal neutralizing antibody responses to infection are common, has proven to be incredibly challenging. Standard vaccine approaches have had limited success, prompting investigation of alternative methods designed to induce more effective host responses. However, progress is incremental for human and animal medicine, perhaps partly due to insufficient viral disease models.

Porcine reproductive and respiratory syndrome virus (PRRSV) is a naturally-occurring, rapidly evolving, RNA virus in the order *Nidovirales*, family *Arteriviridae*, genus *Arterivirus*. PRRSV comprises two genotypic families, type 1 and type 2, that cause a prolonged infection of pigs, and show variable ability to induce neutralizing antibody responses (Martinez-Lobo et al., 2011). The two distinct genotypes share less than 60% nucleotide sequence similarity, and are only weakly immunologically cross-reactive (Murtaugh et al., 1995; Nelsen et al., 1999; Wensvoort et al., 1992). Both PRRSV genotypes have been expanding continuously since their discovery around 1990, and they cause the most significant disease of pigs worldwide (Murtaugh et al., 2010). The prevailing view of immunity to PRRSV is characterized by delayed and ineffectual production of neutralizing antibodies lacking the cross-reactivity necessary for vaccine efficacy. At present, neither vaccines nor exposure through serum inoculation give broadly consistent, predictable protection.

Here, we found high-titer, anti-PRRSV neutralizing activity in sows from two unrelated commercial herds that were exposed to different circulating field strains of PRRSV, either through ongoing virulent virus inoculation or repeated disease outbreaks. Serum neutralized infection of macrophages, the target cell of PRRSV infection, with distantly-related viral strains such as PRRSV VR2332, first isolated in 1989, and Lelystad virus, first isolated in 1990 (Benfield et al., 1992; Collins et al., 1992; Wensvoort et al., 1991). Characterization of broadly anti-PRRSV neutralizing antibodies provides a useful model to further elucidate molecular mechanisms that are exploited in animals against genetically diverse RNA viruses, and to provide insights into ways that more effective human and animal vaccines can be generated.

4.2 Materials and methods

Animals

Serum samples were collected in 2012 from two unrelated commercial sow herds in Minnesota, USA. Both herds had multiple exposures to PRRSV, through natural infection or inoculation of serum containing virulent virus previously isolated on the farm. Herd 1 had a history of disease outbreaks occurring every year from 2006 to 2012, with circulating PRRSV field strains. The herd practiced serum inoculation, exposing incoming PRRSV-negative animals to endogenous PRRSV strains prior to introduction to the herd. Serum samples were collected from 20 cull sows; these animals had been in the herd for a number of years during multiple PRRS virus exposures. However, the outcome of exposure in terms of disease and infection status for individual animals was not known. Five of the twenty sows sampled from this farm were selected that represented a range of titers present in the entire group. Large volumes of serum were collected from these 5 sows for further analysis of neutralizing activity.

Herd 2 was repeatedly exposed to a virulent isolate in an effort to protect against PRRSV outbreaks. Serum samples were collected from 14 breeding-age sows. Detailed outbreak history was not available for this herd, nor were the ages or number of virus exposures or disease outcome for individual animals.

Additional sera were collected from pigs 202 days after experimental infection with PRRSV strain VR2332, and from a commercial sow having a high viral neutralizing titer.

Cell culture

Porcine alveolar macrophages (PAM) were harvested by lung lavage of 12 week old pigs. Briefly, lavage was performed with phosphate buffered saline (PBS) containing 50 ug/ml gentamycin, and when required, red blood cells were lysed in sterile water. Cells were passed through a 70 um

filter, resuspended in 40% RPMI media, 50% fetal bovine serum (FBS) and 10% dimethyl sulfoxide (Fisher Scientific, Pittsburgh PA) for cryopreservation. PAMs were cultured in RPMI medium supplemented with sodium pyruvate, gentamycin sulfate (Cellgro Mediatech, Manassas VA), non-essential amino acids, HEPES buffer, and 5% FBS (Sigma, St. Louis MO). PAM batches were screened for PRRSV permissivity by flow cytometric analysis. Percentage of cells infected were detected by an antibody directed against PRRSV nucleocapsid compared to uninfected control cells, as detailed below under 'detection of PRRSV infected cells by flow cytometry'. Cells in this study showed 35 to 40% infection.

Simian renal epithelial MARC 145 cells were cultured in MEM medium (Gibco, Grand Island NY) supplemented with sodium bicarbonate, non-essential amino acids, HEPES buffer (Sigma, St. Louis MO), and gentamycin sulfate (Cellgro Mediatech, Manassas VA) with 10% FBS (Sigma, St. Louis MO).

PRRSV isolates

North American (type 2) PRRSV isolates VR2332 and MN184 were propagated in MARC 145 cells. Infectious titers ($TCID_{50}$) were determined for each isolate by evaluation of cytopathic effect on MARC 145 cells in 96 well plates and calculated as described (Reed and Muench, 1938).

PRRSV open reading frame (ORF) 5 nucleotide sequences coding for envelope glycoprotein 5, were available for the most recent (within the past 15 months) virulent herd 1 outbreak strains, MN0111 and MN1211. Virus isolates were not obtained. ORF5 sequences from these samples, along with diverse Type 2 PRRSV isolates including lab-adapted VR2332, vaccine strains, field isolates including MN184, European (Type 1) PRRSV isolate Lelystad Virus (LV), and Type 1 field isolates SD 01-08 and 03-15, were aligned in CLC Sequence Viewer version 6.8.2 (CLC

bio/Qiagen, Cambridge, MA). Aligned ORF5 sequences were subjected to phylogenetic tree construction in Geneious R6 version 6.1.7 (Biomatters Ltd., Auckland, New Zealand) Tree Builder using the Tamura-Nei neighbor-joining method with 100 bootstrap resamplings and no outgroup to create a consensus tree. Percent homology between strains was also compared in Geneious R6.

ELISA-based serum neutralization assay

The PRRSV serum neutralization (SN) assay was performed essentially as described (Li and Murtaugh, 2012; Robinson et al., 2013). Briefly, 96-well plates were treated with 25 ug/ml polyethylenimine (Sigma-Aldrich, St. Louis, MO) and PAMs were seeded at 6 x 10^4 cells/well, or MARC 145 cells seeded at 6 x 10^3 cells/well, and cultured for 48 hours. Serum samples were heat inactivated (56°C, 30 min) and serially diluted in culture media without FBS. Diluted sera were mixed with an equal volume of 5 x 10^4 $TCID_{50}$/ml PRRSV in non-adherent 96-well plates and incubated for 1 hour (37°C, 5% CO_2). Serum-virus mixtures containing 5000 $TCID_{50}$ were transferred to duplicate wells of cells for adsorption for 1 h at 37°C. Control wells containing cells only (no serum, no virus), and virus only (no serum) were included on each plate. Cells were washed in PBS, media added, and further incubated for 23 h. Cells were fixed in 3.7% formaldehyde (Sigma-Aldrich, St. Louis, MO) in PBS, washed, permeabilized in 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) in PBS, and blocked in 5% nonfat dry milk, pH 9.6, in PBS for 1 hour, at 37°C. PRRSV monoclonal antibody (mAb) against nucleocapsid protein, SR30-A (Rural Technologies, Brookings SD), was diluted 1:10,000 in 5% nonfat dry milk, pH 7.4, in PBS and added to cells at 100 ul/well for 1 hour at 37°C. HRP-conjugated goat-anti-mouse IgG (H+L) (Bethyl Laboratories Inc., Montgomery TX) 1:10,000 dilution was added at 100 ul/well for 1 hour at 37°C. Color was developed with 100 µl of TMB peroxidase substrate (Kirkegaard &

Perry Laboratories, Gaithersburg MD) for 15 min. Reactions were stopped with 100µl of 1 M phosphoric acid and plates read at 450 nm in a ThermoMax Microplate Reader (Molecular Devices, Sunnyvale CA).

Percent inhibition of viral infection at each dilution was compared with virus only controls after background subtraction of absorbance from uninfected cells. Fifty percent neutralization titer was defined as reciprocal of the highest serum dilution for which 50% inhibition of infection was reached.

Fluorescent focus neutralization (FFN) assay

FFN assays were performed with heat inactivated serum samples diluted in MEM containing 2% horse serum. Virus isolates were added in duplicate to test and virus control wells (100 µl/well at ~100-200 FFU/100 µl) providing a serum dilution series of 1:4 to 1:512. Plates were incubated 1 h at 37°C, transferred to corresponding wells of confluent MARC 145 cells and incubated 24 h at 37°C. Cells were fixed with 80% acetone for 15 min and stained with SDOW-17 or SR-30 FITC conjugated mAb (Rural Technologies, Brookings SD) for 30 min. Fluorescent foci were counted and endpoints determined as the highest serum dilution providing a 90% or greater reduction in infection foci relative to virus only controls.

Detection of PRRSV-infected cells by flow cytometry

To screen for PRRSV permissivity, PAMs were cultured in 5 ml polystyrene snap cap FACS tubes (at 2 x 10^5 cells/tube), and infected or not with PRRSV strain MN184 at a multiplicity of infection of 0.3 for one h. Cells were pelleted at 1200 rpm in an Allegra X-15R centrifuge (Beckman Coulter) for 5 minutes and washed in 1 ml of PBS. After replacing with complete media, cells were incubated for a further 23 h. Cells were washed, stained with 100 µl of 1:500

mouse anti-pig CD172a antibody (AbD Serotec, Raleigh, NC) on ice for 30 minutes, washed, and stained with 100 µl of a 1:200 dilution of APC-conjugated goat anti-mouse IgG antibody (Biolegend) on ice for 30 minutes. After a final wash, cells were fixed and permeabilized in Cytofix/Cytoperm (BD Biosciences, San Diego, CA) then washed twice in Perm/Wash buffer (BD Biosciences, San Diego, CA). After the second wash cells were stained with FITC-conjugated anti-PRRSV nucleocapsid antibody SR30F (Rural Technologies, Brookings, SD) for 30 minutes.

For flow cytometric analysis, 10,000 events were counted in each tube, and compensation calculated based on single-stained controls. Data were analyzed using Flow Jo 10.0.0r2 (FlowJo, Ashland, OR). Permissivity to PRRSV was calculated as percentage of CD172a positive cells that stained positive for nucleocapsid.

Comparison of the ELISA based neutralizing assay with flow cytometry

MARC 145 cells were cultured in a 96-well duplicate plate set up alongside the ELISA-based neutralizing assay. Twenty-four hours after infection, cells were washed and lifted with 150 µl per well of Accumax (Sigma-Aldrich, St. Louis, MO). Cells were then transferred to a non-adherent round bottomed 96 well plate, and pelleted for 5 minutes at 1200 rpm. Cells were resuspended in fixation and permeabilization solution as above, washed twice in Perm/Wash buffer, and stained with FITC-conjugated anti-PRRSV nucleocapsid antibody SR30F (Rural Technologies, Brookings, SD) as described above. Cells were washed, resuspended in Perm/Wash buffer and transferred to FACS tubes. For each tube of cells, 10,000 events were counted. Data were analyzed using Flow Jo. Percent of PRRSV infected cells was determined based on the number of nucleocapsid positive events in the counted population.

Total immunoglobulin isolation by saturated ammonium sulfate precipitation

Saturated ammonium sulfate (Sigma-Aldrich, St. Louis, MO) was slowly added to serum samples with constant mixing to a final concentration of 40%, stirred for 45 min, and centrifuged at 10,000 rpm for 10 min. The precipitation step was repeated twice, with pellets being dissolved in PBS to the original volume. Antibody concentration was measured using a porcine IgG ELISA quantitation kit (Bethyl Laboratories, Inc., Montgomery TX). Samples in 5% β-mercaptoethanol were subjected to denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 10 to 20% gradient Tris-HCl Ready Gels (Bio-Rad Laboratories, Hercules CA) to evaluate purity. Bands were visualized with Coomassie Brilliant Blue staining and destaining in 40% methanol, 20% acetic acid.

4.3 Results

Serum from sows in two independent herds exposed to virulent field strains of PRRSV had unexpectedly high virus neutralization activity against PRRSV strain VR2332 in an ELISA-based neutralization assay on MARC 145 cells. At the lowest serum dilution of 1/4, herd 1 had a mean inhibition of infection of 82% (range 55-100). Herd 2 had a mean of 76% neutralization (range 44-94%) at a 1/3 dilution. Neutralizing activity was dose-dependent and shape of the curves was similar for both herds (Figure 4.1A). While average maximal neutralization was equivalent between herds, herd 1 had a mean reciprocal 50% neutralization titer of 512, the highest individual titer being 2187, and herd 2 had a mean reciprocal 50% titer of 81. Sera from five of the sows from herd 1 were utilized for further analysis. The individual reciprocal 50% neutralizing titers of these sows ranged from 16 to 1024 (Figure 4.1B). All individual sera tested at the minimum dilution from herd 1 (n=20) showed neutralizing activity greater than 50%, as did all but one of the 14 sows from herd 2.

Macrophages are the naturally permissive host cell for PRRSV, but differ from MARC 145 cells in expression of surface proteins implicated in infection. Therefore, virus-neutralizing activity sera of 5 sows in herd 1 were evaluated on macrophages for comparison to MARC 145 cells. Cell-culture adapted PRRSV strain VR2332 has higher growth proficiency on MARC 145 cells than on macrophages (Figure 4.2A). However, virus neutralizing activity was determined by comparing inhibition by serum with virus only control wells and hence normalized these growth differences. The neutralization profiles were similar, with equivalent slopes, suggesting that the mechanism of neutralization was the same in both cell types (Figure 4.2B). Also, there was no evidence of antibody-dependent enhancement of infection at high dilutions in either cell type. PRRSV strain VR2332 is genetically distinct from the herd 1 field isolates MN0111 and MN1211, with ORF5 nucleotide sequence differing by about 15% (Figure 4.3A). Comparison of neutralizing activity of the 5 sow sera shown in Figure 4.1B on strain VR2332 and strain MN184, which is more closely related to MN0111 (94.5% similarity), revealed that cross-neutralizing activity was present against both viruses, with higher activity observed against MN184 (Figure 4.3B).

To further investigate breadth of neutralizing activity, herd 1 sera and herd 2 sera were tested against the genotype 1 strain SD 01-08 (SDEU). Neutralizing activity of herd 1 sera were surprisingly higher against the type 1 strain that type 2 VR2332 (Figure 4.4A), whereas herd 2 sera had similar neutralizing profiles against both strains (Figure 4.4B).

To verify that the presence of high titered neutralizing activity was independent of the assay method, the 5 sera shown in Figure 1B were compared against PRRSV strain VR2332 in both the widely used fluorescent-focus neutralization (FFN) assay, and our FACS-based assay. Correlation of neutralizing titers against VR2332 on MARC 145 cells between the ELISA-based test and the FFN assay was 0.89 (Figure 4.5A). Likewise, neutralizing activity was highly correlated between the ELISA-based method, which measures the average response all cells in an entire well, and

FACS, which is a digital assessment of infection status in 10,000 cells of a tube (Figure 4.5B and C).

Broadly neutralizing antibodies were detected by FFN in all 5 serum samples against a panel of 9 divergent viruses shown in Figure 3A (Table 4.1). Ninety percent neutralization titers were consistently >1/16. Moreover, the highest neutralizing titers were obtained against type 1 PRRSV isolates SD 03-15, SD 01-08 and Lelystad virus (Table 4.1).

PRRSV-neutralizing serum was further characterized to establish that the activity observed in the ELISA-based neutralizing assay was due to antibody-mediated viral neutralization. Precision and inter-assay variation were assessed by comparison of a positive control serum across eight replicate plates. Inter-assay coefficient of variation in the neutralization profile was low across replicates, and increased with serum dilution (Figure 4.6A). Neutralization linearity was observed over a multiplicity of infection (MOI) range of 0.01 to 1.0. Below an MOI of 0.01 the level of infection in positive controls was below the background absorbance of uninfected cells (Figure 4.6B). To determine if neutralization was affected by the amount of virus in the assay, the test was performed over a range of virus concentrations. The proportion of PRRSV infectivity that was inhibited was relatively constant across a 100-fold viral concentration range (Figure 4.6C). This indicates that, within these limits, neutralization conforms to the percentage law, consistent with antibody-mediated neutralization (Andrewes and Elford, 1933). As the virus was diluted below an MOI of 0.01, absorbance values fell outside the sensitivity range of the assay and neutralization could not be assessed (Figure 4.6B, D). To verify that virus-neutralizing activity was due to antibodies, immunoglobulins were isolated and examined for virus-neutralizing activity. Specific virus-neutralizing activity was defined as greater than 30% inhibition of infection at the highest serum concentrations, based on characterization of variation in neutralizing activity from PRRSV-negative serum (data not shown). Ammonium sulfate precipitation removed >99.8% of immunoglobulins from serum based on ELISA, and >80% of

recovered protein was IgG as determined by SDS-polyacrylamide gel electrophoresis (data not shown). Neutralizing activity was present in the immunoglobulin fraction but was absent in the Ig-depleted fraction of a seropositive sow, and all fractions from a seronegative sow were negative (Figure 4.7).

4.4 Discussion

Broadly neutralizing antibodies are central to effective control of genetically variable pathogens and prevention by vaccines against future infection. Inability to elicit broadly neutralizing antibodies is a major contributor to the maintenance of many microbial and parasitic infections in human and animal populations. PRRSV is an example of a rapidly evolving pathogen, many of which are RNA viruses, in which neutralizing antibody responses fail to adequately control and prevent infection due to low titers, insufficiently broad neutralization, or both. In general, PRRSV is difficult to comprehend and control immunologically; there are inconsistencies in our knowledge of PRRSV immunity and a lack of understanding of what constitutes a protective immune response (reviewed in (Murtaugh and Genzow, 2011)). Factors such as extensive viral genetic and antigenic diversity, large populations of animals in regions of dense swine production that facilitate spread of virus, persistence of virus for extended periods in pigs during which it can be transmitted to susceptible animals, and inability of current vaccines to prevent infection in populations all contribute to the problem of PRRSV spread and persistence. Pigs generate a well-documented humoral immune response against PRRSV (Molina et al., 2008; Mulupuri et al., 2008; Nelson et al., 1994). However the slow kinetics, small magnitude and limited breadth of protection observed from the neutralizing antibody response have led to the belief that it is not an important factor, nor realistic approach, for achieving widespread protection against PRRSV (reviewed in (Darwich et al., 2010; Murtaugh and Genzow, 2011)).

Evidence of homologous protection against PRRSV from passive transfer experiments demonstrated proof of principle that serum neutralizing antibodies protect against PRRSV infection in a dose-dependent manner under optimal, controlled conditions (Lopez et al., 2007; Osorio et al., 2002). In an effort to understand the mechanisms of antibody-mediated neutralization of PRRSV, multiple studies have focused on identifying neutralizing epitopes (reviewed in (Darwich et al., 2010; Murtaugh and Genzow, 2011)). Linear epitopes have been reported to exist on glycoproteins 3, 4, 5, and membrane (M) protein, however findings are inconsistent and generally do not evaluate cross-neutralization (reviewed in (Darwich et al., 2010; Murtaugh and Genzow, 2011)). Protection that has been associated with the presence of neutralizing antibodies in short-term experimental studies occurs at low levels, often at or near the reported cut-off titer of 1/8 (Islam et al., 2014; Li et al., 2014). Nevertheless, Lopez *et. al*. (2007) demonstrated that neutralizing antibody titers of 1/8 were sufficient to protect pigs against viremia with homologous virus (Lopez et al., 2007).

The data here show that pigs, the mammalian host for PRRSV, are able to achieve broadly neutralizing antibody activity that, remarkably, is highly effective against type 1 as well as type 2 strains. A second significant feature of the findings is the high titer of anti-PRRSV neutralizing antibodies. High titers were observed in all three assays, i.e. FFN, ELISA and FACS, and the FFN titer determination at 90% inhibition was highly correlated with the titer inhibiting 50% of infection determined by ELISA or FACS. While 50% titers are numerically higher than 90% titers, the FFN results shown in Table 4.1 are exceptional. Previously, reported neutralizing titers using FFN-type assays were consistently low (≤16), or undetectable (Binjawadagi et al., 2014; Kim et al., 2007; Li et al., 2014; Martinez-Lobo et al., 2011; Molina et al., 2008).

The high infection dose (~5,000 $TCID_{50}$) of the ELISA-based assay relative to FFN (~100-200 $TCID_{50}$) is advantageous since it enables optimization of key parameters, including MOI, and testing of the percentage law. The amount of virus in the ELISA corresponds to an MOI of

approximately 0.15, which is in the linear range of the ELISA, as shown in Figure 4.6. The 50% endpoint for neutralization titers was used in place of 90% reduction in FFN units to gain information about antibody reactivity independently of antigen concentration. Virus-antibody interactions can be modeled as antigen-antibody interactions that conform to the law of mass action as a reaction approaches equilibrium (Karush, 1978; Klasse and Sattentau, 2002; Reverberi and Reverberi, 2007; Tyrrell, 1953). Since neutralized and non-neutralized virus is in equilibrium at 50% inhibition of infectivity, mathematical modeling can be used to assess functional qualities of antibodies and antisera that have viral neutralizing activity, as has been shown in HIV, influenza virus, human adenovirus, equine infectious anemia virus, chimeric SIV/HIV, and Newcastle disease virus infections (Klasse and Moore, 1996; McEwan et al., 2012; Nishimura et al., 2002; Schwartz and Smith, 2014; Shingai et al., 2014; To et al., 2012; Tyrrell, 1953; Willey et al., 2010). Characterization of neutralization affinity, reaction kinetics, and avidity cannot be determined under more stringent conditions such as at 90% inhibition which are not at equilibrium. More stringent neutralization readouts may have value for diagnostic purposes or studies in which there is background cross-reactivity such as with dengue virus (Roehrig et al., 2008). However, interfering responses have not been reported to affect PRRSV neutralization assays.

The breadth of neutralization observed here was unexpected due to the widespread perception that PRRSV elicits specific immunity to the same or equivalent viruses, but not to unrelated viruses (Kim et al., 2007; Martinez-Lobo et al., 2011). Consistent with our findings, a recent study demonstrated PRRSV cross-neutralization of a genotype 1 virus with genotype 2 antisera (Binjawadagi et al., 2014). Even so, it was unexpected to find a higher degree of neutralizing activity against genotype 1 viruses compared with genotype 2 for herd 1.

Mechanisms by which high titers of broadly neutralizing antibodies to PRRSV are produced are not known, but may involve multiple exposures to the same or different virus isolates over time.

Consistent with this idea, an increased breadth of neutralization was observed in animals exposed first to a PRRSV vaccine strain followed by heterologous virus challenge compared to those receiving a homologous challenge virus (Li et al., 2014). Additionally, a longer duration of viremia was associated with increased breadth of neutralizing antibodies in a large experimental study using a single PRRSV isolate (Islam et al., 2014). Herd 1 in this study had experienced virulent outbreaks, with two independent isolates, in the previous 15 months, and the second herd was repeatedly exposed to a virulent isolate in an effort to protect against PRRSV outbreaks. It remains to be determined whether time, duration of exposure to antigen, multiple exposures to virus over time, or exposure to genetically or antigenically different variants are critical factors for development of neutralizing breadth against PRRSV. More detailed information relating to virus exposure and clinical outcome of individual animals may help answer some of these questions in the future.

Age by itself is not sufficient for production of high antibody titers, as increased age does not equate to increased SN titers ((Klinge et al., 2009), and unpublished data). Recently, broadly-neutralizing anti-HIV antibodies have been shown to appear after a period of years, during which mutations accrue throughout the molecule to increase affinity (Liao et al., 2013; Walker et al., 2009). PRRSV infection is prolonged although not truly persistent (Murtaugh and Genzow, 2011). Hence, prolonged exposures to antigen in lymphoid tissues or from repeated infection may stimulate affinity maturation through hypersomatic mutation of antibodies directed against conserved neutralization epitopes. In the swine populations sampled for this study, high-titered, broadly neutralizing antibodies were frequently observed, whereas highly active neutralizing antibodies found in humans are rare (Simek et al., 2009). Elucidation of the mechanisms of broadly neutralizing antibody production in swine will be an important area of future investigation, and may provide insights for more efficient enhancement in humans.

Observation of swine with high-titered cross-neutralizing activity has great potential significance for understanding immunity and protection against PRRSV, and establishes a basis for more detailed studies aimed at understanding conditions and mechanisms for their development. At this time, the role that serum neutralizing antibodies play in prevention or protection from re-infection with PRRSV in the field is not clear.

Interpretation of neutralization assays for PRRSV is complicated by substantial variability in porcine macrophage permissiveness, and inefficient culture of field isolates on MARC 145 cells (Bautista et al., 1993; de Abin et al., 2009; Kim et al., 1993; Ludemann and Lager, 2010). However, MARC 145 cells are preferred for neutralization assays due to reproducibility and ease of maintenance. Infection of MARC 145 cells and macrophages occurs by engagement of CD163; an additional role for sialoadhesin (CD169) in macrophages is controversial (Prather et al., 2013; Van Gorp et al., 2008; Welch and Calvert, 2010). The data show, for viral isolates which grow on both cell types, that neutralization characteristics are equivalent among sera tested. This result indicates that the neutralization mechanisms and, by inference, the infection pathway, is the same. We conclude that MARC 145 cells are a valid model for examination of viral neutralization mechanisms.

The findings also show no evidence that PRRSV causes antibody-dependent enhancement of infection (Cancel-Tirado et al., 2004; Yoon et al., 1996; Yoon et al., 1997), since increased infection was never observed in macrophages or MARC 145 cells under the conditions of the study. ADE, known primarily from dengue virus pathogenesis, where subsequent infection by a different serotype may cause severe, life-threatening disease, occurs when weakly neutralizing antibodies bring infectious virus to macrophages via Fc receptor binding, thus increasing uptake (Halstead, 2003). Macrophages are permissive to a range of viruses, many of whose infections are prolonged or persistent in the host. ADE, although reported for a number of viruses *in vitro*, is not a common manifestation of natural infection. While ADE has been reported to occur with

PRRSV, there is no evidence to suggest occurrence in the field (reviewed in (Murtaugh and Genzow, 2011)).

The presence of PRRSV cross-neutralizing activity in commercial sows raises two distinct possibilities, each of which has significant implications for future protection strategies. First, it is possible that generation of relatively low cross-neutralizing antibody titers from multiple exposures to different strains combine to give a broader repertoire of neutralizing activity against diverse strains. Alternatively, individual broadly reactive antibody molecules may be generated over time through prolonged antigen exposure and affinity maturation. In the latter case, it suggests that conserved cross-neutralizable epitopes exist broadly on diverse PRRSV.

PRRSV envelope glycoprotein (GP) 5 and membrane (M) protein, the most abundant envelope proteins, and focus of many earlier studies, interact with macrophage CD169 to mediate attachment (reviewed in (Van Breedam et al., 2010)). More recently, it has been shown that trimers of minor envelope glycoproteins 2, 3 and 4 are critical for permissiveness of cells for viral replication, mediated by interaction with CD163 (Calvert et al., 2007; Das et al., 2010). Conserved epitopes, critical for potent and broad neutralization of other diverse RNA viruses such as HIV and influenza virus, reside in motifs important for binding to host cell receptors (reviewed in (Burton et al., 2012)). Similarly, it is possible that conserved neutralization epitopes may exist on the PRRSV GP2/3/4 trimer in regions critical for CD163 interaction, whereas GP5/M interaction with CD169 is unlikely to affect PRRSV infection *in vivo* (Prather et al., 2013; Van Gorp et al., 2008; Welch and Calvert, 2010). Development of B cell isolation and high-throughput screening methods for neutralizing antibody production (Walker et al., 2009), and the ELISA-based PRRSV neutralizing tests described here will facilitate further characterization of viral infection and neutralization mechanisms. Elucidation of broadly effective antibody mechanisms of PRRSV neutralization is critical to development of more efficacious control of PRRS in pigs.

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* Juan Li conducted immunoglobulin precipitation experiments. Eric Nelson performed fluorescent focus neutralization assays.

Table 4.1 Fluorescent focus neutralization (FFN) cross-reactivity against diverse PRRSV[a]

Genotype	Strain	GenBank accession	Neutralizing titer[b]	ORF5 homology (%)[c]
2	VR2332	U87392	17±1.5	84.7
	MN184	EF442777	56±1.3	94.5
	HV-1	DQ345767	18±1.8	84.7
	SD-23983	U66384	32±2.0	85.1
	PRIMEPAC	AF066384	30±2.1	85.7
	Ingelvac ATP	AY656991	37±2.8	84.9
1	SD03-15 EU	AY395076	84±2.4	62.5
	SD01-08 EU	AY395080	97±1.8	62.0
	Lelystad virus	M96262	84±2.1	61.8

[a] FFN assays carried out at South Dakota State University by EAN.
[b] Reciprocal of the geometric mean titer that neutralized 90% of infectivity (n=5 sows) ± standard deviation. Duplicate tests on each serum sample were run independently on different plates.
[c] Percent homology to herd 1 outbreak strain MN0111.

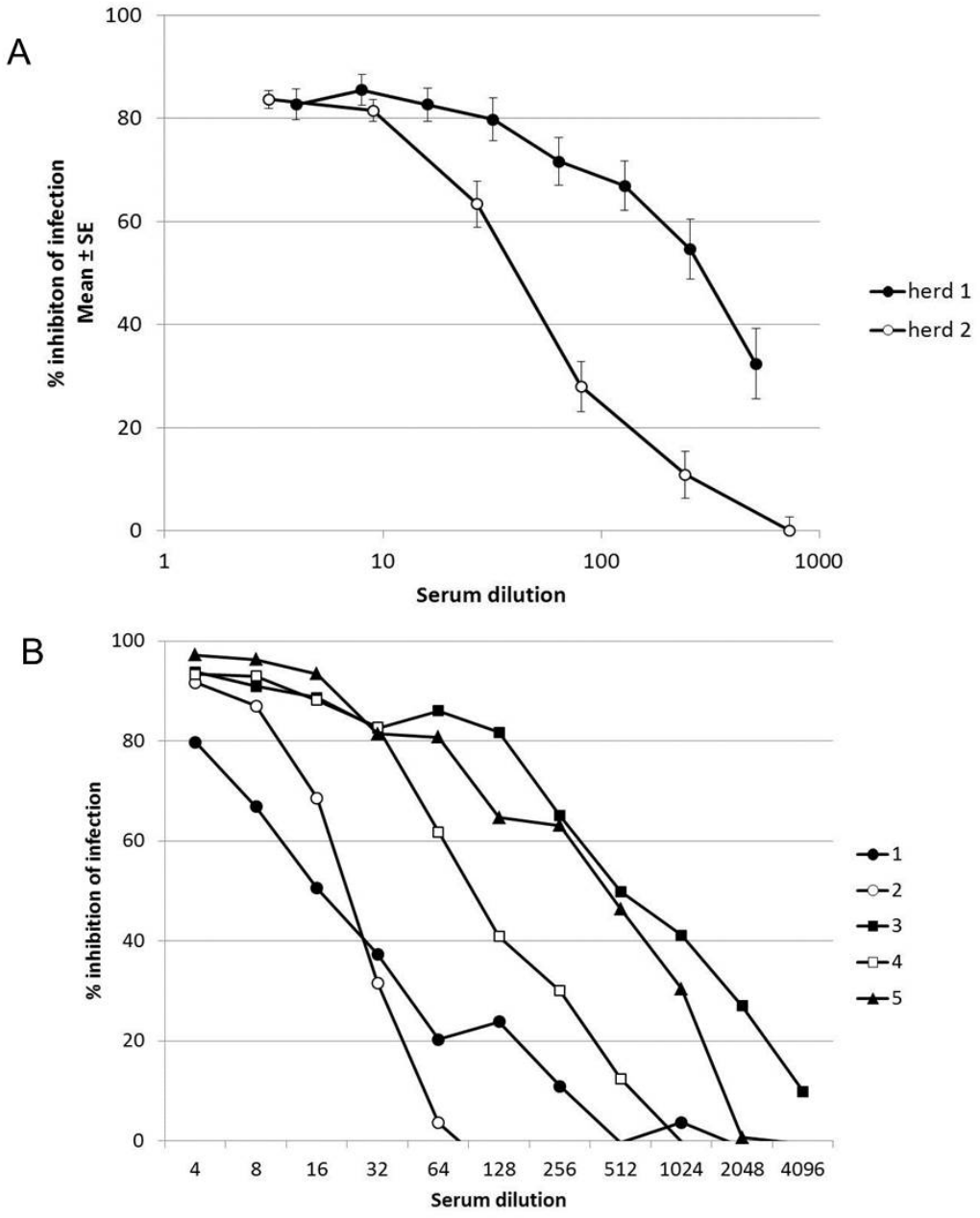

Figure 4.1 Serum neutralization profiles of sows sampled from herds exposed to virulent circulating field strains of PRRSV.

(A) Serum neutralization of PRRSV VR2332 on MARC 145 cells. Sample size from herd 1 n=20, and herd 2 n=14 sows. Data are expressed as the mean percentage inhibition of viral infection ± standard error. (B) Individual neutralizing profiles of 5 herd 1 sow sera.

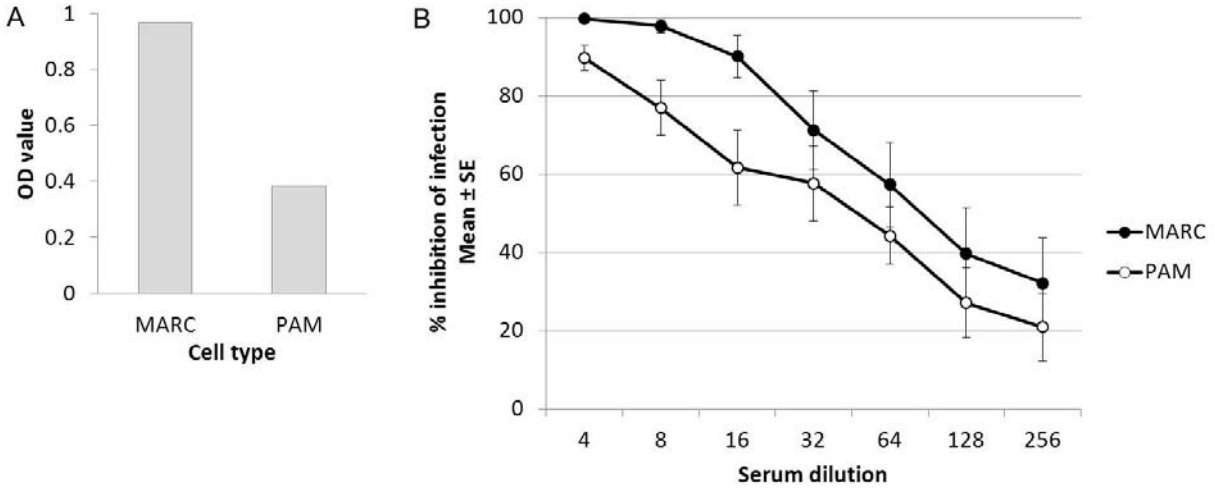

Figure 4.2 Comparison of virus neutralizing activity on MARC 145 cells and porcine alveolar macrophages.

(A) Comparative growth of PRRSV VR2332 on MARC and PAM cells, measured by detection of PRRSV nucleocapsid protein by ELISA in the serum neutralization assay. Data are the absorbance at 450 nm (OD value) of virus only control wells minus background absorbance of uninfected cells. (B) Neutralizing activity of 5 herd 1 sow sera against PRRSV VR2332 cultured on MARC 145 (MARC) cells or alveolar macrophages (PAM). Data are expressed as the mean percentage inhibition of viral infection ± standard error.

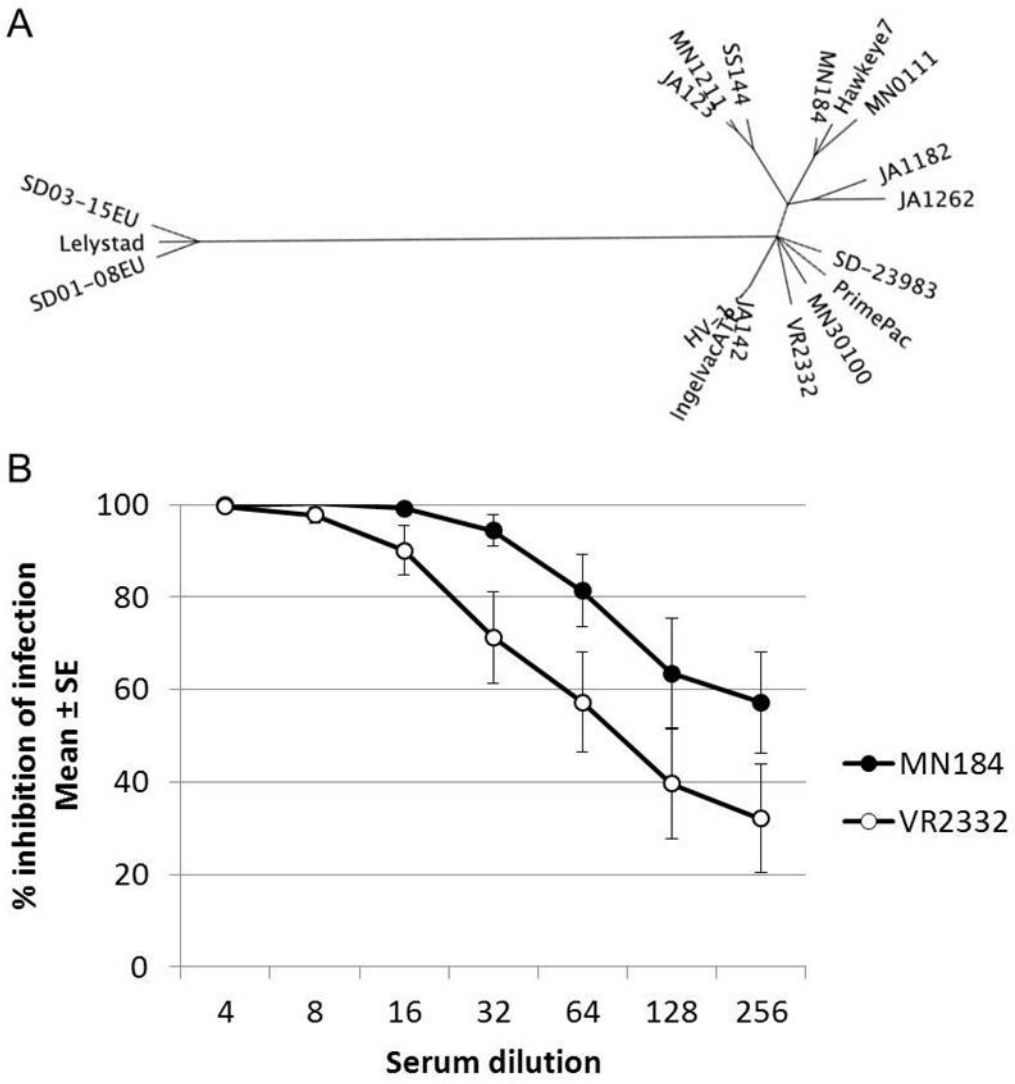

Figure 4.3 Broadly cross-reactive PRRSV neutralization activity.

(A) Phylogenetic tree based on PRRSV ORF5 nucleotide sequences. MN0111 and MN1211 sequences were from outbreaks in herd 1. Pairwise nucleotide similarities are 61% for LV-MN1211, 85% for VR2332-MN1211, and 95% for MN184. (B) Titration of cross-neutralizing activity against PRRSV strain MN184 and VR2332 on MARC 145 cells. Data are expressed as the mean percentage inhibition of viral infection ± standard error.

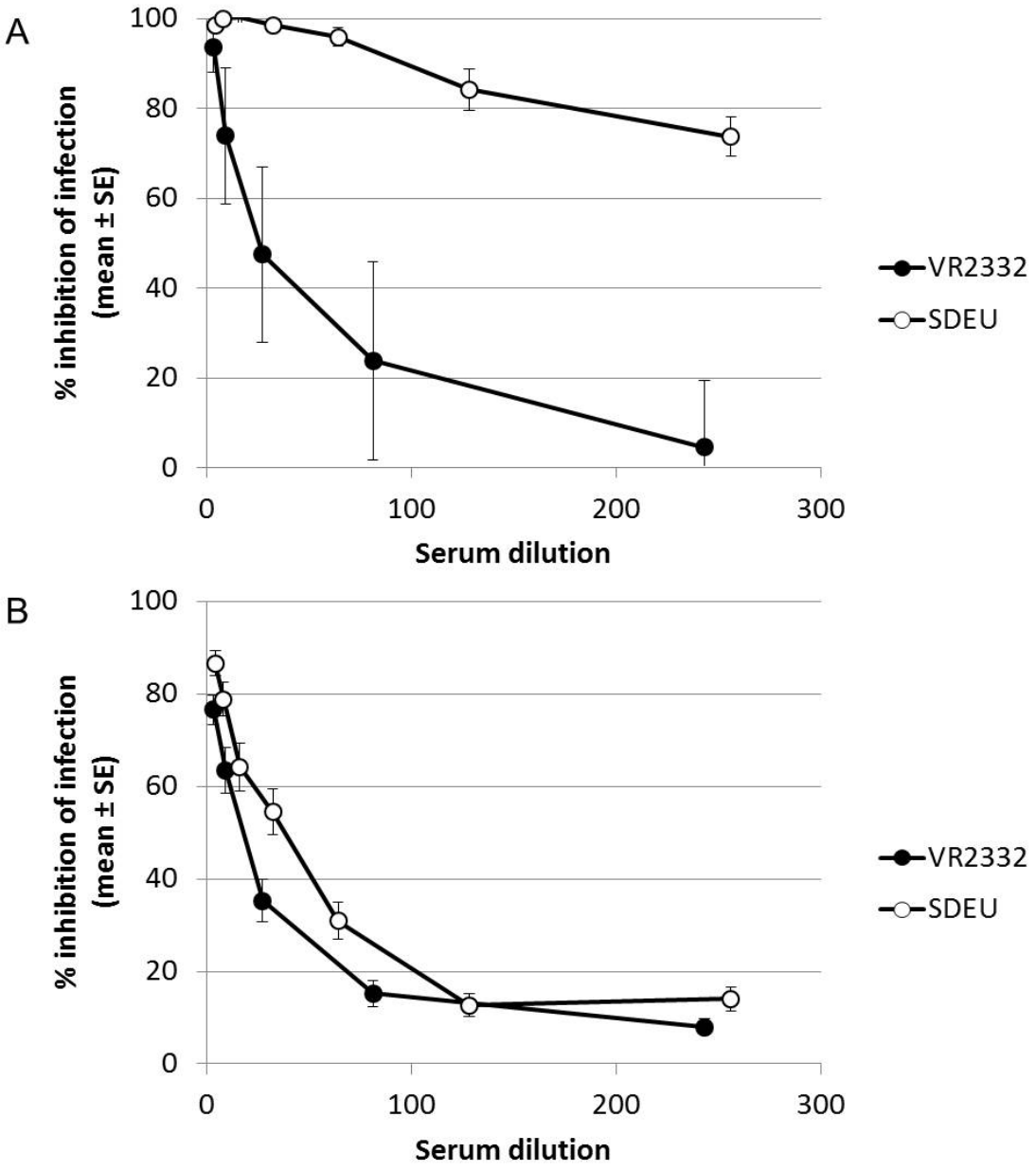

Figure 4.4 Titration of cross-genotypic neutralizing activity against genotype 1 strain SD 01-08 (SDEU), and genotype 2 strain VR2332 on MARC 145 cells.

(A) Herd 1 (n=20). (B) Herd 2 (n=14). Data are expressed as mean percentage inhibition of viral infection ± standard error.

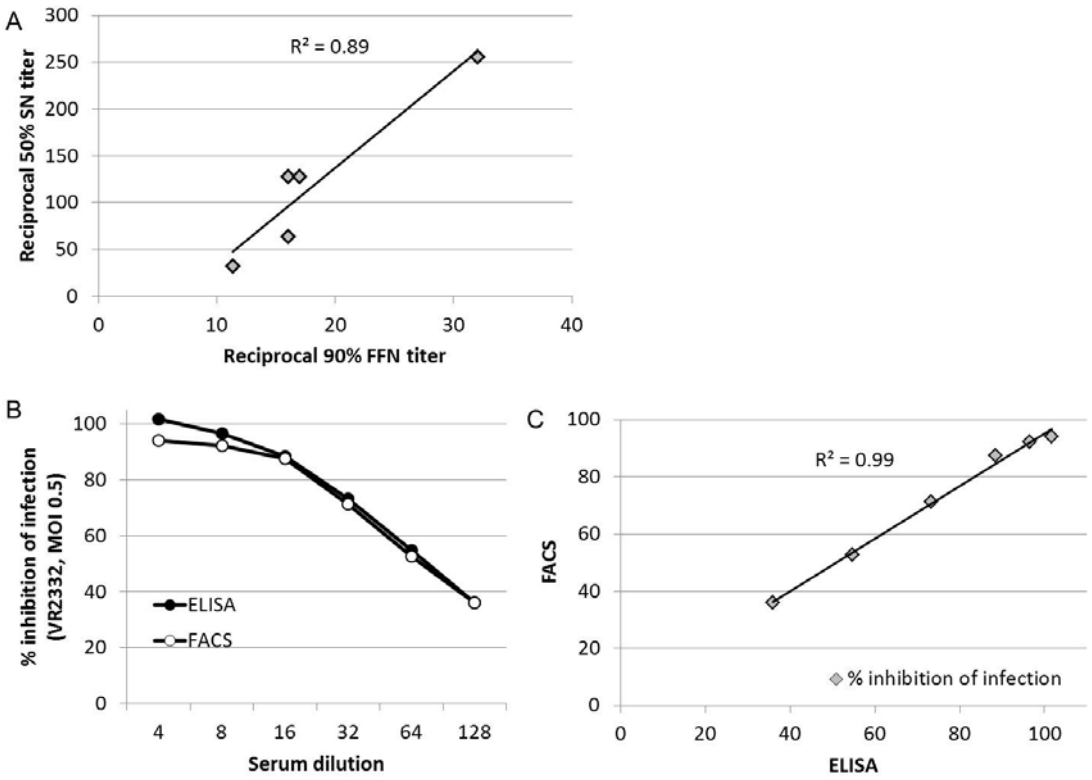

Figure 4.5 Comparison of ELISA-based PRRSV serum neutralizing assay with fluorescent focus neutralizing assay (FFN), and flow cytometry (FACS).

(A) Agreement between the FFN 90% neutralizing titer and ELISA-based 50% neutralizing (SN) titer against VR2332 on MARC 145 cells. Data are individual titers from each of 5 herd 1 sows. (B) Inhibition of PRRSV VR2332 infection at MOI of 0.5 on MARC 145 cells. ELISA and FACS were carried out as described in Materials and Methods on duplicate assay plates, and percent inhibition calculated from ELISA OD values and nucleocapsid-positive cells in FACS. (C) Correlation between percent inhibition of infection measured by ELISA and FACS.

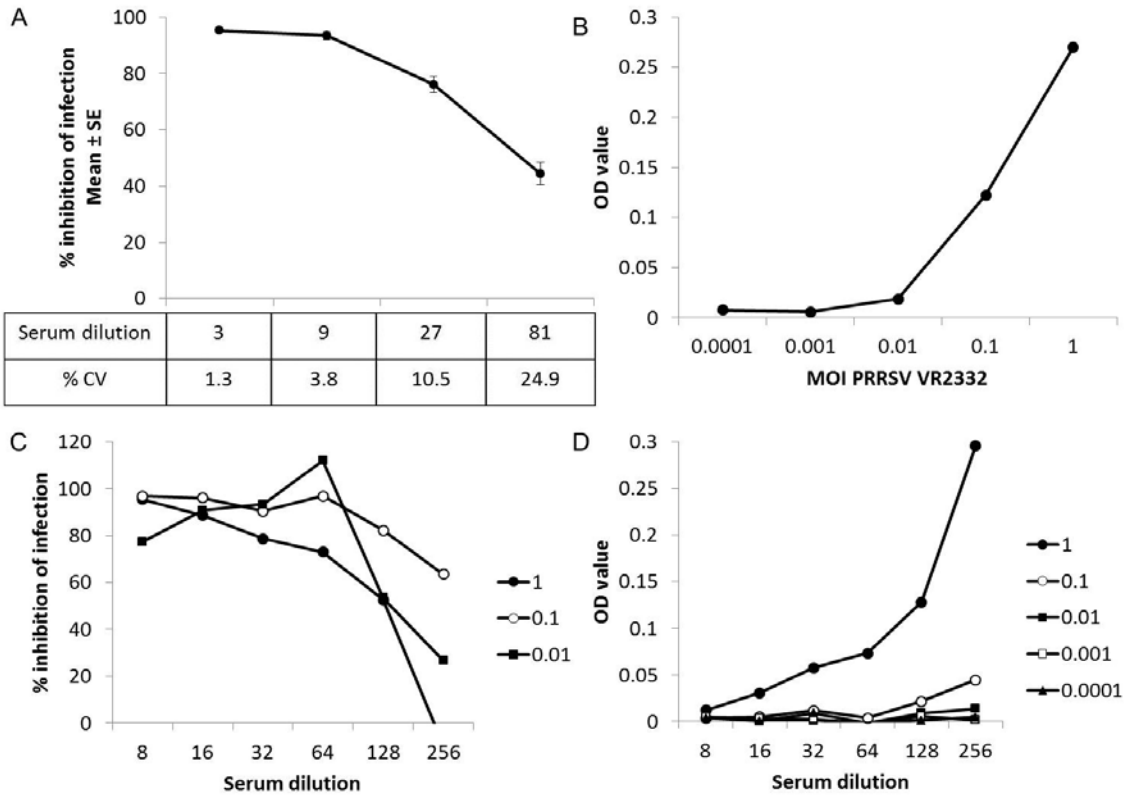

Figure 4.6 Characteristics of ELISA-based PRRSV serum neutralization assay on MARC 145 cells.

(A) Precision of assay demonstrated by minimal variation in neutralization profile of positive control serum assayed on 8 replicate plates. Data are expressed as the mean percentage inhibition of viral infection ± standard error. Percent inter-assay coefficient of variation (% CV) is shown below the graph. (B) Effect of multiplicity of infection (MOI) on assay linearity, measured by absorbance at 450nm. (C) The percentage law; i.e., over a range of virion concentrations, a given concentration of serum neutralizes the same relative proportion of PRRSV infectivity. Proportion of PRRSV infectivity is relatively constant across 100-fold PRRSV mulitplicity of infection (MOI) range. The effect is lost as serum is diluted out and falls outside the detection range of the assay. (D) Effect of MOI on assay sensitivity. As virus is diluted below an MOI of 0.01, the OD value falls below the detection range of the assay.

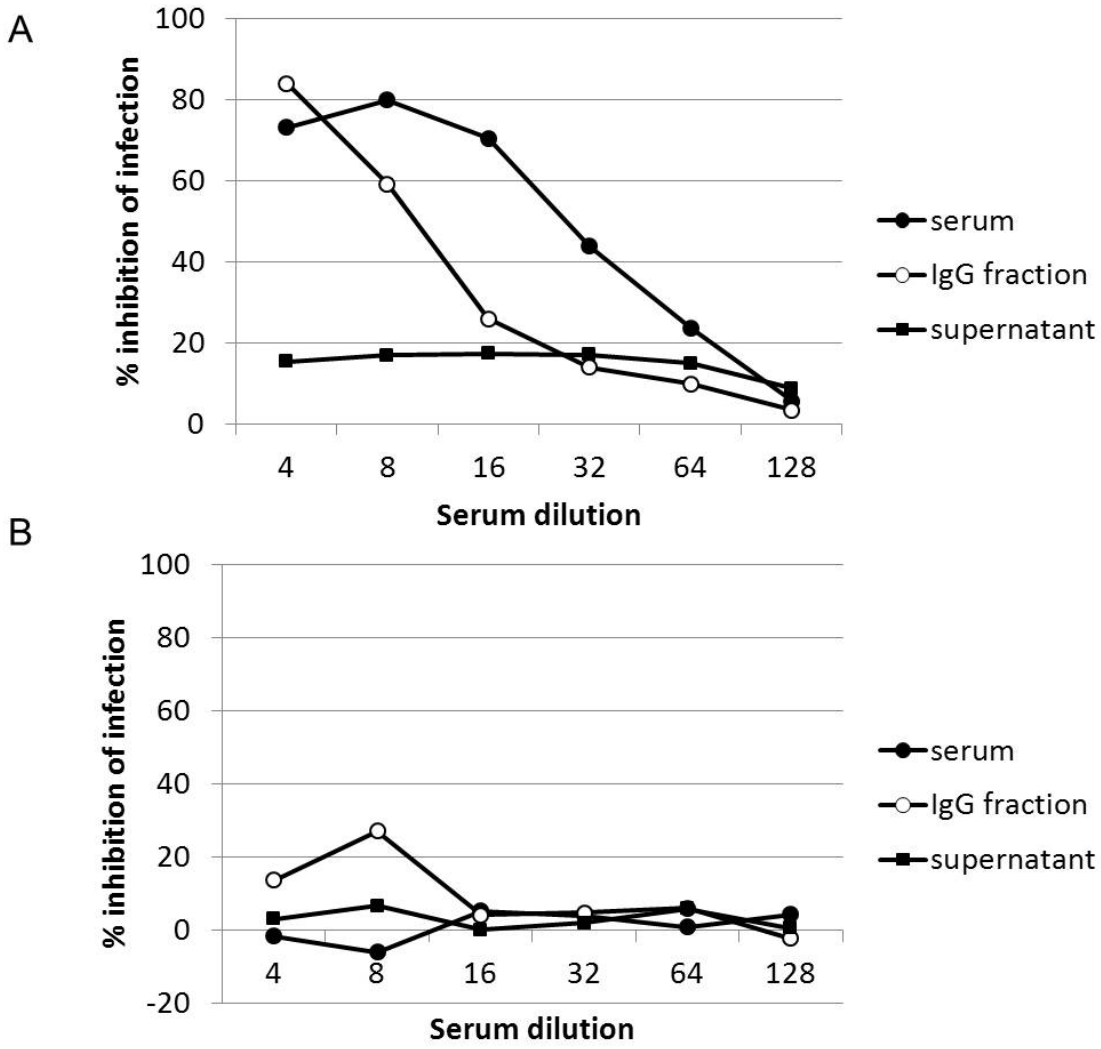

Figure 4.7 PRRSV neutralizing activity is due to immunoglobulins.

Comparison of PRRSV-neutralizing activity in original serum, ammonium sulfate precipitated immunoglobulin fraction (IgG), and IgG-depleted supernatant from a PRRSV-seropositive sow (A), and a PRRSV-negative control sow (B).

Chapter 5 Cross-neutralizing antibodies confer cross-protection against diverse strains of PRRSV

5.1 Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) continues to cause significant disease in swine populations throughout the world despite 25 years of concerted effort to control, prevent or eliminate the virus since its emergence. Strategies for PRRSV prevention, control, and/or elimination can broadly be classified as either management or immune-based. Immunization is the primary immunologic approach to prevent and control PRRSV infection; the two most commonly used are commercial modified live virus vaccines and live virus inoculation (where animals are inoculated with a virulent virus preparation isolated from the resident farm strains). Inoculation can serve to synchronize immunity, expose animals at less critical times such as before entering the herd and prior to breeding, or in the face of an outbreak to reduce dissemination of resident virus (Cano et al., 2007a; Cano et al., 2007b; Linhares et al., 2014; Linhares et al., 2012; Opriessnig et al., 2007). Although immunization undoubtedly has a significant impact on reducing the burden of PRRSV disease, response to vaccination is highly variable, particularly with respect to cross-protection (reviewed in Murtaugh, 2004). Therefore, vaccination does not predictably or reliably prevent infection. Gaps in understanding of PRRSV immunity, particularly with respect to correlates of protection, and how the virus is cleared from the host to achieve sterilizing immunity, are limitations to improving immunologic strategies in PRRSV prevention and control.

Neutralizing antibodies can be highly effective at preventing or controlling viral infections, and some of the most efficacious antiviral vaccines work through induction of neutralizing antibodies (reviewed in Burton, 2002; Burton et al., 2012; Plotkin, 2010). However, to confer reliable

protection, neutralizing antibodies must have sufficient breadth to effectively bind and neutralize the diversity of strains or variants of virus present. It has previously been demonstrated for PRRSV that neutralizing antibodies administered to naïve animals can prevent infection by the homologous PRRSV strain that the neutralizing antibodies were raised against (Lopez et al., 2007; Osorio et al., 2002; Yoon et al., 1996). Although it has been demonstrated that neutralizing antibodies in these and other studies cross-react with heterologous strains *in vitro*, it has only recently been appreciated that high titer cross-neutralizing activity exists under field conditions (Robinson et al., 2015a). It is therefore critical to evaluate whether serum cross-neutralizing activity translates to cross-protection against diverse PRRSV strains *in vivo* in order to understand if neutralizing antibodies play a role in immunity to PRRSV. This study aims to determine if immunoglobulins derived from convalescent sow serum containing high titer cross-neutralizing activity confers passive cross-protection against diverse PRRS viruses in non-immune animals.

5.2 Materials and methods

Sow sample collection

Serum samples from ten sows of parity ≥6 were obtained in 2014 from a herd that had experienced virulent virus outbreaks each year from 2009 to 2013. Blood samples were collected at the farm in serum separator tubes, and shipped to the University of Minnesota. Tubes were centrifuged at 3000 x g for 15 minutes, serum collected and kept at 4°C during testing, and subsequently stored at -20°C. Herd history was obtained as it pertained to PRRSV outbreaks, along with health records for the individual animals tested. Serum samples were evaluated for PRRSV neutralizing antibody profiles detailed in section below: PRRSV ELISA-based neutralizing assay.

Three sows were selected for further evaluation based on their PRRSV neutralizing profile, and were transported to the University of Minnesota where they were euthanized for collection of serum and lymphoid tissues. The study was approved by and conducted under the guidelines of the University of Minnesota Institutional Animal Care and Use Committee, protocol #1402-31319A.

Sera from 22 pigs in the ORF5a immunization study presented in Chapter 2 were used as negative controls in the neutralizing assay. Three animals that were not immunized with ORF5a nor challenged with PRRSV were used as PRRSV negative control animals.

Cell lines

Simian renal epithelial MARC 145 cells were cultured in MEM medium (Gibco, Grand Island NY) supplemented with sodium bicarbonate, non-essential amino acids, HEPES buffer (Sigma, St. Louis MO), and gentamycin sulfate (Cellgro Mediatech, Manassas VA) with 10% FBS (Sigma, St. Louis MO).

PRRSV isolates

PRRSV isolates VR2332 (GenBank U87392), MN184 (EF442777), and SD 01-08 (AY395080) (called 'SDEU' in this study) were propagated in MARC 145 cells. Infectious titers ($TCID_{50}$) were determined by evaluation of cytopathic effect on MARC 145 cells in 96 well plates and calculated by the Reed & Muench method (Reed and Muench, 1938).

Serum samples collected from the sow herd between 2009 and 2013 were submitted to the Veterinary Diagnostic Laboratory at the University of Minnesota, where they were tested by PCR

to detect PRRSV, and ORF5 was sequenced. These sequences, along with reference strains used in the serum neutralizing assays and further isolates representing PRRSV diversity were aligned and subjected to phylogenetic tree construction in Geneious R6 version 6.1.7 (Biomatters Ltd., Auckland, New Zealand) Tree Builder using the Tamura-Nei neighbor-joining method with 100 bootstrap resamplings and no outgroup to create a consensus tree.

PRRSV ELISA

PRRSV nucleocapsid (N) protein was previously expressed and purified for antibody capture ELISA as described (Brown et al., 2009; Johnson et al., 2007). Plates were coated with 100 ng N per well, and serum samples were diluted 1:50 in 5 % non-fat dry milk (NFDM) in phosphate-buffered saline containing 0.05 % Tween 20 (PBST). Detection antibody, horseradish peroxidase-conjugated goat anti-pig IgG (Bethyl Laboratories Inc. Montgomery TX), was used at a 1:100,000 dilution in 5 % NFDM in PBST. Immune complexes were revealed by oxidation with TMB peroxidase substrate (KPL, Gaithersburg MD) for 15 min and stopped with 1 M phosphoric acid. Absorbance was read at 450 nm. Controls for the assay included known PRRSV-positive and negative serum.

ELISA-based PRRSV serum neutralization (SN) assay

SN assays were performed as described in Chapter 4 with the following modifications. Serum (2% FBS) was added to the MEM used for all serum sample and virus dilutions. This helped to maintain a consistent monolayer of MARC 145 cells. Previously, when cells were incubated for one hour in MEM with no FBS, a variable proportion of cells would be washed off the plate following infection or mock infection. All wells in each plate were scored to by light microscopy

to evaluate the condition of the monolayer at the conclusion of the SN assay. In this way, if there was variation in absorbance values for duplicate wells that could be explained by loss of cells in a well, the well was not analyzed.

Cross-reactivity had been observed at low dilutions of neutralizing negative porcine serum samples with the secondary antibody used to detect the murine anti-nucleocapsid IgG antibody. A secondary antibody that is cross-adsorbed against IgG from other species (including pig), was evaluated; HRP-conjugated goat anti-mouse IgG secondary antibody (Bethyl Laboratories, Montgomery, TX) was used at a 1:10,000 dilution for ongoing assays.

The PRRSV multiplicity of infection was re-evaluated. SN assays were performed across a range of MOIs from 4 (128,000 $TCID_{50}$/well) with 2 fold dilutions down to an MOI of 0.008 (250 $TCID_{50}$/well). Subsequent assays were performed with MOI of 0.5, 16,000 $TCID_{50}$/well.

For assays to detect neutralizing activity against the genotype 1 SD 01-08 (SDEU) strain, cells were incubated for 48 hours post infection rather than 24 hours. The longer infection time was necessary as this strain replicates more slowly in MARC 145 cells than genotype 2 viruses (Eric Nelson, *personal communication*).

50% SN titer was determined by the reciprocal of the serum dilution when the inhibition of infection reached 50%.

Immunoglobulin (Ig) isolation from serum

Antibodies were isolated from PRRSV-immune serum from herd 3 sows with high cross-neutralizing activity (sow #7) and negative for cross-neutralizing activity (sow #1). Serum was pooled from three PRRSV-naïve pigs from the ORF5a immunization study in Chapter 2, that

were not immunized with ORF5a nor challenged with PRRSV, and Ig isolated for a negative control.

Total immunoglobulin was isolated from serum by sequential caprylic (octanoic) acid and saturated ammonium sulfate precipitation, modified from (McKinney and Parkinson, 1987) (Figure 5.1). Serum was centrifuged at 10,000 x g for 15 min to reduce lipid fractions, filtered and heat-treated at 56 °C for 30 min. An equal volume of acetate buffer (60mM pH 4.0) was added to 4 °C serum, and pH adjusted to 4.5. Caprylic acid (Sigma-Aldrich, St. Louis, MO) was added 1:15 w/w (where caprylic acid is 0.91 g/ml), and stirred for 30 min at room temperature. The mixture was then centrifuged at 10,000 x g for 30 min and the supernatant (containing the Ig fraction) was poured off and passed through a 70um mesh filter. Tris-HCl (1M pH 8.0) was used to adjust the pH to 7.4.

Saturated ammonium sulfate (Sigma-Aldrich, St. Louis, MO), pH 7.4,was slowly added to serum samples with constant mixing to a final concentration of 40%, stirred at 4 °C overnight, and centrifuged at 10,000 x g at 4 °C for 30 min. Pellets were resuspended in PBS pH 7.0 to 10 to 25 % of the original volume. Purified Igs were dialyzed in PBS pH 7.0, stirring at 4 °C in Spectra/Por 2 membrane dialysis tubing with molecular weight cutoff 12 – 14,000 (Spectrum Laboratories, Rancho Dominquez, CA).

Immunoglobulin concentration was measured using a porcine IgG ELISA quantitation kit (Bethyl Laboratories, Inc., Montgomery TX), with modifications. All washes were in PBS with 0.05% Tween-20 (pH 7.0) (PBST). All washes were done 3 times, with 5 minute incubation in PBST and a further 3 washes in a plate washer. Blocking was 300ul per well of 5% non-fat dry milk (NFDM) in PBST (pH 9.4-9.6) for 1 hour. Antibody, standards and sample dilutions were in 5% NFDM in PBST (pH 7.4-7.6), and stop solution was 1M phosphoric acid.

Samples with and without 5% β-mercaptoethanol were subjected to denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 4 to 20% gradient Mini-Protean Precast Gels (Bio-Rad Laboratories, Hercules CA) to evaluate purity. Bands were visualized with Imperial protein stain (Thermo Scientific, Rockford, IL), and destained in deionized water.

SN assays were performed on purified immunoglobulins as described for serum samples. Two-fold dilutions were made from the purified Ig solution without standardizing the starting concentration.

IgG dose determination

Dose of Ig for administration to pigs was calculated using the number of doses required for each type of Ig, volume of purified Igs, Ig concentrations, dilution factor when absorbed into the blood volume of the pigs and neutralizing activity at that dilution from the neutralizing curve. The goal was to achieve the maximal neutralizing activity of the neutralizing Ig preparation, with an equivalent amount of Ig for each control Ig. Therefore the maximal amount that could be used was based on the most limiting serum.

Passive transfer study

The study was approved by and conducted under the guidelines of the University of Minnesota Institutional Animal Care and Use Committee and Institutional Biosafety Committee (protocol #1310-31023A). The most biologically important parameter for this study was the level of viremia as a measure of infection. Viremia was compared between the groups of interest to determine whether immune immunoglobulins (Ig) were protective against infection, and whether they provided cross-protection against different virus strains compared to animals receiving

control Igs. The number of pigs needed per group was estimated by a power analysis based on mean level of viremia and variation between individuals estimated from previous studies (Robinson et al., 2013). Calculations were based on a 2 log reduction in viremia, (from mean 1,000,000 RNA copies/ml of serum to 10,000, with ±5000 standard deviation in the control group, and ±500,000 in the immune globulin group). To achieve 95% power at 1% alpha level, we calculated that each group would require a minimum of 3 animals (Uitenbroek, 1997). To ensure that valid results would be obtained in case of animals requiring early endpoints, or wider variation between individuals, the study was conducted with 4 or 5 animals per group. In addition, the design allowed for combining groups for statistical analysis based on Ig status. Details of treatment groups is shown in Table 5.1, and timing of procedures in Figure 5.2.

Thirty-two pigs (approximately 3 weeks old) sourced from a PRRSV-negative breeding source were weighed and randomly assigned to seven groups upon arrival at the University of Minnesota isolation facility. Each group of pigs was housed in separate rooms and fed a complete diet ration according to their weight, with *ad lib* access to water. An acclimation period of 2 days was given for pigs to recover from transport, settle into social groups and to ensure health prior to study.

On day -1 of the study (relative to viral challenge) (Figure 5.2), pigs were anesthetized with a combination of Telazol (tiletamine + zolazepam) reconstituted in xylazine by intramuscular injection (to give a final dose of 2.5 - 5 mg/kg tiletamine, 2.5 –5 mg/kg zolazepam, and 1-2 mg/kg xylazine) with use of 0.05 – 0.1ml/kg IM. Blood was collected into serum separator tubes to ensure pigs were not infected with PRRSV (by RT-qPCR), had not previously been exposed to PRRSV (by ELISA), and to measure baseline PRRSV neutralizing activity of serum (by ELISA-based SN assay). Pigs were injected with either PRRSV-neutralizing antibodies purified from sow serum (Neutralizing Ig) or an equivalent amount (determined by Ig concentration) of non-

neutralizing Ig, PRRSV-negative Ig, or no Ig via a 14 gauge catheter designed for intravenous use, into the peritoneal cavity.

Twenty-four hours following immunoglobulin administration, blood was collected from all pigs to evaluate the PRRSV-neutralizing activity in serum at time of viral challenge (day 0 with respect to challenge). At this time, pigs in groups 2-7 were infected by intramuscular inoculation of PRRSV MN184 or SDEU clarified and filtered tissue culture supernatant (at a dose of 1 x 10^5 $TCID_{50}$ in a volume of 1 ml tissue culture media) in the muscles of the neck. Uninfected animals were sham-inoculated with the same volume of tissue culture media intramuscularly. All animals were evaluated daily thereafter for clinical signs of illness such as fever, coughing/sneezing, lethargy, reduced activity, and reduced appetite. Blood samples were collected from all animals on days 3, 7, 10, and 14 post viral challenge. At 21 days post infection, pigs were weighed and anesthetized by intramuscular injection of Telazol as described above. Blood was collected under anesthesia prior to euthanasia by intravenous barbiturate overdose (sodium pentobarbital 390 mg + sodium phenytoin 50 mg/ml) 0.22 ml/kg IV (~86 mg/kg pentobarbital). Necropsies were performed on all animals for evaluation of gross lung morphology and collection of lung, spleen, tonsil and lymph nodes into RNA stabilization reagent (RNAlater, Qiagen). Tissues were kept at 4 °C overnight and frozen to -20 °C. Blood samples were collected in serum separator tubes, and were centrifuged to collect serum which was frozen at -20 °C for further analysis.

Viral RNA isolation and reverse transcription quantitative PCR (RT-qPCR)

Quantification of virus in serum was performed essentially as described for genotype 2 PRRSV (Robinson et al., 2013). Separate assays were used in this study for detection of genotype 1 and genotype 2 viruses, although all samples were run on both assays irrespective of their viral

challenge status. Viral RNA was isolated from serum using a QIAmp Viral RNA Mini kit (Qiagen, Valencia CA) eluted into 50 ul of RNase-free water and stored at -80 °C. Complementary cDNA was synthesized with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad CA) with random hexamer primers. Primer sequences TCA ACT GTC CCA GTT GCT GG (forward) and AAA TGT GGC TTC TCA GGC TTT T (reverse) amplified a 96 bp ORF7 fragment for genotype 1 PRRSV. Primer sequences AAC CAC GCA TTT GTC GTC (forward) and TGG CAC AGC TGA TTG ACT GG (reverse) amplified a 198 bp ORF6-7 fragment for genotype 2 PRRSV. PCR reactions were performed in a total volume of 20 ul, containing 5 ul of cDNA, 10 ul of SYBR Green PCR Master Mix (PerfeCTa SYBR Green FastMix, Quanta Biosciences, Gaithersburg MD) and 200 ng of each primer. Reactions were run in a Stratagene Mx3000P thermal cycler (Agilent Technologies, Inc. Santa Clara CA), with activation at 95 °C for 1 minute, 35 cycles of denaturation at 95 °C for 3 seconds and annealing/extension at 60 °C for 25 seconds, followed by a dissociation step. All samples were run in duplicate.

PRRSV was quantified using the standard curve method as described (Wasilk et al., 2004). A standard curve was generated from gel purified PCR products from PRRSV genotype 1 and 2 strains used to infect the pigs using the same method of viral RNA isolation, cDNA synthesis and primers for PCR described above. After purification, cDNA concentration was estimated by spectrophotometry and number of copies of the template calculated (Staroscik, 2004). Serial 10-fold dilutions were used to construct a standard curve, ranging from $>10^6$ to <1 copy per reaction. Identification of positive samples was determined by the presence of a quantification cycle value and analysis of the dissociation product. Negative controls without cDNA were used in all PCR plates.

Statistical analysis for differences in mean levels of viremia between groups at each timepoint was conducted by the Wilcoxon Rank Sum Test (Mann-Whitney U Test). Conservative p values of ≤ 0.1 were considered significant. Area under curve analysis to determine percent reduction in total viremia was performed in GraphPad Prism (GraphPad Software, La Jolla, CA).

5.3 Results

Optimization of neutralizing assay

Non-specific background inhibition of infection became evident at low serum dilutions when PRRSV-negative or non-neutralizing samples were tested on the ELISA-based neutralizing assay. To test the hypothesis that it was a result of secondary antibody cross-reactivity, serum was tested in the assay across a range of dilutions, in the presence or absence of PRRSV with HRP-conjugated goat-anti-mouse IgG (H+L) (KPL, Gaithersburg MD). Reactivity was compared between primary (SR30A mouse anti-nucleocapsid IgG) antibody alone at 1/10,000 dilution, secondary antibody alone at 1/500 dilution, and assay with primary and secondary antibodies, where the secondary was evaluated at 1/500 and 1/1000. A high degree of cross-reactivity was evident at low serum dilutions in the absence of virus, which was reduced in magnitude in the presence of virus (Figure 5.3A). This phenomenon was also evident in the presence of secondary antibody where no primary antibody was present (data not shown). An alternative HRP-conjugated goat-anti-mouse IgG secondary antibody was evaluated that had been cross-adsorbed to IgG of unintended target species, including porcine IgG (Bethyl Laboratories, Montgomery, TX). The characteristics of the Bethyl antibody enabled it to be used at a 20-fold higher dilution than the KPL antibody, with 1/10,000 being sufficient for detection of virus, with low background absorbance (Figure 5.3B). Direct comparison of cross-reactivity to porcine serum in

the absence of virus between the two secondary antibodies, at the dilution used for optimal detection in the neutralizing assay, showed a marked difference. Cross-reactivity was high with the KPL antibody, and Bethyl cross-reactivity with porcine serum was not evident (Figure 5.3C). The amount of virus per well was re-evaluated with the new assay conditions using the Bethyl secondary antibody, and addition of 2% FBS to MEM used for virus and dilutions. The addition of FBS was evaluated and was found to greatly improve the maintenance of MARC145 monolayers during the SN assay, without otherwise influencing assay results (data not shown). PRRSV VR2332 could be detected above background absorbance in a dose-dependent manner from $TCID_{50}$/well of 2000 up to 128,000 (corresponding to MOI of 0.06 to 4) (Figure 5.4A). Neutralizing activity was constant across an MOI range of 0.125 to 0.5, with inhibition of infection reduced in a dose-dependent manner with addition of more virus (Figure 5.4B).

PRRSV neutralizing activity of sow serum

Neutralizing activity was evaluated against PRRSV VR2332 on MARC cells in a third independent sow herd (in addition to the two sow herds presented in Chapter 4, and the neutralizing negative animals from the ORF5a immunization study in Chapter 2) (Figure 5.5). Herd 3 sows had been exposed to multiple PRRSV outbreaks between 2009 and 2013, as well as a PRRSV modified live vaccine. Mean herd neutralizing activity against PRRSV VR2332 was lower for herd three (mean 50% SN titer of 16) than that of herds 1 and 2, but more variation was evident between individuals (Figure 5.5A). Although fewer animals were evaluated from herd 3, the herd characteristics and exposure history were similar (Figure 5.5B).

Herd 3 sow sera were confirmed to have PRRSV-specific antibodies by ELISA (Figure 5.6A). The variation in neutralizing activity of individual sows was not related to the magnitude of the PRRSV-specific antibody response (Figure 5.6B).

ORF5 sequences were available for outbreak strains between 2009 and 2012 (Figure 5.7A). To evaluate the breadth of neutralizing activity, sera from the 10 sows were tested against 3 PRRSV strains representing maximal diversity from the outbreak strains (Figure 5.7A). The degree of neutralizing activity was proportional to relatedness of virus strains to the herd outbreak strains, with greatest activity against the more closely related MN184 strain, followed by VR2332, and genotype 1 strain SDEU (Figure 5.7B). The cumulative 50% neutralizing titers against each of the three virus strains was compared, revealing three of the ten sows (sows 4, 7 and 10) had high level broadly neutralizing activity (Figure 5.7C). A further three (sows 5, 8, and 9) had lower level activity primarily against MN184 with some reactivity against VR2332 and genotype 1 SDEU. Sows 2 and 3 had low neutralizing activity, with some cross-reactivity against genotype 2 strains, and sows 1 and 6 had low to negative neutralizing activity (Figure 5.7C).

Immunoglobulin isolation from serum

Immunogloblins were isolated from serum of sow 1 (non-neutralizing), sow 7 (cross-neutralizing) and from pooled serum of PRRSV-negative animals. Enrichment of immunoglobulins from the original serum samples was evident in the final dialyzed Ig preparation by SDS-PAGE, with bands corresponding to the molecular weight of IgG and IgM heavy and light chains (Figure 5.8A). Ig products were present as anticipated in the caprylic acid supernatant, and absent in the saturated ammonium sulfate (SAS) supernatant. Under non-reducing conditions, bands of the expected size of intact Ig molecules are present (Figure 5.8B). PRRSV-specific antibodies were

detected in the purified immunoglobulins of the PRRSV-immune but not the PRRSV-negative animals by ELISA (Figure 5.9A). IgG concentration, measured by ELISA, increased as a result of the purification/enrichment process (Figure 5.9B). Accounting for volumes of starting serum and purified Ig, 30-35 % of IgG was recovered from original serum (Figure 5.9C). Although no Ig bands were observed by SDS-PAGE in the SAS supernatant after immunoglobulins precipitation, the 30-35 % recovery indicates loss of Ig during the purification process. Neutralizing activity was present in the SAS supernatant at lower levels than the precipitated Ig fraction, consistent with incomplete precipitation (Figure 5.10A and B). However, it was not detectable by the IgG ELISA (Figure 5.10C). Sow 1 serum had barely detectable neutralizing activity, which became apparent with enrichment of the Ig (Figure 5.10A). In contrast, the SAS supernatant of the sow with high serum neutralizing activity (#7) had lower neutralizing activity than the serum or purified Igs (Figure 5.10B). The neutralizing activity of the three immunoglobulin preparations was compared. Ig from sow 7 maintained high neutralizing activity, and sow 1 Ig had comparatively lower activity (Figure 5.11). Non-specific inhibitory activity was present in the PRRSV-negative Ig at low dilutions (Figure 5.11).

Passive transfer study

The passive transfer study was designed to address the hypothesis that cross-neutralizing PRRSV immunoglobulins protect pigs against infection from diverse strains of PRRSV. Purified immunoglobulins were passively transferred to naïve pigs, subsequently challenged with either a genotype 1 or 2 PRRSV, and level of viremia monitored compared with positive and negative controls (Figure 5.2 and Table 5.1).

Average weight of the 32 three week old pigs on arrival was 6.6 kg, ranging from 5.5 – 7.7 kg, with standard deviation of 0.6 kg. Blood volume of the pigs was estimated at 420ml (7% of body weight). The immunoglobulin dose was determined by calculating the maximum attainable amount of Ig per pig based on the volume of each Ig available and number of doses required for each Ig preparation such that an equivalent amount of Ig would be present in each dose based on the most limiting Ig preparation (Table 5.2). This resulted in a dose of 242mg Ig per animal (0.58 mg/ml or 40mg/kg). The dilution factor could be estimated based on absorption of Ig volume in the blood volume of the pig (Table 5.2). Based on the Ig dilution factor, the expected PRRSV neutralizing activity was estimated from the Ig neutralizing curves (Figure 5.11).

Animals were monitored clinically following viral challenge. Infection resulted in subclinical infection with both SDEU and MN184 virus strains, as pigs had no clinical signs of respiratory disease such as increased respiratory rate, coughing, sneezing, fever or lethargy. There were no differences in average daily weight gain between groups over the study duration (data not shown).

Neutralizing activity was present in the serum of pigs that received neutralizing Igs at the time of viral challenge (24 hours post Ig administration), but not in those receiving non-neutralizing, PRRSV negative or no Igs (Figure 5.12). Pigs that received neutralizing Igs had significantly lower levels of SDEU (Figure 5.13A) and MN184 (Figure 5.13B) viremia compared with pigs receiving PRRSV negative Ig or no Ig. Overall levels of viremia were higher for MN184 infected pigs than SDEU (Figure 5.13A and B). There was no difference in levels of viremia between pigs receiving non-neutralizing antibodies and negative or no antibodies for either virus (Figure 5.13A and B). Reduction in total viral load attributable to neutralizing Ig administration, determined by area under the curve, was 43% for SDEU and 83% for MN184 (Figure 5.13C). The *in vitro*

neutralizing activity of sow 7 serum from which the neutralizing antibodies were isolated was not appreciably different between that against SDEU and MN184 (Figure 5.7C and Figure 5.14).

Pigs that received passively transferred Igs achieved low neutralizing titers *in vitro* (Figure 5.15). There was no neutralizing activity present in serum prior to Ig administration in any of the animals (Figure 5.15A). Twenty-four hours post Ig administration (at the time of challenge) there was a distinct increase in neutralizing activity observed in the pigs that received neutralizing Igs compared with all other groups (Figure 5.15B). At this time, the maximal level of inhibition of infection was between 20 and 40 %, and decreased in a dose-dependent manner. At 15 and 22 days post Ig administration, neutralizing activity remained higher in the animals that received neutralizing Ig (>20 %) than non-neutralizing, negative, and no Ig (<20 %) (Figure 5.15 C and D).

5.4 Discussion

The ELISA-based PRRSV serum neutralizing assay has a number of advantages over traditional PRRSV neutralizing assays. The quantitative readout can be used to generate neutralizing curves which provide a more complete picture than a single endpoint titer. It allows more in-depth analysis and comparison between samples or populations, as the shape and slope of curves are informative (for example, (Honnen et al., 2007; Klasse, 2014; Magnus, 2013; Trautman, 1979)). Importantly, it provides an unbiased analysis of neutralization, as the biologic significance of specific endpoints such as 90% reduction, while useful for diagnostic comparisons, does not facilitate biological or biochemical analysis. The absorbance of individual wells is measured in an automated microplate reader, and thus does not require manual counting of microscopic foci in each well, saving time and eliminating subjectivity. It was important to optimize and standardize

the assay conditions to ensure robustness and to provide a validated assay that can serve as a resource to others. The assay initially developed by Juan Li was modified in Chapters 2 and 4, and further optimization has been demonstrated here. Addition of 2% FBS to culture media for dilutions made a significant difference in maintaining MARC 145 cell monolayers. The secondary antibody was replaced by one with improved performance for this assay. Previously, in the absence of the preferred high-affinity target (mouse IgG antibody to detect PRRSV nucleocapsid), the secondary antibody cross-reacted with porcine IgG in the assay serum samples in a dose-dependent manner.

As discussed in Chapter 4, the amount of virus used in the ELISA-based PRRSV neutralizing assay is substantially greater than in the widely-used PRRSV FFN assay (Wu et al., 2001). The goal for the FFN assay is to have a manageable number of fluorescent foci to count in a well, such that 90% reduction can easily be identified. For the ELISA-based assay, the goal is to have a difference in OD values between virus only control wells and background from cell (± serum) only control wells sufficient to accurately discriminate between samples with varying neutralizing activities, that falls within linear/proportional range of the assay, and within the range that conforms to the percentage law of antibody neutralization. With this in mind, virus was titrated to determine a standard amount with the updated assay parameters. An MOI of 0.5, 16,000 TCID50/well was selected for ongoing assays. As amount of virus was increased beyond this, absorbance values were still appropriate, but above MOI of 1 there was a virus dose-dependent reduced proportional inhibition of infection as the amount of virus overwhelms available neutralizing antibodies. Conditions were evaluated with PRRSV VR2322 at 24 h post-infection, as per the standard assay. For ongoing assays to evaluate breadth of neutralization, a standard panel of viruses needs to be tested in this manner. For assays using PRRSV SDEU in this study, virus was used at lower MOI because stock virus was low concentration and therefore limited by

volume that could be added to wells. This was overcome by allowing infection to proceed for 48 hours; virus is amplified by additional rounds of infection to compensate for the lower starting amount of virus. This likely introduces complexity to the system, as the fraction of the virus population that is resistant to neutralizing antibodies will be selectively amplified, and depending on the proportion of virus this represents with any given sample, could result in either increase or decrease in level of infection.

Sows in this study from a third independent breeding herd had lower mean neutralizing activity compared with herds 1 and 2 (from Chapter 4), but the degree of individual variation was greater. The simplest explanation may be the smaller number of animals sampled from this herd, however it may also be a result of less uniform immunity in the herd, or less uniform cross-neutralization against the VR2332 strain for which the comparison was made. From what is understood of the exposure history of the different herds, herd 3 potentially had less exposure to modified live vaccine, which is derived from the parental VR2332 strain (Ingelvac PRRS® MLV, Boehringer Ingelheim, St. Joseph, MO). ELISA against N suggests variable serologic response to PRRSV amongst the 10 sows, which was not correlated with neutralizing activity. Although this only reflects N-specific antibodies, correlation between PRRSV-specific antibody responses and serum neutralization was evaluated in another study using a commercial ELISA with whole virus coated on wells (Appendix 1: Comparison of PRRSV neutralizing activity with PRRSV ELISA). PRRSV specific ELISA results were well correlated with PRRSV N specific results, but were not associated with neutralizing activity against SDEU, VR2332, or MN184.

Variation in level and breadth of neutralization between herd 3 individuals was observed. Three of the ten PRRSV immune sows from herd 3 had high levels of broadly cross-neutralizing activity against diverse strains of PRRSV, including across genotypes. ORF5 sequences of the herd outbreak strains from 2009 – 2013 showed they belong to genotype 2, and there was no

history of the herd having any exposure to genotype 1 strains. The sows had multiple exposures to virulent and vaccine PRRSV strains over time, however as they were sampled in a cross-sectional manner, the outcome of each PRRSV exposure for individuals with respect to infection, disease, and response to subsequent exposure are unknown. The *in vitro* observations raised the question of whether serum neutralizing characteristics are predictive of cross-protection *in vivo*. The capacity for neutralizing antibodies from the serum of these sows to provide cross-protection was determined in the passive transfer study.

A recent study demonstrated that animals with longer duration of viremia were more likely to develop cross-neutralizing antibodies from a primary immune response to PRRSV infection (Islam et al., 2014). In the study, around 40% of the 454 animals had cross-neutralizing activity against other genotype 2 viruses that differed by at least 10% from the inoculation strain. Seven percent of the pigs were able to neutralize each of the 3 different PRRSV strains as well as the inoculation strain. The animals were not subsequently challenged, so it is not known if cross-neutralizing activity in serum was predictive of protection. Whether *in vitro* serum cross-neutralizing activity can serve as a correlate of protection will need to be assessed in future studies.

A proportion of the PRRSV-specific B cells from sow 7 with broad cross-neutralizing activity bound both type 1 and type 2 virus (Michael Rahe, *personal communication*). It is not yet known whether the proportion of cross-reactive B cells differs between the highly neutralizing and non-neutralizing animals but may suggest that the antibodies bind epitopes that are conserved across genotypes. It raises the possibility that conserved cross-neutralizing epitopes exist, and that individual cross-reactive B cells secrete broadly neutralizing antibody molecules.

Antibodies were purified from serum of one of the sows with high neutralizing activity and one with low neutralizing activity. In addition, antibodies were purified from a PRRSV-negative animal (from an unrelated herd). Visualization of the Ig purification products revealed 3 major bands under reducing conditions. The bands represent Ig light chain at ~25 kDa, and IgG and IgM heavy chains at ~50 and 70 kDa respectively (Pescovitz, 1998). This is supported by the non-reducing gel, where the major product was intact IgG at ~150 kDa, and pentameric IgM is observed at the very top of the gel, as its size is ~900 kDa. It would be possible to confirm the identity of bands by immunoblot with IgG and IgM specific antibodies, and quantification of IgM by ELISA. The vast majority of serum proteins were removed to leave a relatively pure enriched Ig preparation. Recovery of IgG was 30-35%, with some loss of Ig in the caprylic acid precipitated pellet along with most other serum proteins. There was no Ig detectable by ELISA or SDS-PAGE in the SAS supernatant; inhibition of infection demonstrated in the SN assay from this fraction may instead have been due to remaining presence of ammonium sulfate (for example, inhibition may have been a result of pH change). Data from the PRRSV-negative control Ig fractions is lacking, without which it cannot be determined if inhibition of infection was a result of neutralizing activity due to loss of Ig in the SAS supernatant in sow 7, concentration of low level neutralizing Ig in sow 1, or non-specific inhibition from the ammonium sulfate. Despite the 30-35% recovery, Ig was enriched in the purified product compared with serum. It was important that the volume injected into the weaned pigs be minimized, since systemic absorption from the peritoneal cavity is efficient with lower Ig volumes (Barrett et al., 1991).

Neutralizing activity was retained in the Ig fraction following purification. Inhibition of infection was evident in low dilutions of purified Ig fraction from the non-neutralizing and negative animals. This may result from concentration of low-level neutralizing antibodies in the non-

neutralizing serum upon purification. However, inhibition of infection at low dilutions of the purified PRRSV-negative Ig suggests that it is non-specific inhibition resulting from excessive immunoglobulin in the assay. In support of this, no reduction of viremia resulted from the non-neutralizing Ig compared with no or negative Ig.

Antibodies were administered by intraperitoneal administration, such that the same amount of immunoglobulin was administered to each animal. PRRSV neutralization curves from purified Ig predict 80%, 40% and 7% inhibition of infection at the dilutions expected in the blood volume of the naïve pigs. Neutralizing activity was present in serum at time of viral challenge, but levels were lower than expected. Previous studies have shown that immunoglobulins are absorbed from the peritoneal cavity to produce maximal serum titers by 24 hours post-administration (Barrett et al., 1991; Trautman and Bennett, 1979; Yoon et al., 1996). Neutralizing activity levels at 8, 15 and 22 days post administration showed the expected pattern whereby nAb > non nAb> neg Ab> no Ab, but did not exhibit clear dose-dependent reduction in activity. Antibody half-life for PRRSV-specific neutralizing antibodies is around 6-10 days (Lopez et al., 2007; Osorio et al., 2002; Senn et al., 1998; Yoon et al., 1996). Given that maximal neutralizing titers were expected to occur by 24 hours post administration, with steady decline thereafter, neutralizing activity was likely insufficient to neutralize virus in the SN assay.

Pigs receiving neutralizing antibodies had reduced viremia from both SDEU and MN184 challenge compared with animals receiving non-neutralizing, negative, or no Ig. The 83% reduction in MN184 viremia by neutralizing Ig was as expected based on the predicted value of 80% inhibition of infection (Table 5.2). In contrast, reduction in SDEU viremia was around half that expected at 41% even though Sow 7 serum demonstrated equivalent neutralization against the two virus strains *in vitro*. MN184-challenged pigs had higher levels of viremia compared with SDEU, but had greater proportional reduction. Although the proportional reduction in viremia

was not as great for SDEU, viremia was suppressed or delayed from the earliest timepoint in animals receiving neutralizing antibodies. The discrepancy between predicted inhibition from the *in vitro* neutralizing data and response *in vivo* for SDEU may be related to the amount of virus that was used in the SDEU assays. As discussed earlier, neutralization may have been more efficacious in the assay because of the lower amount of virus used. Alternatively, the neutralizing antibodies may have lower affinity for the genotype 1 virus that did not manifest in the SN assay but was important *in vivo*. This theory could be tested by ELISAs designed to measure avidity. Plates could be coated with purified virus (MN184 or SDEU) and binding of neutralizing versus non-neutralizing Ig measured in the presence or absence of urea or thiocyanate (Binjawadagi et al., 2014; de Souza et al., 2004; Prince et al., 2014). Virus levels in lymphoid tissues have not yet been evaluated, but may assist in understanding the infection dynamics of each viral strain in the presence and absence of neutralizing antibodies. As infection in this study was subclinical, it was not possible to use clinical disease as a measure of protection. No antibody dependent enhancement of infection was observed *in vivo* with sub-neutralizing levels of sow 1 cross-neutralizing antibodies or during decline of sow 7 neutralizing Ig to sub-neutralizing levels as described by Yoon et al. (1996).

Compared with the IgG concentration in the serum of the original animal (sow 7) of 16.13 mg/ml, the animals in the study received 0.58 mg IgG/ml of blood if complete absorption occurred from IP administration. Even at approximately 1/28th the amount of neutralizing antibodies, viremia was reduced, demonstrating that cross-neutralizing antibodies confer cross-protection. The dose required for complete cross-protection against productive infection by the different strains was not determined in this study, and it is not possible to predict if the donor animals would have been protected if challenged. In the PRRSV passive transfer study by Osorio et al. (2002), sows were administered ~11mg/kg of Ig (approx. 20 times more than this study), resulted in an endpoint

serum neutralizing titer of 1:16 and complete protection of sows against homologous viral challenge (Osorio et al., 2002). Lopez et al. (2007) determined that 90% serum neutralizing titer of 1:8 was protective against homologous viremia, but not spread to lymphoid tissues or transmission, and 1:32 titer conferred sterilizing immunity in 3 of 6 animals (Lopez et al., 2007). The major differences between this and the aforementioned PRRSV passive transfer studies were i) evaluation of cross-protection, ii) by Ig isolated from commercial sows under typical field conditions (compared with those experimentally induced by intensive hyperimmunization including Freund's adjuvant), and iii) discrimination between PRRSV immune neutralizing and non-neutralizing Igs . The findings from this study suggest neutralizing antibody mediated cross-protection is possible under field conditions. To gain a complete understanding of neutralizing activity as a correlate of protection, it will be necessary to understand how *in vitro* serum neutralizing titers are related to cross-protection in individual animals.

In summary, this study confirms existence of high titer cross-neutralizing antibodies to PRRSV in the field. Variation in neutralizing breadth and activity exists between individuals in a relatively uniform population with respect to age, genetics, husbandry and viral exposure conditions. Neutralizing antibodies mediated cross-protection against diverse PRRSV strains across genotypes. Collection of serum and B lymphocytes from sows with cross-neutralizing and non-neutralizing activity provides a valuable resource for ongoing studies to identify features and mechanisms of antibody-mediated neutralization of PRRSV.

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Table 5.1 Treatment groups for passive transfer of immunoglobulins

Group	Category	Viral challenge	Treatment (number of pigs)
1	Uninfected negative control	None	Neutralizing Ig (n=2) Non-neutralizing Ig (n=2)
2	Neutralizing Ig + PRRSV	SDEU	Neutralizing Ig (n=5)
3	Non-neutralizing Ig + PRRSV	SDEU	Non-neutralizing Ig (n=5)
4	Infection positive control	SDEU	Negative Ig (n=2) No Ig (n=2)
5	Neutralizing Ig + PRRSV	MN184	Neutralizing Ig (n=5)
6	Non-neutralizing Ig + PRRSV	MN184	Non-neutralizing Ig (n=5)
7	Infection positive control	MN184	Negative Ig (n=2) No Ig (n=2)

Table 5.2 Immunoglobulin dose determination

Ig source	# doses needed	Available volume (ml)	[IgG] (mg/ml)	Conc. rel to sow 7	Volume required/ ml of sow 7 Ig	Dose (ml/pig)	IgG (mg)*	Dilution (factor) in blood volume	% inhibition of PRRSV infection
PRRSV neg (ORF5a)	4	58	17.58	0.73	1.38	13.8	242	13.8/420 (30)	7
Sow #1 (non neut)	12	250	28.21	1.16	0.86	8.6	242	8.6/420 (49)	40
Sow #7 (neut)	12	220	24.20	1	1	10	242	10/420 (42)	80

* 0.58 mg/ml or 40 mg/kg

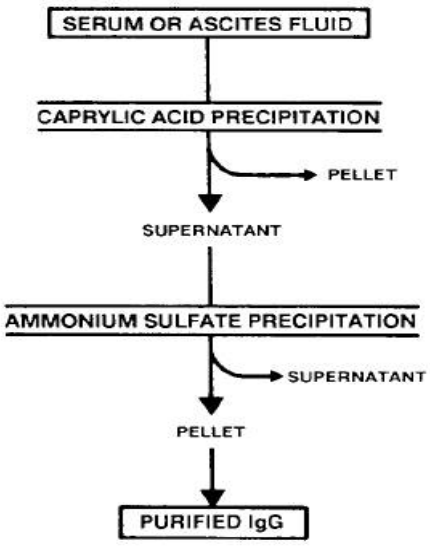


Fig. 1. Outline of the procedure to purifity IgG.

Figure 5.1 Purification of immunoglobulins by sequential caprylic acid – saturated ammonium sulfate precipitation. From: McKinney and Parkinson (1987).

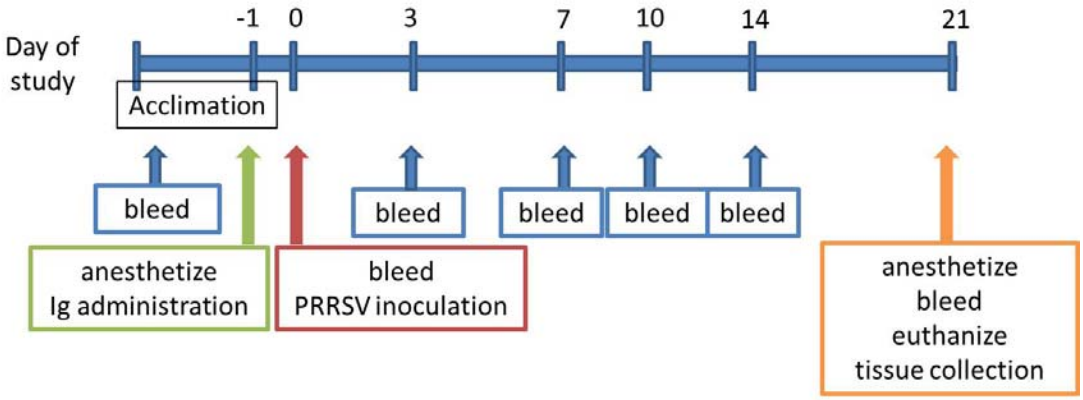


Figure 5.2 Passive transfer study timeline

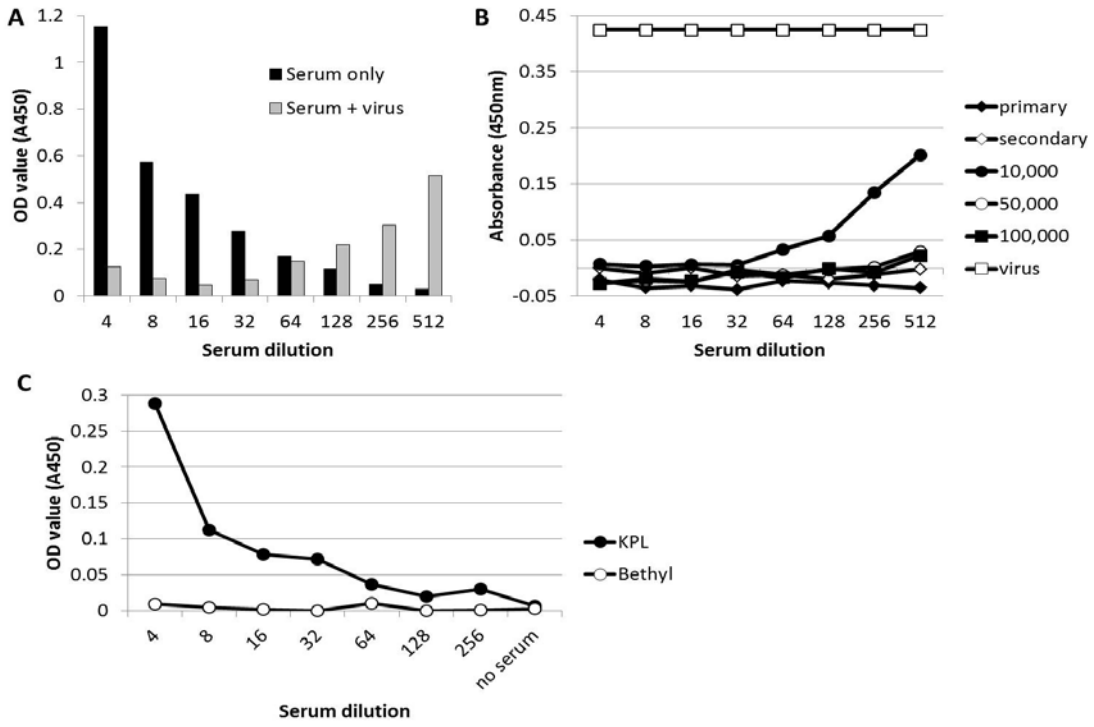


Figure 5.3 Cross-reactivity of secondary antibody in the PRRSV serum neutralizing assay

ELISA-based PRRSV serum neutralizing assays on MARC 145 cells with PRRSV VR2332 and PRRSV neutralizing serum. OD values represent A_{450} minus background absorbance of cell only control wells. A) Detection of secondary antibody reactivity in the presence and absence of virus with 1:500 dilution of HRP-conjugated goat-anti-mouse IgG (KPL). B) Optimization of cross-adsorbed HRP-conjugated goat-anti-mouse IgG (Bethyl) concentration. C) Direct comparison of the KPL (1:500) and Bethyl (1:10,000) HRP-conjugated goat-anti-mouse IgG reactivity to porcine serum.

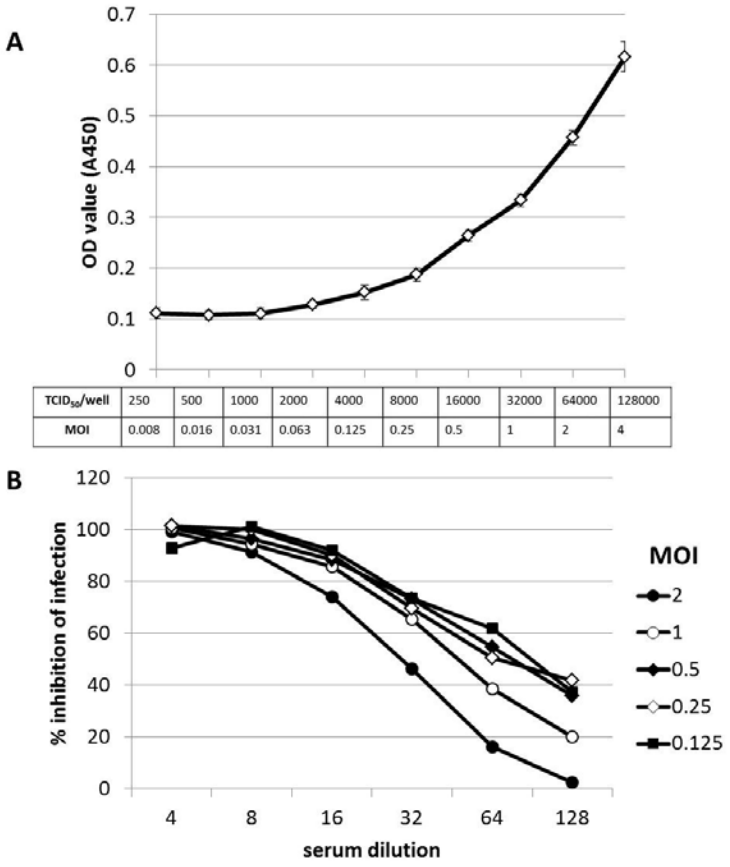
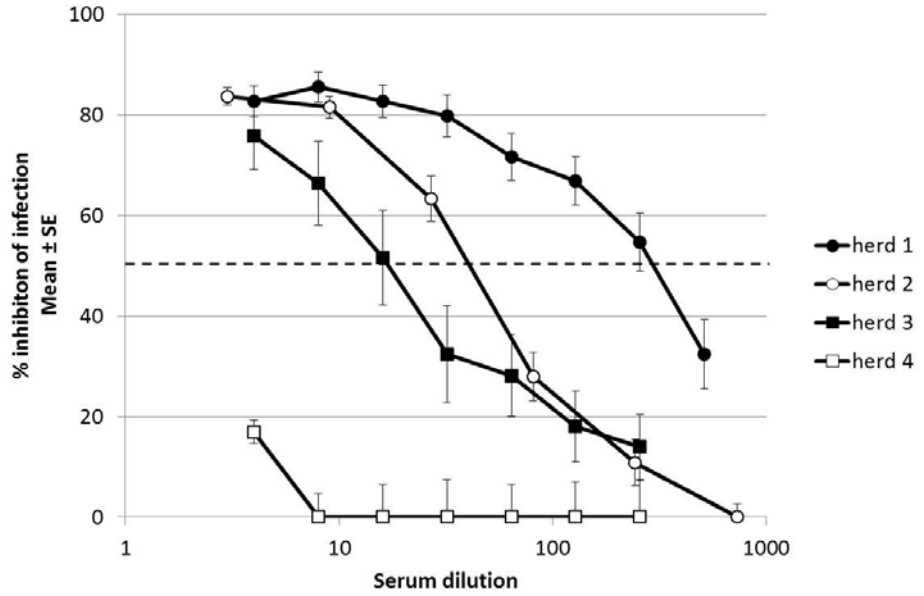


Figure 5.4 Optimization of serum neutralizing assay virus concentration with PRRSV VR2332 on MARC 145 cells

A) Relationship between of OD value and amount of virus input in the ELISA-based serum neutralizing assay with increasing amount of virus in the absence of serum samples. OD values represent A_{450} minus background absorbance of cell only control wells. B) Serum neutralization curves with increasing amount of virus to evaluate conformity to the percentage law.

A



B

Herd	N	Type of exposure	Virus strain	Day post infection	Neutralizing activity
1	20	Field	Multiple	Unknown	High
2	16	Field	Multiple	Unknown	Moderate
3	10	Field	Multiple	Unknown	Variable
4	22	Experimental	VR2332	21	No

Figure 5.5 Neutralizing characteristics of different pig populations

A) Comparison of serum neutralizing activity against VR2332 on MARC 145 cells between four herds of pigs. Dashed line indicates 50% inhibition of infection. B) Infection characteristics of the four populations.

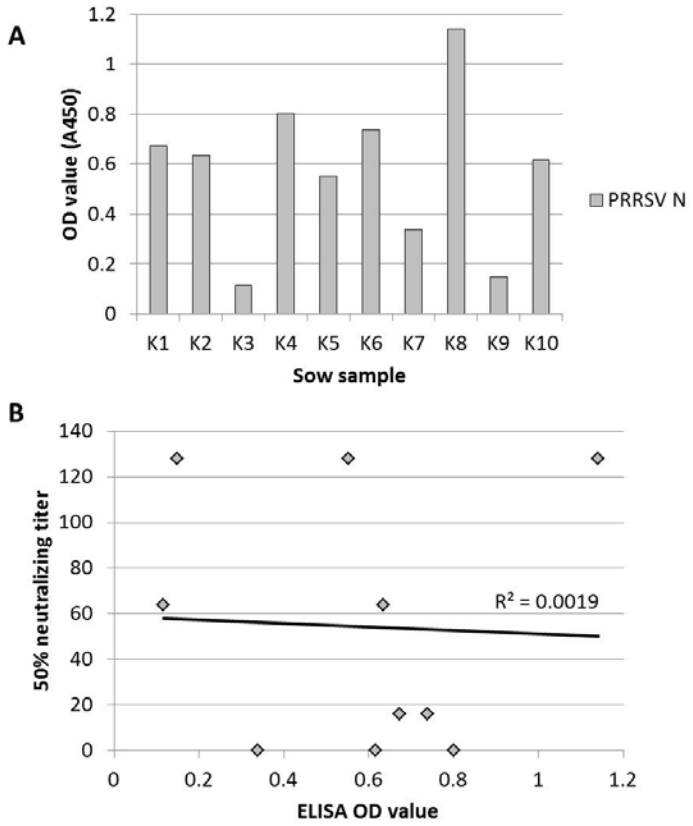


Figure 5.6 PRRSV-specific antibody response does not predict neutralizing titer

A) ELISA to detect PRRSV nucleocapsid-specific antibody reactivity for the 10 sows from herd 3. B) Correlation analysis between PRRSV nucleocapsid ELISA OD value and 50% neutralizing titer against PRRSV MN184 for the 10 sows from herd 3.

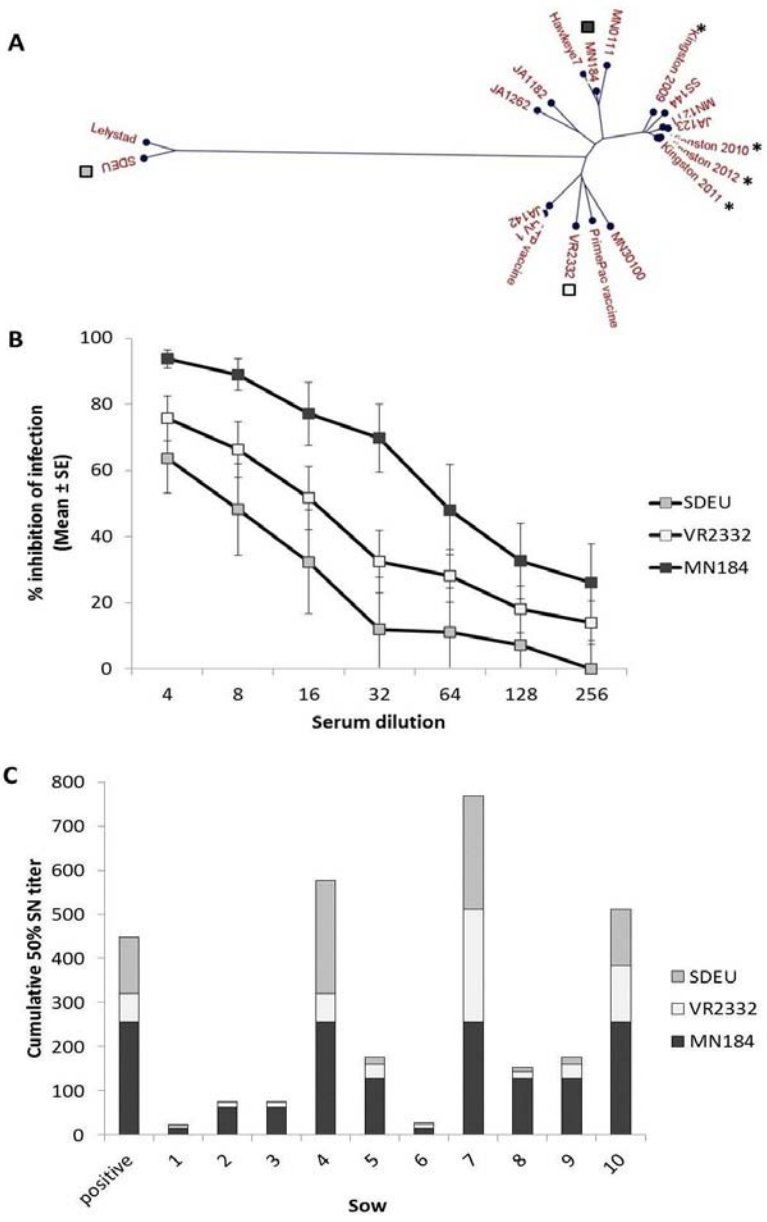


Figure 5.7 Broadly neutralizing activity in sow herd 3 serum

A) ORF5 phylogeny of diverse PRRSV isolates. Herd 3 isolates from 2009 – 2012 are marked with *. Viruses representing maximal PRRSV diversity used in neutralizing assays are marked with squares. B) Comparative neutralizing activity of 10 sows from herd 3 against 3 diverse PRRSV strains. C) Cumulative 50% neutralizing titers against 3 diverse PRRSV strains for the 10 individual animals from herd 3. Positive control was a sow from herd 1 previously tested for neutralizing activity in Chapter 4.

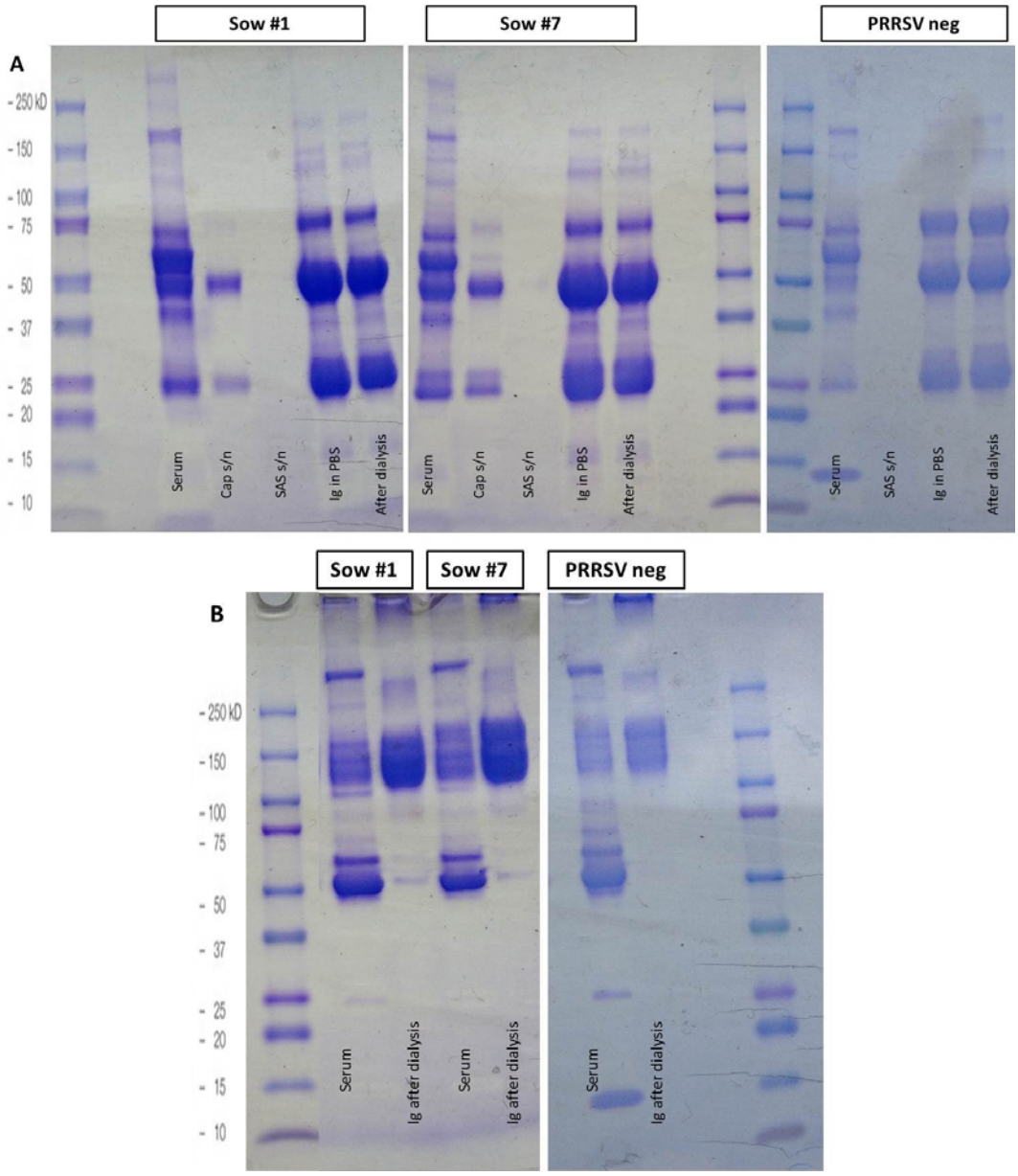
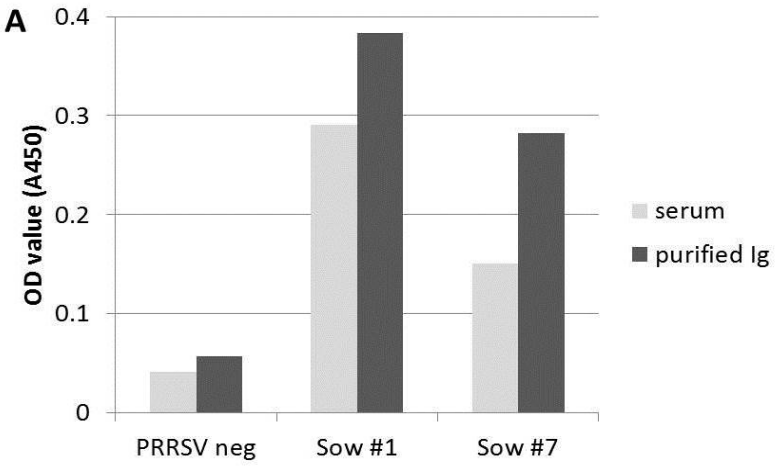
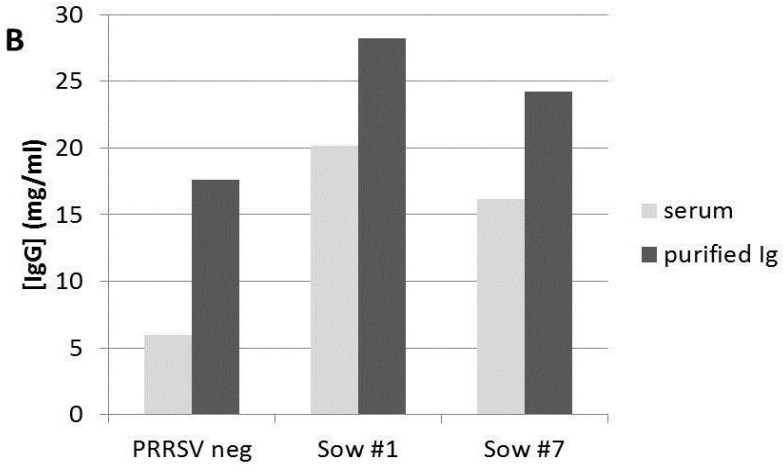


Figure 5.8 Purification and enrichment of immunoglobulins from serum

Gel electrophoresis (SDS-PAGE) of serum and immunoglobulin purification fractions for Sow 1, 7, and PRRSV negative control under A) reducing with addition of β-mercaptoethanol, or B) non-reducing conditions. Molecular weight markers are shown on each gel. Fractions are the starting serum, caprylic acid supernatant (Cap s/n), saturated ammonium sulfate supernatant (SAS s/n), SAS precipitated Ig resuspended in PBS before (Ig in PBS), and after (Ig after dialysis) dialysis into PBS. All fractions were drop-dialyzed against distilled water prior to electrophoresis.





C

Sample	Total IgG (mg)		% recovery IgG
	serum	purified Ig	
PRRSV-neg	3399	1020	30
Sow 1	20151	7054	35
Sow 7	16133	5331	33

Figure 5.9 Quantification of purified immunoglobulins

A) PRRSV nucleocapsid ELISA on serum and purified immunoglobulins. OD value represents A450 with background absorbance subtracted. B) Porcine IgG ELISA. C) Total IgG yield for each sample. Starting volume for PRRSV-neg serum was 567ml, compared with 1000ml for sows 1 and 7.

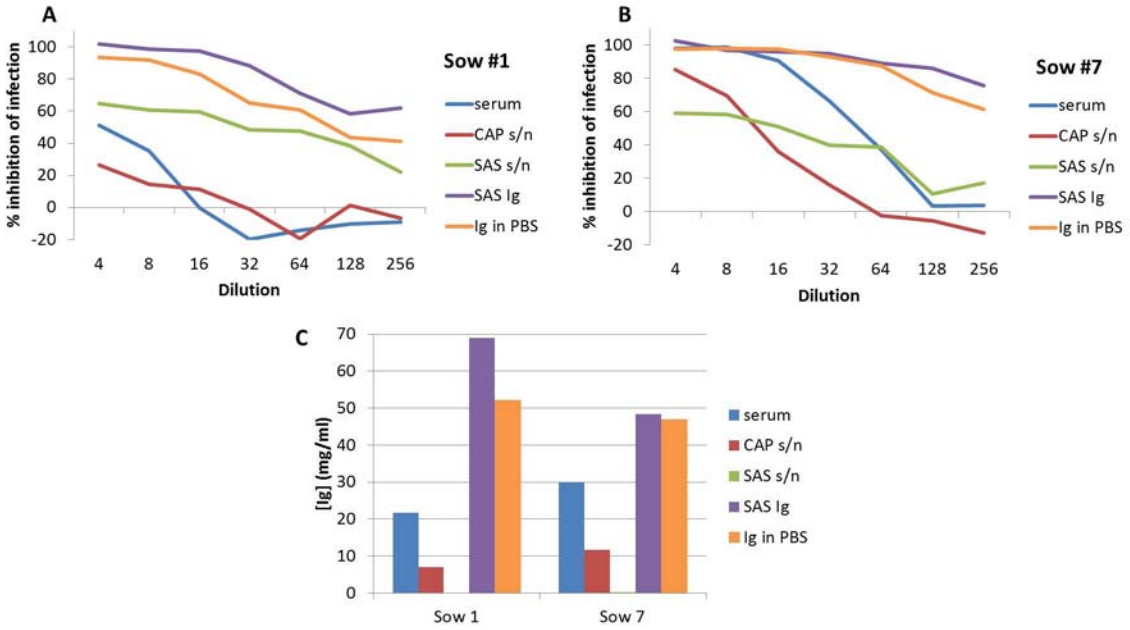


Figure 5.10 Concentration and neutralizing activity of immunoglobulin purification fractions

ELISA-based neutralizing assay of Ig purification fractions against PRRSV VR2332 on MARC 145 cells for A) non-neutralizing PRRSV-immune sow 1, and B) neutralizing PRRSV-immune sow 7. C) Porcine IgG ELISA on Ig purification fractions to determine IgG concentration. Caprylic acid supernatant (CAP s/n), saturated ammonium sulfate supernatant (SAS s/n).

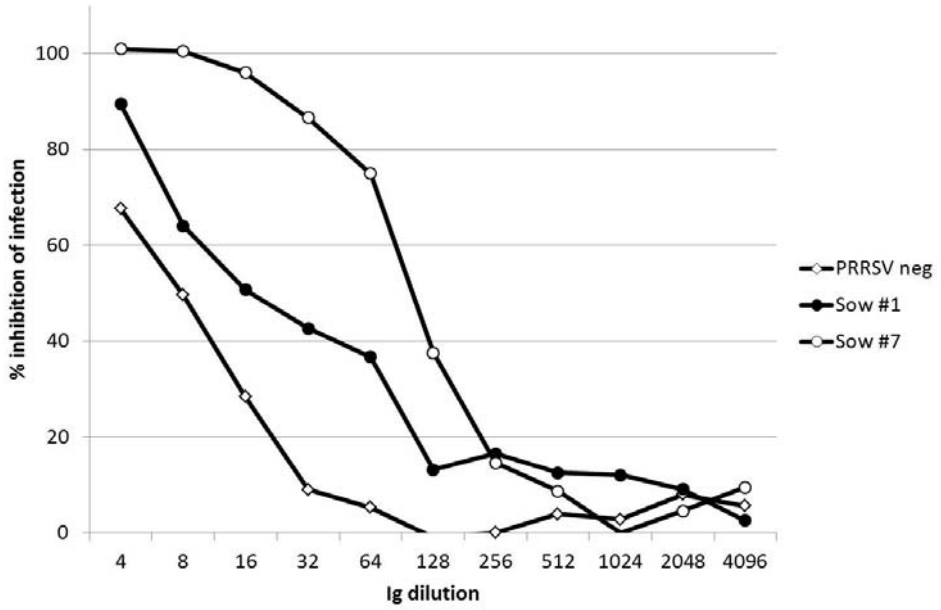


Figure 5.11 Neutralizing activity of purified immunoglobulins.

ELISA-based neutralization assay against PRRSV VR2332 on MARC 145 cells for PRRSV-negative, PRRSV-immune non-neutralizing sow 1, and PRRSV-immune neutralizing sow 7.

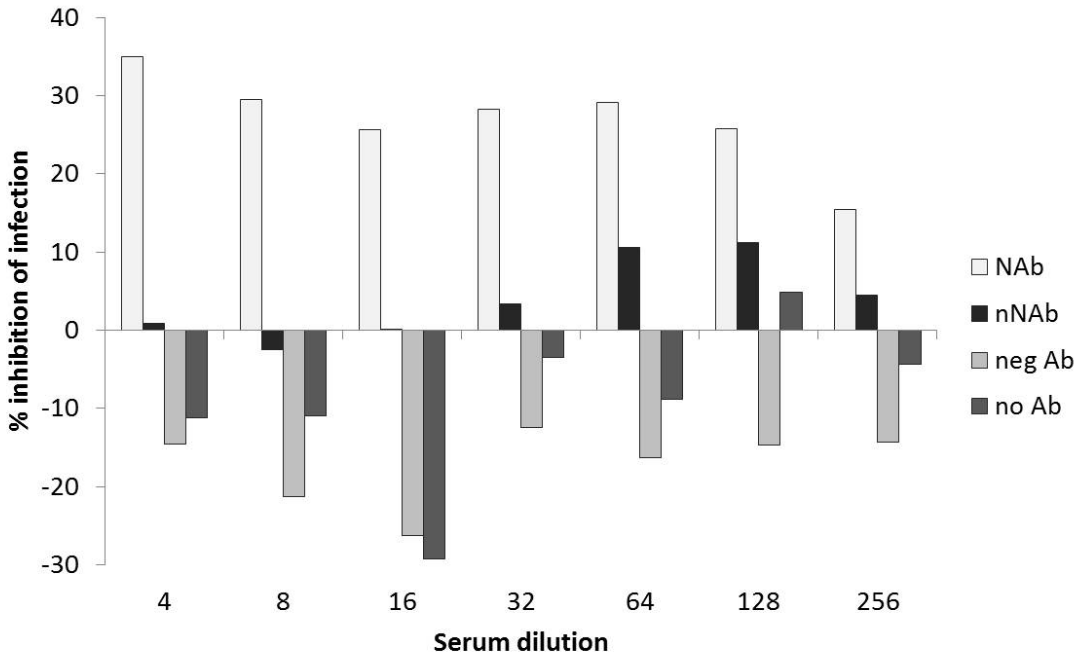
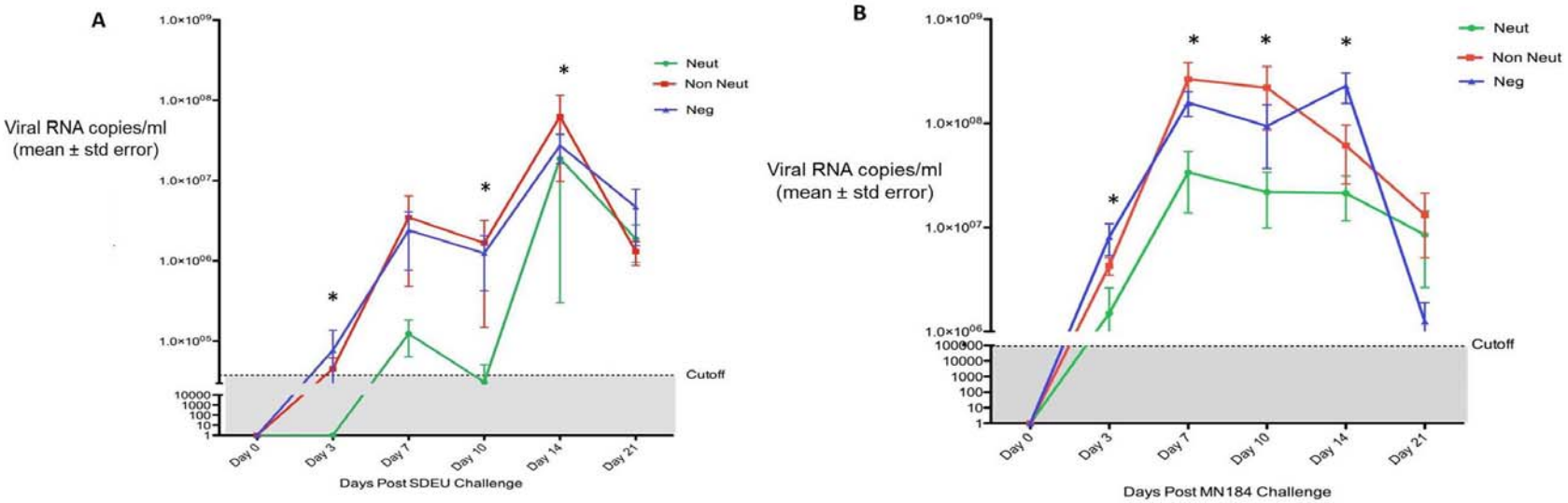


Figure 5.12 Neutralizing activity in serum at time of viral challenge

ELISA-based serum neutralizing assay of PRRSV VR2332 on MARC 145 cells from 32 animals in passive transfer of immunoglobulin study. Data is the difference between neutralizing activity 24 h post Ig administration and baseline neutralizing activity in serum at time of injection, for pigs receiving neutralizing (NAb n= 12), non-neutralizing (nNAb n= 12), PRRSV-negative (neg Ab n= 4) or no (no Ab n= 4) immunoglobulins.



C

Challenge virus (genotype)	% reduction viremia AUC
SDEU (1)	41
MN184 (2)	83

Figure 5.13 Passive transfer of neutralizing antibodies reduces viremia

PRRSV RNA quantification from serum by RT-qPCR for A) genotype 1 SDEU, and B) genotype 2 MN184 in PRRSV-naïve pigs administered neutralizing Ig (Neut, n= 12), non-neutralizing Ig (Non Neut, n= 12), PRRSV-negative or no Ig (Neg, n= 8). Difference between mean viremia in groups receiving neutralizing Ig and negative Ig was compared by Wilcoxon Rank Sum Test, with p values ≤ 0.1* considered significant. C) Percent reduction in overall viremia by area under curve analysis (to PCR assay cutoff value).

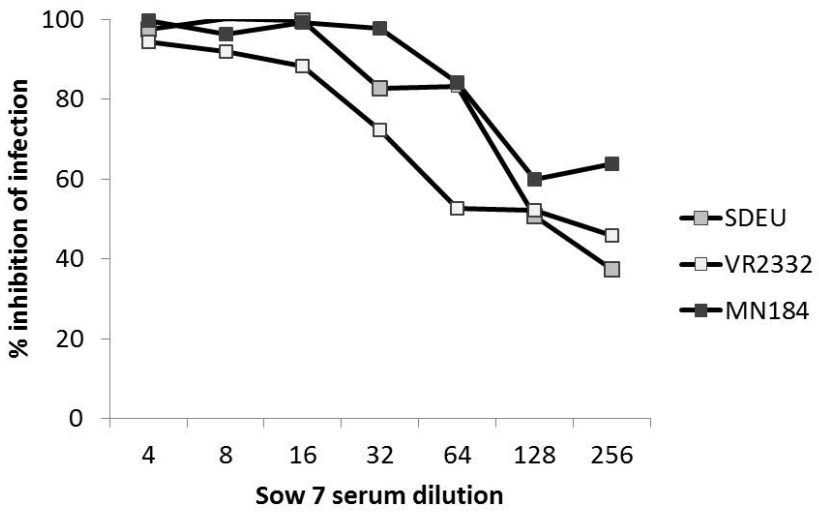


Figure 5.14 Cross-neutralizing activity of sow 7 serum. ELISA-based serum neutralization assay against PRRSV SDEU, VR2332 and MN184 with sow 7 (herd 3) serum.

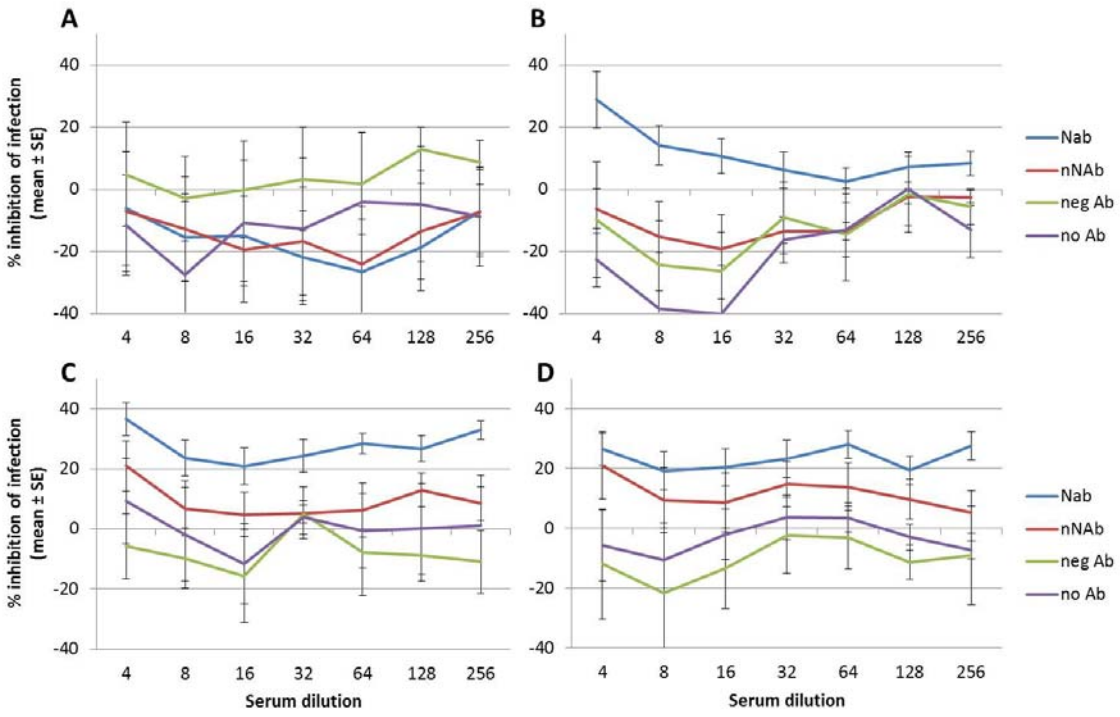


Figure 5.15 Neutralizing activity after immunoglobulin transfer

ELISA-based serum neutralizing assays against PRRSV VR2332 on MARC 145 cells from 32 animals in passive transfer of immunoglobulin study. Neutralizing activity at A) time of Ig administration, B) 24 hours post Ig administration (time of viral challenge), C) 15 days, and D) 22 days post Ig administration, for pigs receiving neutralizing (NAb n= 12), non-neutralizing (nNAb n= 12), PRRSV-negative (neg Ab n= 4) or no (no Ab n= 4) immunoglobulins.

Chapter 6 Targets of PRRSV broadly cross-neutralizing antibodies

6.1 Introduction

With evidence that neutralizing antibodies play a role in cross-protection against PRRSV (Chapter 5), it is important to investigate the underlying mechanisms of neutralization and characteristics of neutralizing antibodies. Mechanistic understanding of PRRSV neutralization will be required to develop strategic approaches aimed at inducing protective cross-neutralizing responses. Determining viral targets of cross-neutralization is a critical step towards further understanding neutralization mechanisms, isolating and identifying B cells that produce neutralizing antibodies, and for inducing neutralizing antibody responses to PRRSV in pigs. Identification of a PRRSV epitope that elicits broadly neutralizing antibodies would provide us with a specific antigen whose delivery to pigs in an appropriate vector or adjuvant could protect pigs effectively and broadly against most or all PRRSV strains, and provide a correlate of cross-protection.

For many years, the major envelope glycoprotein GP5 was the focus of investigations aimed at elucidating PRRSV neutralization targets. It is the most abundant envelope protein in PRRSV, existing as a dimer with M protein (Dea et al., 2000; Mardassi et al., 1996; Meulenberg et al., 1995; Wissink et al., 2005). In addition, the GP5 homologues in related Arteriviruses equine arteritis virus and lactate dehydrogenase elevating virus have been shown to contain a major neutralizing epitope and induce neutralizing antibody production, thus GP5 was predicted to be a critical target in PRRSV (Faaberg et al., 2011). Neutralization epitopes have been described in PRRSV structural proteins GP3, GP4, GP5 and M, however there are inconsistencies between the findings and more comprehensive studies are required to understand the nature of neutralizing epitopes and their contribution to a protective immune response (reviewed in Darwich et al.,

2010; Murtaugh and Genzow, 2011). Importantly, epitopes that induce or are susceptible to cross-neutralizing antibodies have not been evaluated.

PRRSV has a limited cell tropism. In the host, PRRSV primarily infects porcine macrophages, but infection of monocytes and dendritic cells has also been demonstrated (Baumann et al., 2013; Silva-Campa et al., 2010; Wang et al., 2007; Wensvoort et al., 1991; Yoon et al., 1992). Growth on continuous cell lines is limited to simian renal epithelial CL2521/MA104 and the derivative MARC 145 (Collins et al., 1992; Kim et al., 1993). It was previously thought that that GP5-M interaction with sialoadhesin on macrophages was responsible for PRRSV infectivity (Delputte and Nauwynck, 2004; Van Breedam et al., 2010; Van Gorp et al., 2008). It was subsequently demonstrated that CD163 (scavenger receptor cysteine rich family) was sufficient for PRRSV infection, and explained permissivity of simian MARC 145 cells which express CD163, but not sialoadhesin (Calvert et al., 2007; Duan et al., 1998; Van Gorp et al., 2008; Wissink et al., 2003b). The role of GP5 sialic acids binding sialoadhesin on macrophages remained controversial, with conflicting evidence as to whether it facilitates infection, with some studies showing higher titer of infection in the presence of CD169 and CD163 compared to CD163 alone, with others showing no difference (Van Gorp et al., 2008; Welch and Calvert, 2010). Recent studies have demonstrated that CD169 is not necessary for infection *in vivo* with CD169 knockout pigs, and removal of N-glycans does not reduce viral infectivity (Li and Murtaugh, 2015; Prather et al., 2013). GP5-M ectodomains have now been shown to be unimportant for PRRSV neutralization in genotype 1 and 2 PRRSV (Li and Murtaugh, 2012; Vanhee et al., 2011). Consistent with this, neutralizing curves shown in Chapter 5 suggest the mechanism of neutralization is the same for PAMs and MARCs, which supports the notion that the mechanism of PRRSV neutralization does not involve sialoadhesin.

Minor envelope glycoproteins GP2, GP3 and GP4 form a heterotrimer, and GP2 and GP4 have been shown to interact with the CD163 receptor, and are the major determinant of viral tropism in cell culture (Das et al., 2010; Tian et al., 2012). Based on broadly-neutralizing epitope targets identified in other RNA viruses such as influenza and HIV (reviewed in Laursen and Wilson, 2013; West et al., 2014), PRRSV cross-neutralizing targets would reasonably be anticipated to be conserved structural or functional regions critical for essential pathogen/host cell interactions. Currently, there is no structural information regarding PRRSV envelope proteins apart from that predicted from their amino acid sequences (Dokland, 2010).

The approach for this study was to determine whether conserved regions in PRRSV glycoprotein ectodomains may be targets for *cross-neutralization*. The hypothesis is that conserved ectodomain regions of GP2, GP3 and/or GP4 are targets of cross-neutralizing antibodies. Peptides containing epitopes critical for neutralization would be expected to compete with their counterpart on the surface of the virion for antibody binding in a dose-dependent manner to abolish neutralizing activity. If CD163 –interacting regions are the targets, peptides from envelope glycoproteins GP2, GP3 and/or GP4 that are conserved between diverse isolates are predicted to block the ability of neutralizing serum to neutralize infection by divergent strains of PRRSV. The peptides also would compete with virus for binding a cell surface receptor, so would be predicted to directly block viral infection.

6.2 Methods

Identification of conserved ectodomain peptides

Whole genome sequences from 58 unique PRRSV strains, including genotypes 1 and 2 were obtained from GenBank. Sequences of ORF2a, ORF2b, ORF3 and ORF4, representing GP2, E,

GP3, and GP4 respectively were concatenated for each PRRSV strain, aligned using Geneious R6 version 6.1.7 (Biomatters Ltd., Auckland, New Zealand) and translated to demonstrate conservation at the amino acid level. Predicted ectodomain regions were identified, based on (Dokland, 2010). Within the ectodomain fragments, the most conserved linear peptides regions were identified.

Peptide synthesis and characteristics

Six linear peptides, one each from GP2 and E, and two each from GP3 and GP4, were synthesized by GenScript (Piscataway, NJ). Solubility testing was performed at GenScript, and based on this, peptides were solubilized to 1 mg/ml in either 0.1M PBS (pH 7.4) (GP2, E, GP4a), N-methyl-2-pyrrolidone (NMP) (GP3a, GP3b), or dimethyl sulfoxide (DMSO) (GP4b). Further dilutions from the 1 mg/ml stock were made in MEM containing 2% FBS.

Cellular receptor competition assay.

Each of the 6 peptides, a mixture of the 6 peptides, and vehicle only controls were diluted to 10 ug/ml in MEM 2% FBS. Five-fold serial dilutions of the peptides were made in MEM 2% FBS to 0.016 ug/ml. The 5 dilutions, and control wells with no peptides (containing MEM 2% FBS) or vehicle only (at the equivalent dilution for the most concentrated condition of 10ug/ml) were added to duplicate wells of MARC 145 cells and incubated at 37°C for 1 hour. PRRSV strain VR2332 was added at an MOI of 0.5 in the presence of peptides and incubated at 37°C for a further hour. The peptide/virus mixture was decanted, cells were washed once with warmed PBS, and complete MEM with 10% FBS was added for 23 hours further incubation. At this time, cells were washed with PBS, and treated as per the PRRSV neutralizing assay described in Chapter 5 with respect to fixation, permeabilization and ELISA to detect PRRSV infection in cells.

Neutralizing serum competition assay.

Each of the 6 peptides, a mixture of the 6 peptides and control wells with no peptides (containing MEM 2% FBS) were added to duplicate wells at 10 ug/ml or 1ug/ml to a fixed concentration equivalent to a 1/32 dilution of either PRRSV neutralizing serum, PRRSV negative serum, or no serum (MEM 2% FBS), and incubated at 37°C for 1 hour. All serum was previously heat treated at 56 °C for 30 min. A constant amount of PRRSV VR2332 to achieve an MOI of 0.5 was added and incubated for a further hour, before transferring the peptide/serum/virus mixture to cells. Additional wells with cells in MEM 2% were included as a control to determine background absorbance. Plates were incubated for a further hour before washing off with PBS and allowing a further 23 hours of incubation. At this time, cells were washed with PBS, and treated as per the PRRSV neutralizing assay described in Chapter 5 with respect to fixation, permeabilization and ELISA to detect PRRSV infection in cells.

6.3 Results

Predicted ectodomain regions were identified (Figure 6.1A) and mapped to the minor envelope protein sequence alignment (Figure 6.2A). The alignment included 58 PRRSV of maximal diversity, and identified regions of high conservation (Figure 6.2A). Within the ectodomain fragments, the most conserved linear peptides regions were identified in GP2, E, GP3, and GP4 and synthesized (Figure 6.2B).

Individual synthetic peptides from GP2, E, GP3 and GP4 were evaluated for evidence of neutralizing epitopes by two complementary approaches. Firstly, peptides were added to cells before addition of virus: if peptide(s) were to bind cellular CD163 (mimicking virus binding), they would compete with virus in a dose-dependent manner to result in reduced infection (Figure

6.3A). The second approach was to add peptides to serum, prior to addition of virus before being placed onto cells: if peptides were to bind neutralizing antibodies, they would compete with virus in a dose-dependent manner for binding and make more virus available for infection i.e. would inhibit neutralizing activity and result in increased infection (Figure 6.3B).

None of the 6 peptides or peptide mixture inhibited infection in a dose-dependent manner beyond that resulting from the vehicle alone (Figure 6.4). Although there was a pattern of increased viral infection with peptides used at 10 ug/ml compared to 1 ug/ml in the presence of neutralizing serum, the increase did not exceed the level of infection when no peptides were present (Figure 6.5). With addition of 1 ug/ml of peptides, there was greater than 60% reduction in infection.

6.4 Discussion

This study screened linear peptides from highly conserved regions of minor envelope proteins for evidence of competition with cellular receptors of MARC 145 cells or to neutralizing antibodies for infection. However, under the conditions evaluated, none of the peptides evaluated competed with PRRSV for CD163 or neutralizing antibody binding either individually, or as a mixture. The preliminary nature of these experiments preclude robust conclusions to dismiss GP2, GP3 and GP4 ectodomain regions as being important for cross-neutralization.

The primary limitation of the experimental approach relates to the peptides being linear, which does not represent the conformational nature of the peptide sequences in the native protein structure. Conformational epitopes may require this structure to bind to neutralizing antibody targets. Also, epitopes may be non-contiguous, where antibody molecules bind residues that span different proteins. Glycoproteins 2, 3 and 4 associate to form trimeric complexes in the viral envelope, which has been shown to interact with E protein, ORF5a protein, and the GP5-M dimer

(Figure 6.1B) (Das et al., 2010; Sun et al., 2015). Therefore, the essential binding features of neutralizing epitopes may bind components of more than one protein in the complex. Therefore, negative results from the experiments in this study using linear peptides do not exclude the possibility of minor envelope protein contributions to neutralization.

Physical evidence for structure of PRRSV minor envelope proteins is extremely limited, so the field is currently reliant on bioinformatic predictions (Dokland, 2010). Amino acid residues on GP2 and GP4 critical for CD163 interactions are not yet known. This gap in knowledge limits the ability to predict regions that might be likely neutralizing targets based on structural interactions.

PRRSV E protein has few exposed ectodomain residues, so is unlikely to be a major determinant for neutralization (Figure 6.1A). However, it was included in the initial screen due to its interaction with the minor envelope glycoprotein complex, and therefore possible contribution (Figure 6.1B). ORF5a protein has also recently been shown to interact with the complex via E and GP4 (Figure 6.1B) (Sun et al., 2015). PRRSV ORF5a protein is 43-51 amino acids, smaller than E protein, with an ectodomain region of just 6 or 7 amino acid residues. We demonstrated in Chapter 2 that pigs immunized with synthetic ORF5a had a robust antibody response to the protein that had no neutralizing activity (Robinson et al., 2013). It was therefore not included in this study, but could be utilized as a negative control protein in future experiments. GP5 and M proteins were not included as they were not relevant for the aim of this study, and recent evidence shows that GP5-M ectodomains are not important for neutralization (Li and Murtaugh, 2012). As with ORF5a, the GP5-M ectodomain recombinant protein may be a useful reagent for future experiments.

A potential confounding factor in this study is the use of polyclonal neutralizing serum. Although serum is heat treated to inactivate heat labile fractions such as complement, serum has many

components along with PRRSV-specific neutralizing antibodies. As such, there may be unappreciated interactions occurring between serum components, for example competition of PRRSV-specific but non-neutralizing antibodies for binding of neutralizing epitopes. A partial solution, and next step in this study, is to test the peptides against the purified immunoglobulins with cross-neutralizing activity described in Chapter 5 to eliminate some of these variables. Monoclonal cross-neutralizing antibodies have not yet been isolated for PRRSV, but constitute a future goal of this research.

Here, we have taken a simple and straightforward approach to look for evidence of neutralization targets. Alternative approaches to identify targets of cross-neutralizing antibodies are briefly described:

i. ELISA to compare reactivity and avidity. Addition of urea in an ELISA can evaluate avidity of binding of cross-neutralizing versus low-neutralizing immunoglobulins to recombinant PRRSV envelope protein ectodomains. To assess cross-reactive targets, proteins would be from an unrelated viral strain to which the antibodies were generated. This approach selects for cross-reactive antibodies, to determine whether cross-neutralizing antibodies preferentially bind or have higher affinity binding to particular proteins. It can then be determined from a larger set of serum samples if a relationship exists between cross-neutralizing titers and avidity to particular proteins.

ii. Mass spectrometry to identify viral epitopes. Purified immunoglobulins from cross-neutralizing versus non-neutralizing immune serum can be compared for binding to divergent PRRSV targets by a subtractive approach that identifies epitopes bound by cross-neutralizing antibodies that are not present in non-neutralizing serum. This approach allows binding of antibodies to the virion in its native structure, and also has the advantage of identifying non-contiguous epitopes.

iii. Deep sequencing of viral isolates to look for evidence of escape mutants under selective pressure of neutralizing antibodies. If viruses escape neutralization (evidenced by an increase in viral growth in presence of neutralizing antibodies compared with controls), mutations will be informative in identifying critical neutralizing targets. Neutralizing targets can then be confirmed by genetic manipulation to evaluate components that are critical for reproducing the escape phenotype.

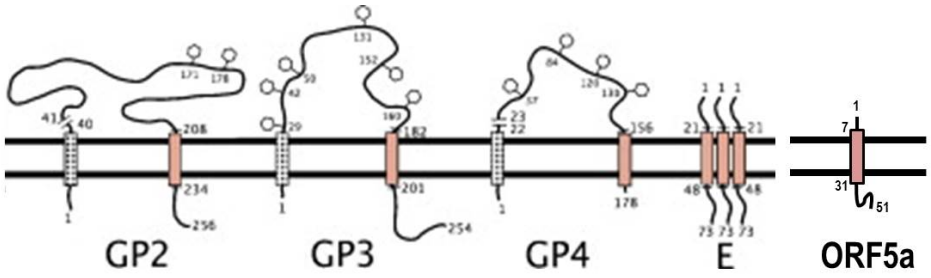
iv. Generation of monoclonal cross-neutralizing antibodies. B lymphocytes or plasma cells are isolated from an animal with cross-neutralizing activity, and screened for cross-reactivity to different strains of fluorescently labeled PRRSV by FACS. Cross-reactive cells are sorted into individual wells, induced to proliferate and secrete antibodies. Supernatants are then evaluated for cross-neutralizing activity. Relevant heavy and light chain pairs can be sequenced and cloned to make recombinant antibodies to elucidate targets on virions by either of the above approaches.

In summary, further experiments are needed to identify viral targets of cross-neutralization against diverse PRRSV strains and to determine if these are conserved regions with essential roles in infections such as for structure or binding to cellular receptors. Only through elucidation of viral targets of cross-neutralization will it be possible to realize the potential for induction of cross-protective neutralizing antibodies through new immunization approaches, and to develop reliable correlates of neutralizing antibody-mediated cross-protection.

Acknowledgements

Xiong Wang assisted with compilation and alignment of concatenated minor envelope protein sequences, identifying conserved peptide regions, and preparing the image demonstrating the degree of conservation.

A



PRRSV ORF	Protein	Length (aa)	Ectodomain residues	Ectodomain size (aa)
2a	GP2	251-256	41-208	168
3	GP3	254-265	29-182	154
4	GP4	178-183	23-156	134
2b	E	70-73	1-21	22
5a	ORF5a	43-51	1-7	7

B

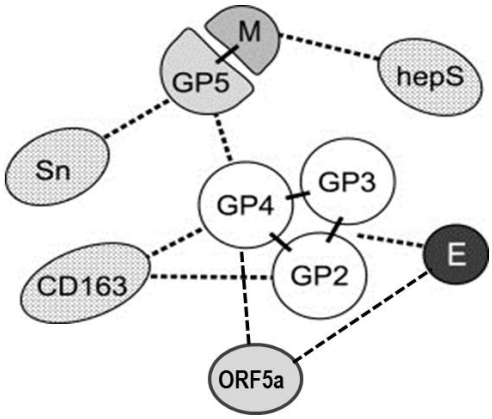
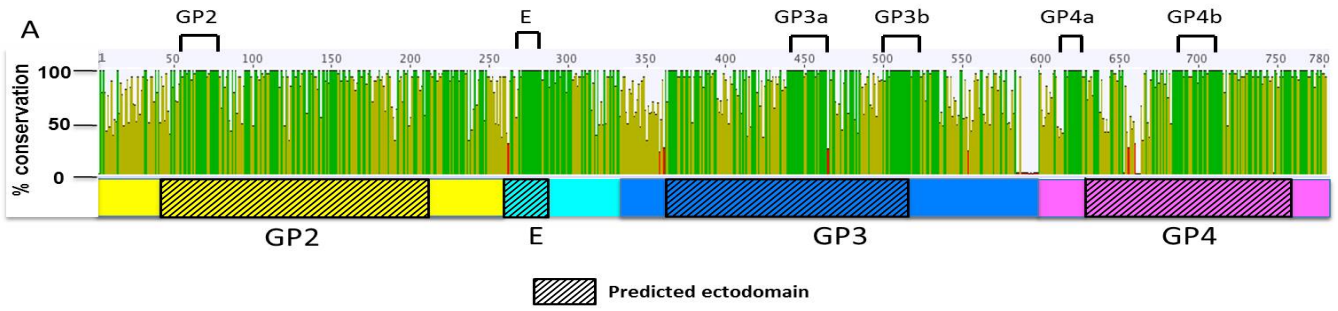


Figure 6.1 Minor envelope proteins of PRRSV. Adapted from Dokland, (2010) with information from Johnson et al. (2011) and Sun et al. (2015).

A) Structure and topology of proteins in the PRRSV envelope predicted from amino acid sequence. Transmembrane domains are represented as rectangles crossing the lipid bilayer, with stippled boxes being signal peptides and cleavage sight indicated by broken line. Glycosylation is shown as hexagons. Length is shown as a range resulting from variation between genotype 1 and 2. Ectodomain aa residues and size shown here are based on genotype 2 isolates. B) Interactions between PRRSV envelope proteins and host cell surface proteins. Solid lines indicate the well-established, possibly covalent interactions of GP5-M dimers and GP2-GP3-GP4 timers. Dashed lines indicate non-covalent interactions.



B

Peptide	Position*	Length (aa)	Sequence	Solubility
GP2	52-76	25	SDWFAPRYSVRALPFTLSNYRRSYE	PBS^
E	13-25	13	QLFVDAFTEFLVS	PBS^
GP3a	108-133	26	YAWLAFLSFSYTAQFHPEIFGIGNVS	NMP†
GP3b	169-192	23	YQHQVDGGNWFHLEWLRPFFSSWL	NMP†
GP4a	20-28	9	AFACKPCFS	PBS^
GP4b	82-113	32	TANVTDENYLHSSDLLMLSSCLFYASEMSEKG	DMSO‡

* aa position in full length protein sequence
^ 0.1M Phosphate buffered saline pH 7.4
† N-methyl-2-pyrrolidone
‡ Dimethyl sulfoxide

Figure 6.2 Conserved PRRSV minor envelope ectodomain peptides.

A) Alignment of 58 PRRSV sequences (56 from genotype 2, 2 from genotype 1). Open reading frames 2, 3, and 4 sequences were concatenated and translated. GP2 (yellow), E (aqua), GP3 (blue), and GP4 (pink) are represented. Sequence conservation is expressed as a percentage, with 100% meaning sequences are identical or completely conserved. Predicted ectodomain regions are marked with hashed boxes. Ectodomain peptide sequences with maximal conservation selected are marked with black lines at top of the figure. B) Conserved ectodomain peptide position, length and sequences. Peptides were synthesized by GenScript (Piscataway, NJ). Solubility testing was performed by GenScript, and the solvent used for initial dilution to 1mg/ml is indicated for each.

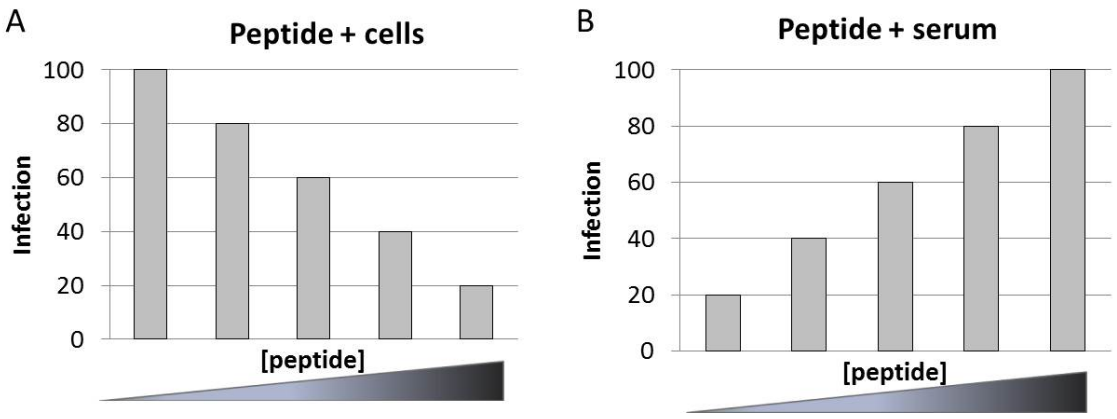


Figure 6.3 Expected outcome from peptide competition with neutralizing targets.

A) Depiction of predicted outcome of peptides added to cells before addition of virus. If peptide(s) bind cellular CD163 (mimicking virus binding), they will compete with virus in a dose-dependent manner to result in reduced infection with increasing concentration of peptide. B) Predicted outcome of peptides added to serum prior to addition of virus and infection of cells. If peptides bind neutralizing antibodies, they will compete with virus in a dose-dependent manner for binding and make more virus available for infection i.e. will inhibit neutralizing activity and result in increased infection with increasing concentration of peptide.

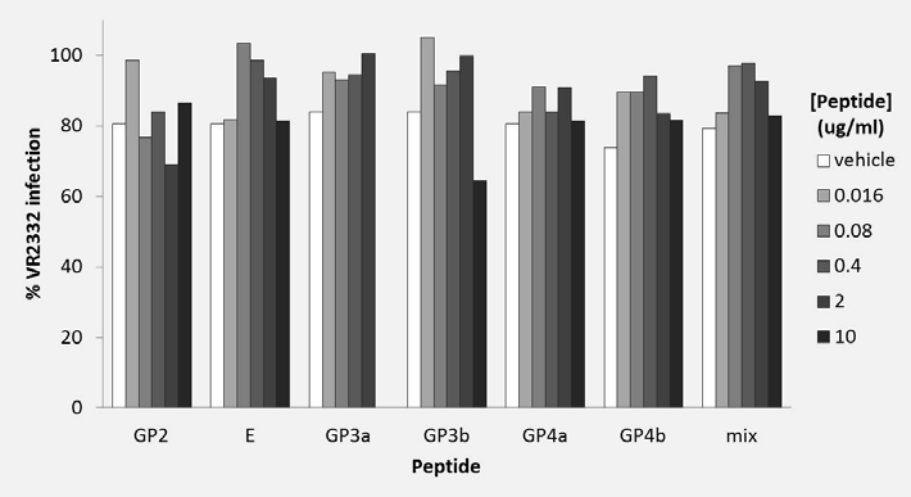


Figure 6.4 Peptide competition for cellular receptors. Effect of increasing concentration of six synthetic peptides and mixture of the six peptides on infection of MARC 145 cells by PRRSV VR2332 (MOI of 0.5) relative to infected control wells with no peptides. Vehicle only controls are shown for reference.

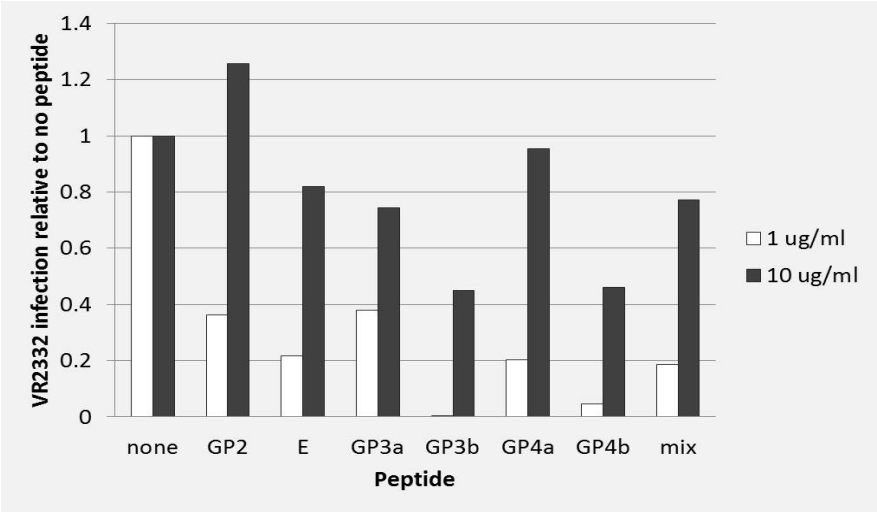


Figure 6.5 Peptide competition for neutralizing antibodies**.** Effect on PRRSV infection by two different peptide concentrations (1 and 10 ug/ml) for each of six synthetic peptides, and mixture of the six peptides, incubated with PRRSV-neutralizing serum relative to controls with no peptides added. PRRSV VR2332 (MOI 0.5) was incubated with the peptide-serum mix prior to inoculation of MARC 145 cells.

Chapter 7 Perspectives on immune protection against PRRSV

The aim of this dissertation was to re-evaluate aspects of PRRSV immunity in light of contemporary information for novel clues to identify mechanisms of protection. The approach was to investigate the role of recently discovered PRRSV protein ORF5a in immunity, and to re-evaluate the role of neutralizing antibodies in PRRSV cross-protection in light of new information emerging from other rapidly evolving RNA viruses.

Identification of ORF5a protein was significant because it is the first example of a PRRSV protein discovered using unbiased screening instead of rote prediction by sequence alignments. Previously, PRRSV structural proteins were based on comparisons with other previously characterized arteriviruses. Prior to ORF5a, the ORF2b product or E protein, was most recently identified in 2001, following its discovery in EAV as the first bicistronic overlapping arterivirus ORF (Snijder et al., 1999; Wu et al., 2001). ORF5a was unknown prior to 2011. Being small and in relatively low abundance, it had not been observed by virion protein electrophoresis, and was not known to exist in the other arteriviruses. It was therefore important to examine the role of ORF5a protein in PRRSV immunity, given the potential to discover much needed new targets or mechanisms of immunity.

Building on the pivotal work of Johnson et al. and Firth et al. describing the presence of ORF5a (Firth et al., 2011; Johnson et al., 2011), and in establishing its necessity (Sun et al., 2013), we have demonstrated further evidence for its importance in the extreme balancing act of concurrently maintaining functional regions of overlapping ORF5a and GP5. This finding has implications for understanding N-linked glycosylation site variability in GP5 in a new context. The necessity for glycosylation residues in PRRSV envelope glycoproteins is not well understood. They are not required for infection, but important roles may include protein structure, virion assembly, and host immunity (Li and Murtaugh, 2015). Evidence for an immunologic role

such as shielding of neutralizing epitopes is conflicting, and will likely be resolved as a more mechanistic understanding of PRRSV antibody neutralization is developed. ORF5a immunization did not elicit protection against PRRSV despite a robust ORF5a antibody response that was not virus-neutralizing. T cell responses to ORF5a were not evaluated since there was no indication of a protective effect. While this study does not strictly exclude a role for ORF5a in PRRSV immunity, it is highly unlikely to be a target of neutralizing antibodies because the predicted ectodomain is so small. ORF5a protein has recently been shown to interact with itself, as well as E protein and GP4 (Oh and Lee, 2012; Sun et al., 2015). Evidence from our study suggests the conserved RQ-rich region is of functional significance, and it appears likely to be involved in virion assembly through interaction with other envelope proteins or binding viral RNA. It will be informative to evaluate progeny virion structure and morphology in ORF5a deficient clones.

Discovery of ORF5a raises the question of whether other potential small overlapping PRRSV proteins exist. Further investigation is indicated, since small accessory proteins can have vital functions in countering host antiviral responses, as demonstrated for the HIV accessory proteins vif, vpr, vpx, vpu, and nef (Collins and Collins, 2014; Harris et al., 2012). Subgenomic mRNA 5 was a relatively easy target to investigate for PRRSV owing to the vast number of ORF5 sequences available; it is the primary means to identify viral variants by examining change over time in a herd to determine whether an outbreak is due to the same virus or a new introduction. Other PRRSV ORFs have been predicted or identified (Juan Abrahante and Michael Murtaugh, *personal communication*). The accessibility and decreasing cost of next-generation sequencing technology has led to a rapidly increasing number of whole PRRSV genomes available in recent years (Brar et al., 2015). This may facilitate identification of potential ORFs in other genomic regions and provide greater capacity to identify signatures of selection and minor population variants (Lu et al., 2014).

Discovery of broadly neutralizing antibody activity against PRRSV under typical field conditions has significant implications for understanding PRRSV immunity. Demonstration that the cross-neutralizing activity confers cross-protection against diverse PRRSV strains confirms the importance of this observation, and offers potential for new immunologic approaches to elicit cross-protection. Previously described PRRSV neutralizing epitopes were strain-specific, or epitopes implicated in cross-protection are sparsely described (Trible et al., 2015). Just as information on neutralization of other RNA viruses prompted re-evaluation of the situation for PRRSV, it has also shown us that conserved regions critical for receptor binding or structure where mutations are deleterious can be targets of broadly neutralizing antibodies (Corti and Lanzavecchia, 2013; Laursen and Wilson, 2013; West et al., 2014). With this in mind, we sought to evaluate conserved regions in minor envelope protein ectodomains as targets of neutralization. Although the simplistic peptide competition approach yielded negative results, it ruled out the possibility that linear peptides were neutralization targets. More sophisticated approaches will be required to determine viral targets of cross-neutralizing antibodies.

The greatest factor limiting mechanistic understanding of PRRSV neutralization is that individual neutralizing antibody molecules have not yet been identified. Polyclonal serum and immunoglobulin preparations have complexities that preclude understanding specific interactions (Klasse, 2014). Therefore, a major goal of ongoing research is to identify and isolate individual B cells and neutralizing antibodies, as has been achieved for other RNA viruses (Corti and Lanzavecchia, 2013). First and foremost, it will allow understanding as to whether truly broadly neutralizing antibody molecules to PRRSV exist, or whether antibodies from B cells with different specificities combine to confer breadth. Genetic and structural features of potent neutralizing antibodies can be determined through sequencing of immunoglobulins, and comparing them to PRRSV-specific, but non-neutralizing antibodies. Monoclonal neutralizing

antibodies will also enable identification of viral targets of neutralization. Such mechanistic studies are likely to provide solutions by informing design of new vaccines or strategies to promote efficient formation of broadly-protective neutralizing antibodies.

In addition to detailed mechanistic studies, investigating conditions that facilitate efficient neutralizing antibody development in experimental and field studies offers the potential for short-term solutions by better utilization of existing immunologic tools. Specifically, relationships between degree of cross-neutralization and age/parity of sows, number of exposures to PRRSV or vaccine, and diversity and virulence of virus strains will be evaluated. We aim to use this information to determine how long it takes for cross-neutralization to develop in sows, if cross-neutralization development is related to the number or diversity of virus exposures, and if conditions required for development of PRRSV cross-neutralizing antibodies in sows under field conditions can be rapidly applied to develop improved immunization strategies against PRRSV with existing tools (vaccines and/or targeted exposure to virus). The immediate goal is to provide better protection against the diverse virus strains that sows encounter in the field, and hence reduce production losses associated with this terrible disease.

Moving forward, it will be important to develop a framework for evaluating neutralizing activity as a correlate of cross-protection for assessing and predicting PRRSV immune/protected status. Initially, the relationship between cross-neutralizing antibodies and protection will be evaluated in a herd outbreak scenario, comparing serum samples from sows with clinical PRRS disease, and an equal number of neighboring sows without clinical signs of disease. Viremia and cross-neutralizing antibodies will be analyzed by logistic regression with two classes, clinical disease and absence of disease, and the continuous variable, neutralizing antibody titer. As viral targets of cross-neutralization are understood, this model can be refined to identify specific correlates of cross-protection.

From a practical standpoint, the problems with PRRSV immunity identified in Chapter 1 are maintenance of virus in population by transmission to naïve animals, or re-introduction of PRRSV to a recovered herd owing to population turnover and incomplete immunity. How can this be overcome with induction of a cross-neutralizing antibody response? Susceptible young animals can be protected by colostrum-derived neutralizing antibodies. This is feasible if neutralizing antibodies can be induced reliably in sows. With greater understanding, perhaps sows can be strategically boosted to provide high level to piglets. It is important to consider whether there is a risk of antibody-dependent enhancement of infection during period of waning maternal immunity. Further studies are required to establish the likelihood of this phenomenon. A concern for protection of piglets is the time it takes for neutralizing antibody breadth to develop, possibly leaving a window of vulnerability following decline of maternally-derived neutralizing antibodies. With current tools and knowledge, it would require that piglets be infected with replicating virus and hence likely experience disease. However, it may be possible in the future to induce a robust cross-neutralizing antibody response in the absence of live virus, through piglet immunization with targeted neutralizing epitope subunit vaccines, or induction of recombinant broadly neutralizing antibodies that are expressed by host cells. In addition, better immunization regimens are needed for gilt acclimation that induces cross-neutralizing antibodies to provide protection during the vulnerable period of herd entry. The goals of these strategies are to reduce the overall viral burden and disease in a herd, support elimination of PRRSV and prevent introduction of new strains and PRRSV outbreaks.

Conclusion

The studies herein took an exploratory approach to investigate new findings in PRRSV research and question how these fit the existing paradigm of PRRSV immunity. Our findings suggest that ORF5a is not important for immune protection against PRRSV but plays a critical role in the virus life cycle. It will be important to further understand its role in PRRSV biology, and to investigate other proposed novel PRRSV ORFs. The serologic cross-neutralization of diverse PRRSV demonstrated in these studies are significant findings that have come from taking a fresh look at PRRSV immunity in light of contemporary techniques and comparative systems, opening the door for invigorated research into cross-neutralizing antibodies to PRRSV. It is crucial to understand how broadly neutralizing antibodies can be induced and harnessed for protection against PRRSV. It will be important to determine if conserved epitopes exist on diverse PRRSV strains that are targets for cross-neutralization. This will require a more detailed understanding of individual neutralizing antibody molecules, facilitated by individual B cell isolation and culture. It is hoped that understanding features of cross-neutralizing antibodies and their viral targets will facilitate improved strategies for robust and predictable PRRSV cross-protection that will assist in PRRSV elimination, and ultimately, eradication efforts.

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Appendix 1 Comparison of PRRSV neutralizing activity with PRRSV ELISA

Purpose of study

To compare nucleocapsid and BioChek PRRSV ELISA, and evaluate with serum samples of known neutralizing characteristics in order to determine whether the BioChek ELISA measures neutralizing antibodies.

Approach

PRRSV nucleocapsid and BioChek ELISA were compared using serum from PRRSV immune herds with high and moderate neutralizing activity and also to PRRSV immune animals with no neutralizing activity. Results were used to evaluate correlation between neutralizing activity and BioChek ELISA.

Table 1: Characteristics of animals/herds for the samples analyzed

Herd	N	BioChek (+)	Type of exposure	Virus strain	Day post infection	Neutralizing activity
1	20	20	Field	Multiple	Unknown	High
2	16	14	Field	Multiple	Unknown	Moderate
3	10	10	Field	Multiple	Unknown	Variable
4	22	15	Experimental	VR2332	21	No
5	15	7	Experimental	Lelystad	26	Undetermined*

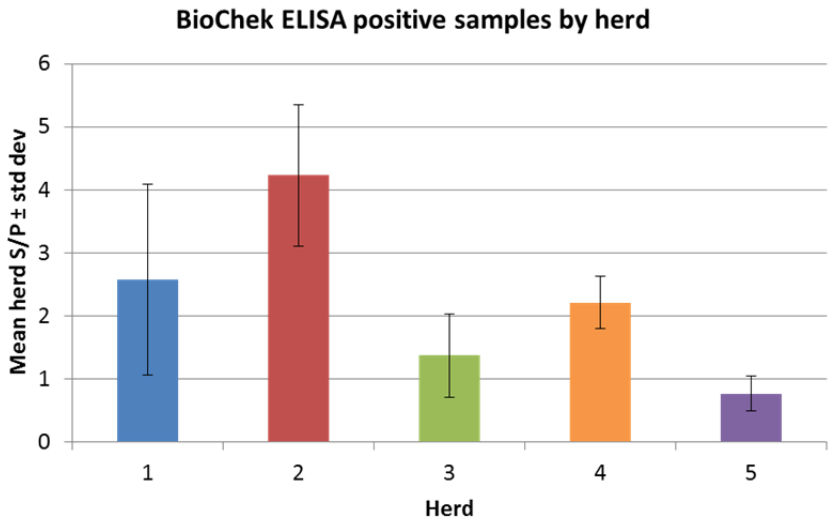
* Neutralizing activity was not evaluated in these animals. However they would be expected to have low to no cross-neutralizing activity based on having a single strain experimental infection and samples being from 26 days after challenge.

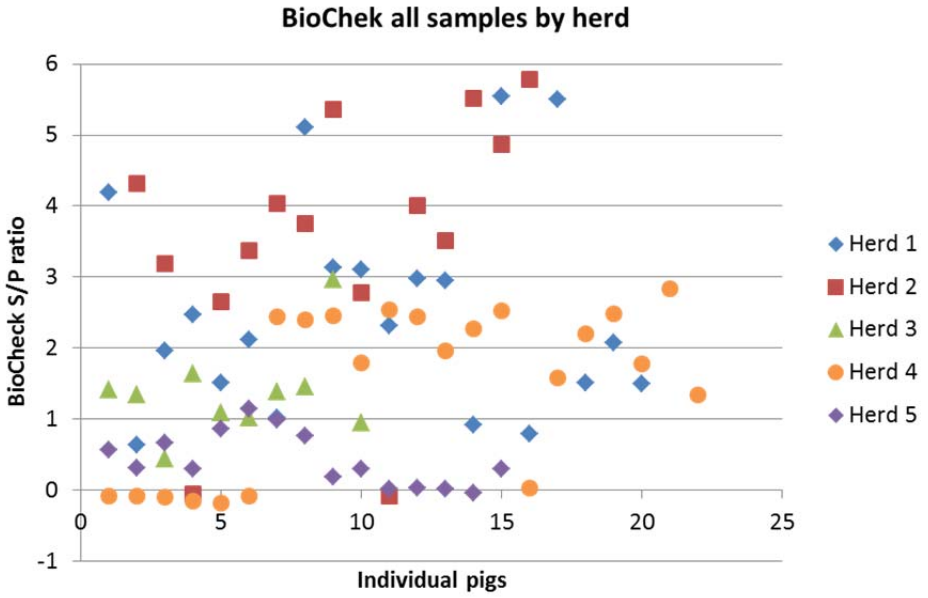
Findings

BioChek S/P ratios are presented below for 83 serum samples from 5 herds described in Table 1. All samples were run in duplicate according to the kit protocol. Samples from herds 1-3 were run on one plate, with those from 4 and 5 on a second plate. Data from both plates were considered valid as defined by the kit protocol (Table 2).

Table 2: Absorbance values for control samples to determine validity of assay

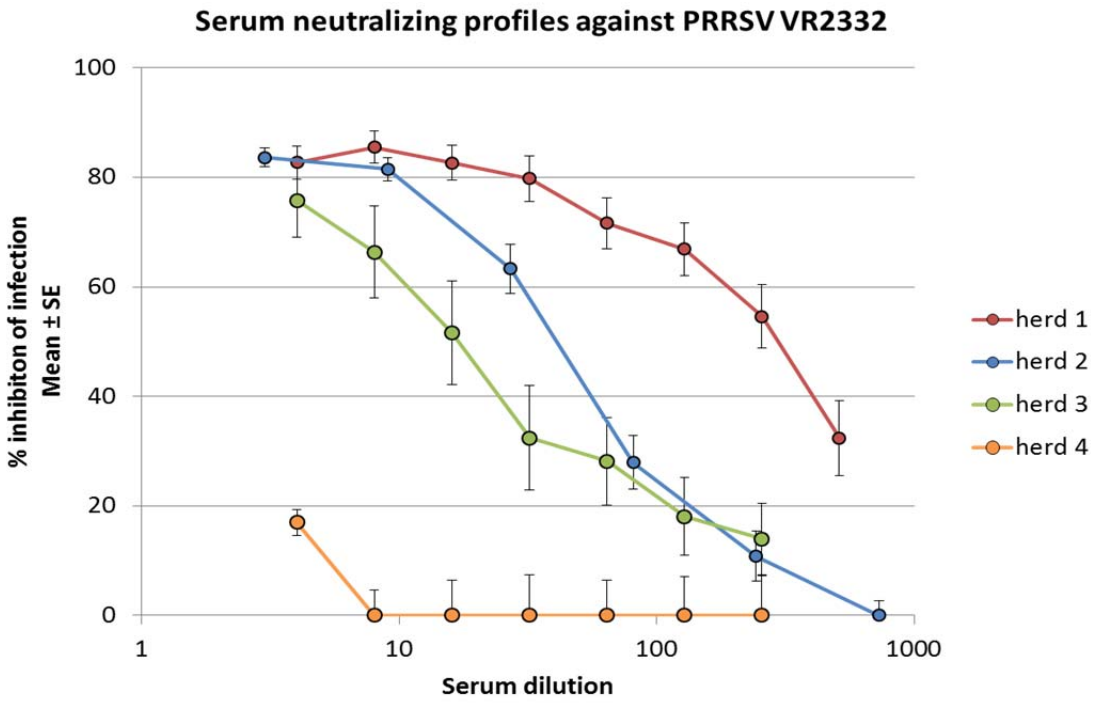
Absorbance (405nm)	Positive control	Negative control	Difference	Valid
Plate 1	0.418	0.150	0.269	Yes
Plate 2	0.437	0.162	0.274	Yes
Cut-off to determine validity		<0.35	>0.15	

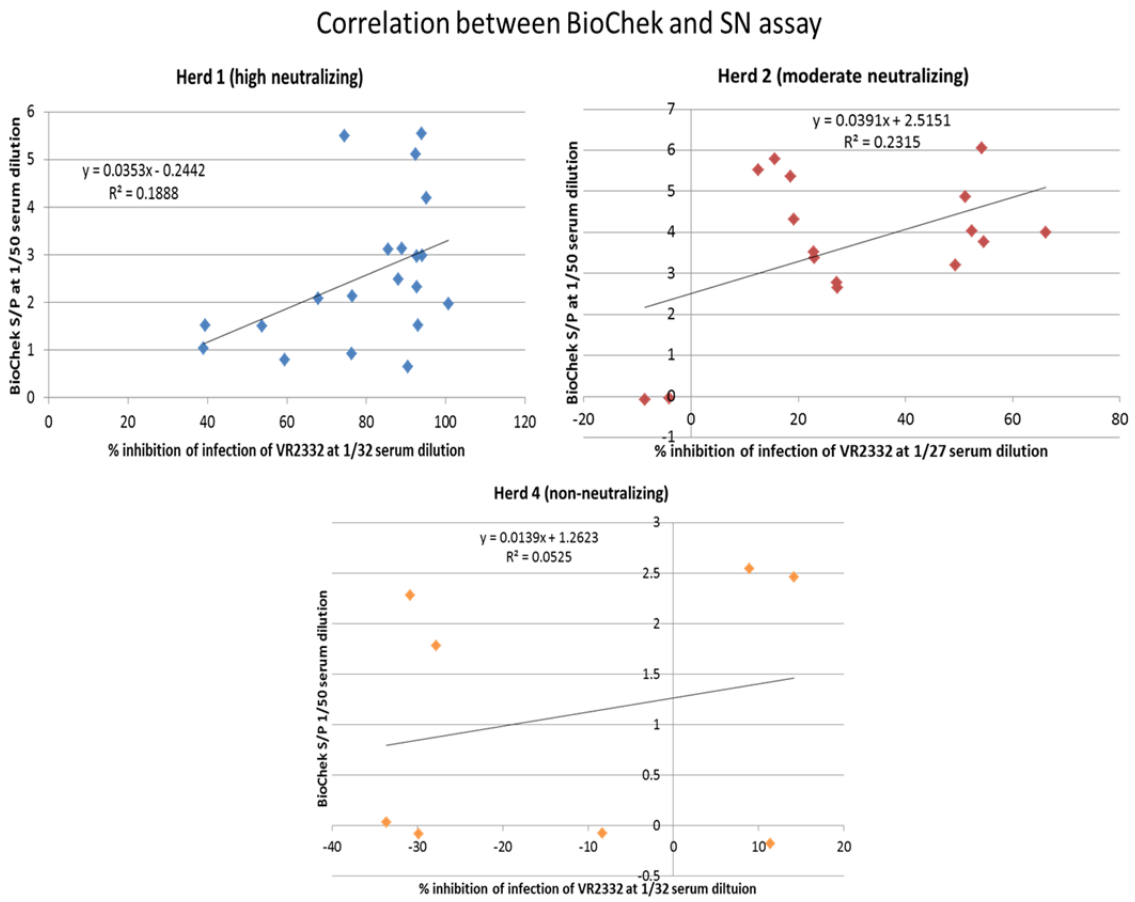

BioChek ELISA positive samples by herd
Mean herd S/P ± std dev
6
5
4
3
2
1
0
1
2
3
4
5
Herd


BioChek all samples by herd
BioCheck S/P ratio
6
5
4
3
2
1
0
-1
0
5
10
15
20
25
Individual pigs
Herd 1
Herd 2
Herd 3
Herd 4
Herd 5

Correlation analysis between BioChek and serum neutralizing assay

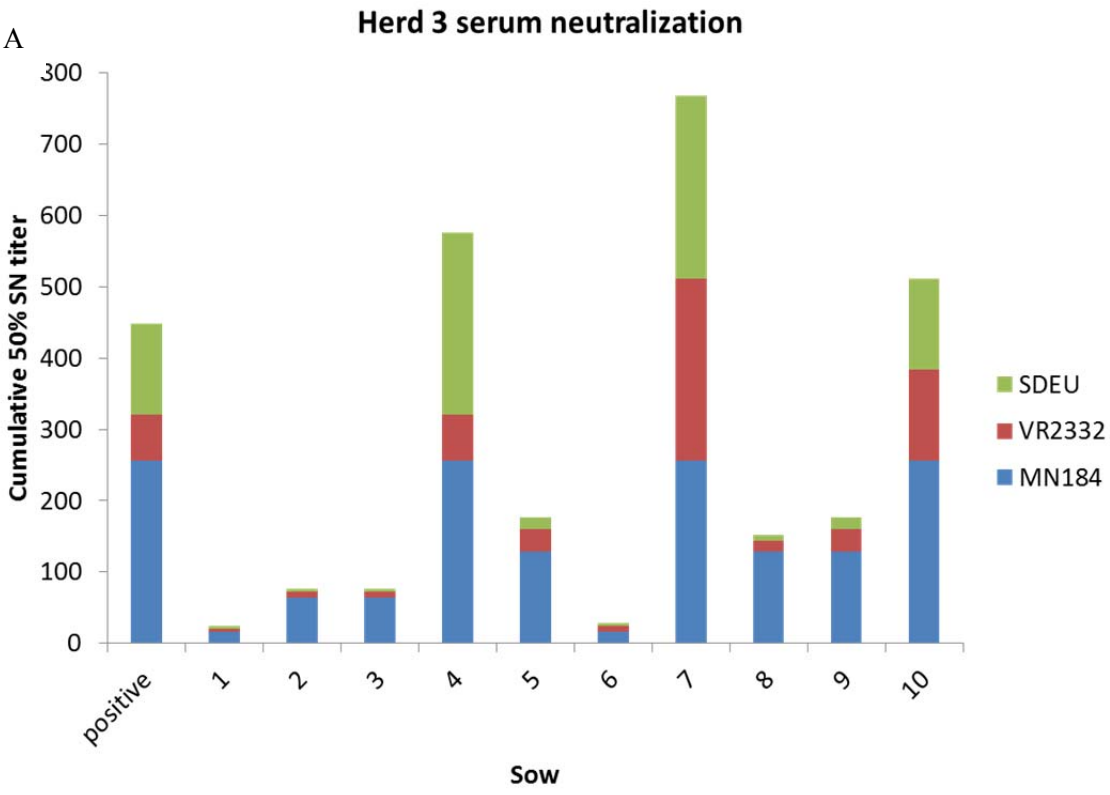
Mean serum neutralizing profiles are shown below for herds 1-4. No neutralizing data is available for herd 5 at this time. Correlation analysis between BioChek ELISA and neutralizing activity was performed using neutralizing data for 1/32 serum dilution (shown as % inhibition of VR2332 infection). Neutralizing data was generated on MARC 145 cells in an ELISA-based serum neutralization assay.



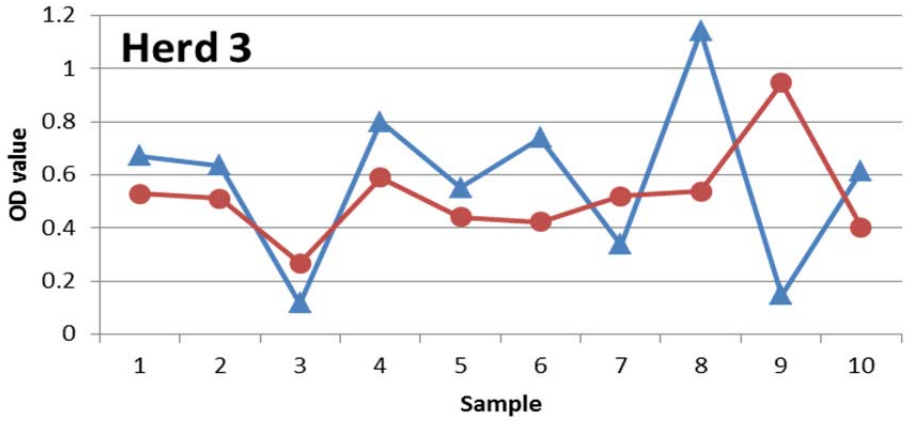


Herd 3 was further analyzed, as more individual variation in neutralization activity existed. The figures below show neutralizing activity of 10 herd 3 sows against three diverse PRRSV strains, and the corresponding ELISA OD values (BioChek in red circles, nucleocapsid in blue triangles) (A). Variability in neutralization between sows was not mirrored in ELISA OD values (B).

A


Herd 3 serum neutralization
Cumulative 50% SN titer
300
700
600
500
400
300
200
100
0
positive
1
2
3
4
5
6
7
8
9
10
Sow
SDEU
VR2332
MN184

B


Herd 3
OD value
1.2
1
0.8
0.6
0.4
0.2
0
1
2
3
4
5
6
7
8
9
10
Sample

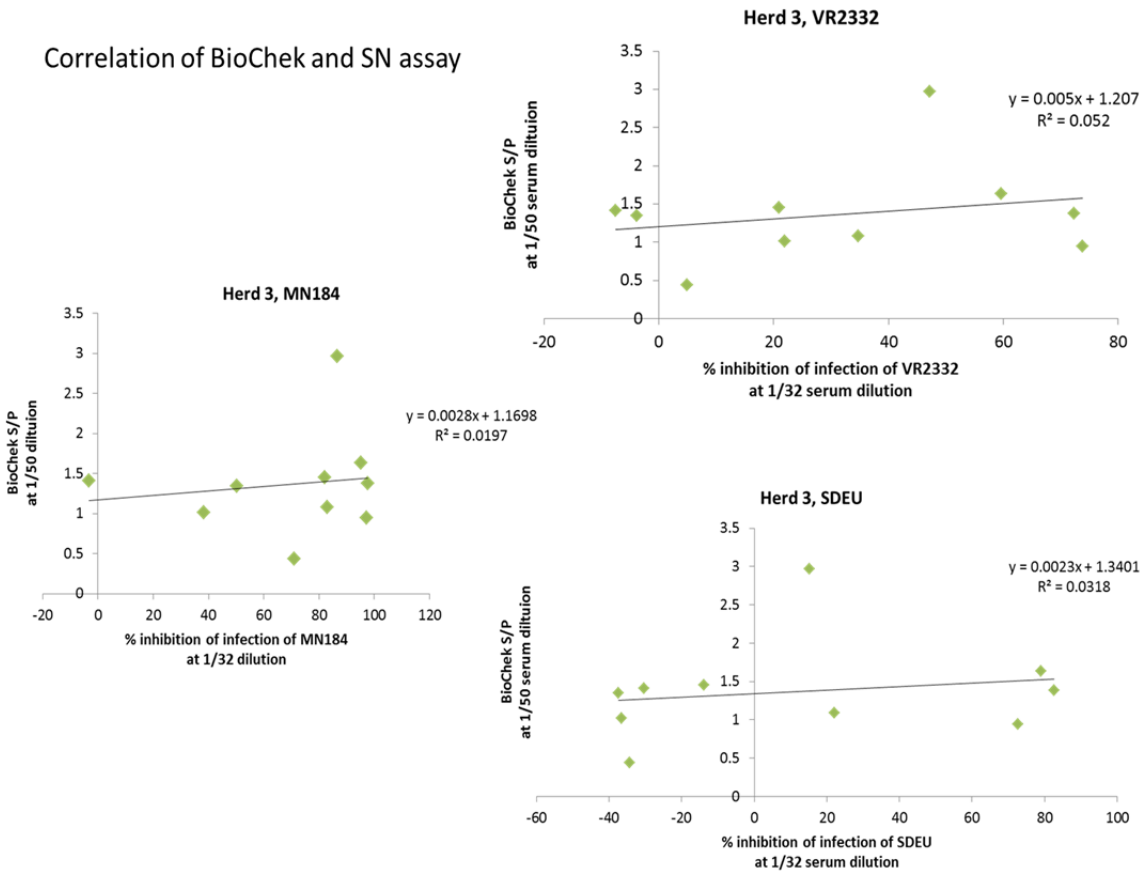


Table 3: Correlation of herd 3 neutralizing activity and BioCheck ELISA

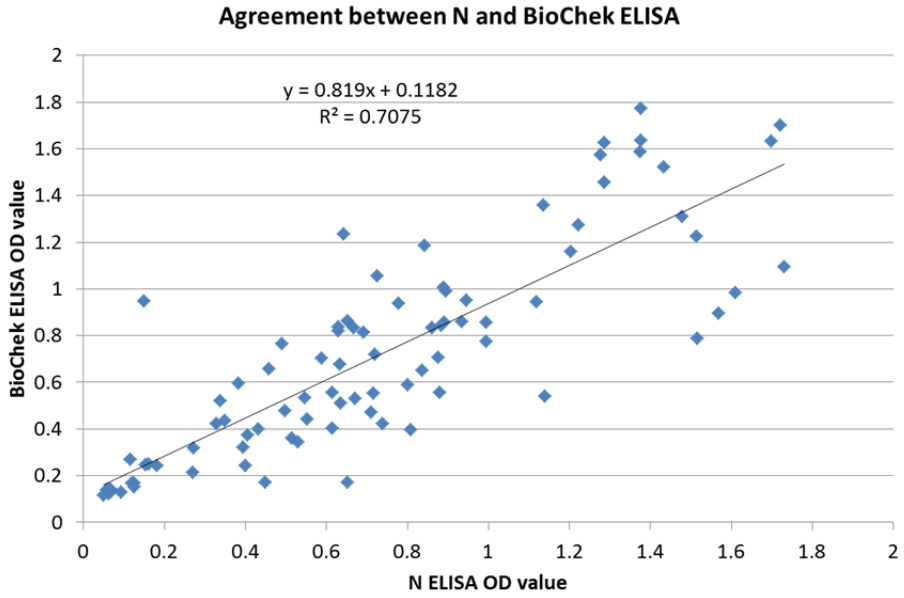
PRRSV strain	R-squared
VR2332 (genotype 2)	0.05
MN184 (genotype 2)	0.02
SDEU (genotype 1)	0.03

Correlation analysis of herd 3 ELISA and neutralizing activity against three diverse virus strains revealed no relationship between the two assays. Looking at the above data, and the summary of corrlation in Table 4, there is a lack of agreement between ELISA and neutralizing activity.

Table 4: Summary of correlation between BioChek ELISA and neutralizing activity against PRRSV VR2332

Herd	Neutralizing activity	R-squared
1	High	0.19
2	Moderate	0.23
3	Variable	0.05
4	Negative	0.05

Comparison of BioChek and nucleocapsid ELISA. The Murtaugh Lab PRRSV ELISA uses a bacterially-expressed recombinant PRRSV VR2332 nucleocapsid protein to coat plates, serum at 1/50 dilution, and a HRP conjugated secondary antibody. OD values were compared between BioChek and nucleocapsid ELISAs, and correlation between the two, determined by R-squared, was 0.71. Overall agreement was determined by the PRRSV positive and negative status of each sample by each assay using BioChek S/P cutoff value of 0.4, and nucleocapsid OD cutoff value of 0.16.



Agreement between BioChek and N ELISA

		Nucleocapsid		
		(+)	(-)	Total
BioChek	(+)	64	2	66
	(-)	5	12	17
	Total	69	14	**83**

$$p_o = \frac{64+12}{83} = \frac{76}{83} = 0.92$$

Overall agreement = 92%

In conclusion, BioChek and PRRSV nucleocapsid ELISA assays show 92% agreement overall for determining seroconversion to PRRSV. The magnitude of absorbance values was similar between the two assays for most samples. This suggests either the BioChek ELISA is predominantly measuring the same antibody response to nucleocapsid, or to other proteins with a similar magnitude of response. Discordant samples were not further analyzed by ELISA to other recombinant PRRSV proteins. Neither nucleocapsid, nor BioChek ELISA values were correlated with PRRSV neutralizing activity in serum. Our analysis suggests the BioChek ELISA is not preferentially detecting PRRSV neutralizing antibodies.