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Characterization of the molecular mechanisms underlying neutralization, immune escape, and inhibition of porcine reproductive and respiratory syndrome virus

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Characterization of the molecular mechanisms underlying neutralization, immune escape, and inhibition of porcine reproductive and respiratory syndrome virus

by

Alyssa Bennett Evans

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

DOCTOR OF PHILOSOPHY

Major: Genetics

Program of Study Committee:
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Iowa State University
Ames, Iowa
2016

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# TABLE OF CONTENTS

**LIST OF TABLES**

iv

**LIST OF FIGURES**

v

**ACKNOWLEDGEMENTS**

vi

**ABSTRACT**

viii

**CHAPTER 1: GENERAL INTRODUCTION**

1

Introduction 1
Overall Goal 3
Specific Aims 3
Dissertation Organization 4
Literature Cited 5

**CHAPTER 2: LITERATURE REVIEW**

9

Viral Variation and its Consequence on Infection 9
The quasispecies structure of RNA virus populations 9
Antigenic variation and immune escape 11
Antiviral drug resistance 16
Emergence of new viral diseases 17
Summary of viral variation 20
Porcine Reproductive and Respiratory Syndrome Virus 20
Genome and virion structure 21
Replication and molecular pathogenesis 22
Immune response to PRRSV 27
PRRSV antigenic variation and immune escape 29
Vaccines and antivirals 32
Summary of porcine reproductive and respiratory syndrome virus 33
Literature Cited 33

**CHAPTER 3: PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS UTILIZES MULTIPLE GENETIC PATHWAYS OF IMMUNE ESCAPE DURING INFECTION IN VIVO**

52

Abstract 52
Importance 53
Introduction 54
Materials and Methods 56
Results 64
Discussion 76
Literature Cited 81
Tables 87
Figures 93
<table>
<thead>
<tr>
<th>Chapter Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHAPTER 4: CHARACTERIZATION OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS NEUTRALIZATION AND REPLICATION PHENOTYPES OF NEUTRALIZING ANTIBODY ESCAPE VARIANTS</td>
<td>100</td>
</tr>
<tr>
<td>Abstract</td>
<td>100</td>
</tr>
<tr>
<td>Introduction</td>
<td>101</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>105</td>
</tr>
<tr>
<td>Results</td>
<td>110</td>
</tr>
<tr>
<td>Discussion</td>
<td>116</td>
</tr>
<tr>
<td>Literature Cited</td>
<td>121</td>
</tr>
<tr>
<td>Tables</td>
<td>125</td>
</tr>
<tr>
<td>Figures</td>
<td>126</td>
</tr>
<tr>
<td>CHAPTER 5: IDENTIFICATION AND CHARACTERIZATION OF SMALL MOLECULE INHIBITORS OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS</td>
<td>130</td>
</tr>
<tr>
<td>Abstract</td>
<td>130</td>
</tr>
<tr>
<td>Introduction</td>
<td>131</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>134</td>
</tr>
<tr>
<td>Results</td>
<td>140</td>
</tr>
<tr>
<td>Discussion</td>
<td>147</td>
</tr>
<tr>
<td>Literature Cited</td>
<td>150</td>
</tr>
<tr>
<td>Tables</td>
<td>154</td>
</tr>
<tr>
<td>Figures</td>
<td>156</td>
</tr>
<tr>
<td>CHAPTER 6: GENERAL DISCUSSION</td>
<td>164</td>
</tr>
<tr>
<td>General Summary</td>
<td>164</td>
</tr>
<tr>
<td>Importance</td>
<td>164</td>
</tr>
<tr>
<td>Limitations and Alternative Strategies</td>
<td>169</td>
</tr>
<tr>
<td>Future Work</td>
<td>173</td>
</tr>
<tr>
<td>General Conclusions</td>
<td>175</td>
</tr>
<tr>
<td>Literature Cited</td>
<td>176</td>
</tr>
</tbody>
</table>
LIST OF TABLES

**Table 3.1**: Summary of sequenced clones and viral variation 87

**Table 3.2**: Summary of significant nucleotide variants and associated amino acid changes in ORF6-6 and nsp2 88

**Table 3.3**: Predominant ORF6-6 haplotypes within late day virus samples 90

**Table 4.1**: Summary of chimeric viruses 125

**Table 5.1**: Summary of anti-PRRSV compounds’ potency and cytotoxicity 154

**Table 5.2**: Summary of anti-PRRSV potency of compounds up to $10^5$ FFU virus 155
LIST OF FIGURES

Figure 3.1: Viremia and neutralization profiles in selected pigs 93
Figure 3.2: Temporal changes in the population structure in nsp2 and ORF2-6 94
Figure 3.3: ORF2-6 quasispecies structure and late day virus haplotypes 95
Figure 3.4: Neutralization phenotypes of rebound haplotypes against autologous rebound sera 96
Figure 3.5: The effect of single amino acid variants from ORF2-6 rebound haplotypes on resistance to autologous neutralizing serum 98
Figure 3.6: Immune escape variants are sensitive to neutralization by broadly neutralizing sera 99
Figure 4.1: Neutralizing activity of PHGC pooled serum versus FL12, escape, and non-escape viruses 126
Figure 4.2: Attachment and PRRSV RNA replication assays ± pooled serum 127
Figure 4.3: Particle to infectivity ratios for FL12 and the NAb escape and non-escape viruses 128
Figure 4.4: Replication kinetics assays measuring virion production and FFU production of FL12 and the NAb escape variants 129
Figure 5.1: Synthesis routes for atracylodinol and analogs 156
Figure 5.2: Anti-PRRSV activity screens of the fifteen synthesized compounds 159
Figure 5.3: Inhibition assays to determine IC<sub>50</sub>s of the eight compounds with inhibitory activity of PRRSV 160
Figure 5.4: Potency assays of the eight anti-PRRSV compounds up to 10<sup>5</sup> FFU 161
Figure 5.5: Mechanism of action of compound 19 162
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ABSTRACT

The rapid mutation rate of RNA viruses leads to significant genetic and antigenic variation. This diversity presents great challenges for the development of effective vaccines and treatments. These challenges highlight the need for a better understanding of the molecular mechanisms underlying antigenic variation and alternative treatment strategies. While significant research to address these problems has been undertaken in human diseases, less is known about these processes in many veterinary diseases, including the economically significant porcine reproductive and respiratory syndrome virus (PRRSV). Although immune selection has been a proposed mechanism for the diversity in PRRSV and its continual circulation within and between herds, direct evidence of immune escape variants arising during the course of infection in vivo has never been shown. To examine the role of immune selection and escape during PRRSV infection, we examined genetic and antigenic variation of PRRSV using retrospective, sequential samples from five experimentally infected pigs with differing virological and immunological outcomes, including three pigs that experienced a rebound in viremia, suggesting viral escape from host immune control. Sequence analyses revealed limited genetic variation during acute PRRSV infection. However, distinct envelope protein-encoding ORF2-6 haplotypes were identified in each pig’s late day virus. Chimeric viruses containing all or part of predominant ORF2-6 haplotypes were tested in virus neutralization assays using autologous sera, and in four viruses, genetic variation in ORF2-6 resulted in antigenic variation and immune escape. Immune escape mapped to multiple envelope proteins, including GP5 and/or GP2, GP3, and GP4 and usually, but not always, required a
combination of amino acid changes. Importantly, the key amino acid changes and/or combination of changes that mediated escape differed by pig and by virus haplotype. To test if the mutations that mediated immune escape resulted in a reduction in replication fitness of the viruses, we performed a series of infectivity and replication kinetics assays using the escape variants and the parental virus. Our results showed that one of the four escape variants had reduced infectivity compared to the parental virus, but none of the escape variants had markedly reduced growth rates in MARC-145 cells. PRRSV pooled neutralizing antibody was found to primarily target a post-attachment, pre-genome synthesis step of replication. These data demonstrate that PRRSV envelope proteins are under immune selection that contributes to the emergence of replication-fit immune escape variants. The diverse strategies of immune escape likely contribute to difficulties in producing effective vaccines for PRRSV, and therefore alternative control strategies are needed. Antivirals have proven to be an extremely successful treatment strategy for several human viral diseases, and have the potential for use in the treatment of PRRSV. Previously, the natural compound atracylodinol was reported to have anti-PRRSV activity in vitro. Atractylodinol and fourteen analogs were synthesized and their anti-PRRSV activity was characterized in vitro. Seven of the analogs had potent inhibitory activity against 5-log_{10} infectious units of PRRSV at low μM concentrations. Analog compound 19 was shown to inhibit PRRSV primarily at a post-attachment step during PRRSV entry. These results provide evidence that the atracylodinol analogs are promising antiviral candidates for trials in pigs. Overall, the work from this dissertation indicates that selective pressure by the immune system contributes to the vast genetic diversity of PRRSV, and there is potential for the treatment of PRRSV outbreaks with antivirals.
CHAPTER 1: GENERAL INTRODUCTION

Introduction

The rapid rate of viral evolution and quick accumulation of mutations is a major contributing factor to the lack of effective vaccines for many viral diseases, both for human and animal infections. Some of the most researched human diseases, including human immunodeficiency virus type 1 (HIV-1), hepatitis C virus (HCV), and influenza virus, still lack effective vaccines due to the high mutation rate of these viruses and their ability to evade the host immune response (1-5). The high amount of genetic variation within these viruses makes developing vaccines that are effective against diverse strains of the virus extremely challenging. However, in recent years classes of broadly neutralizing antibody (bNAb) have been discovered targeted against HIV-1 or influenza A that are capable of neutralizing diverse strains within these viruses (6). The broadly acting nature of the bNAbs is due to their targeting of epitopes within critical functional domains of the virus, such as the highly conserved stem region of influenza’s hemagglutinin (HA) envelope protein that is critical for fusion of the virus with the host’s endosomal membrane (7, 8), and the receptor and co-receptor binding sites of HIV-1’s envelope protein (6, 9, 10). The discovery of these bNAbs provides hope for the development of “universal vaccines” against HIV-1, influenza, and other viruses that have thus far been elusive. However, bNAb escape mutants do arise, and in fact are commonly found in chronically infected HIV-1 patients. However because escape is mediated through viral variation in functionally important domains, the bNAb escape variants are usually associated with decreased
replication fitness (6, 11). Therefore, understanding the specific interactions between
viruses and hosts that mediate immune escape, and the effect these mutations have on viral
replication will provide crucial information into viral pathogenesis that can lead to better
treatment and vaccine strategies.

Porcine reproductive and respiratory syndrome virus (PRRSV) has remained an
economically significant disease of swine since its emergence in both Europe and the
United States in the late 1980's (12-14). Despite nearly thirty years of research, there is
currently no PRRSV vaccine available that is effective against heterologous strains of the
virus (15, 16). As with HIV-1 and influenza A, the lack of an effective vaccine is largely due
to a high degree of genetic variation among PRRSV strains (17). However, unlike in HIV-1
and influenza A, the role of immune escape variants in infection is poorly understood, and
no direct evidence of immune escape in PRRSV infection in vivo currently exists. There is
some indirect evidence of immune selection via characterization of in vitro escape variants
and bioinformatics-based approaches (18-20). However, several studies of PRRSV variation
in vivo have found no evidence of immune selection or immune escape (21-23). The lack of
evidence of immune selection and immune escape may be due in part to the weak adaptive
immune response elicited by PRRSV (24, 25). In order to evaluate the existence of PRRSV
immune escape variants, the work described in this dissertation utilized retrospective
samples available from the PRRS Host Genetics Consortium from experimentally infected
pigs that experienced rebound in viremia following an initial clearance of the virus from
the bloodstream (26, 27). Rebound viremia suggested escape from the initial host immune
control, providing a unique sample set to identify immune selection pressures and escape
variants. Further characterization of the identified escape variants was carried out in order
to identify the mechanism of neutralization of PRRSV, and the effect of immune escape on viral replication fitness. In addition, because of the weak immune response and difficulty in making effective vaccines against PRRSV, the use of antiviral drugs may be a viable treatment option. To date, few anti-PRRSV compounds have been identified, and currently none are used in the field. Therefore, we identified and characterized small molecules for anti-PRRSV activity *in vitro*.

**Overall Goal**

To address the knowledge gap in the role of immune escape in PRRSV and to increase potential treatment options for PRRSV infection, the overall goal of this dissertation research was to understand the immune selective pressures underlying PRRSV variation, the consequences of variation on viral pathogenesis within pigs during infection, and the susceptibility of PRRSV to synthesized small molecule compounds.

**Specific Aims**

In order to achieve the overall goal, the specific aims of this dissertation research are:

1. Characterize genetic variation and immune selection of PRRSV within experimentally infected pigs.

2. Evaluate PRRSV envelope protein variants for escape from neutralizing antibody.

3. Characterize the mechanism of neutralization and consequences of neutralizing antibody escape on virus replication fitness.
4. Identify and characterize small molecule compounds for inhibitory activity of PRRSV.

**Dissertation Organization**

This dissertation is organized into six chapters. Chapter 1 includes a general introduction, and the overall goals and specific aims of the dissertation research. A review of the literature pertaining to viral variation and PRRSV pathogenesis is described in Chapter 2. A manuscript submitted to the Journal of Virology describing the evaluation of immune selection during PRRSV infection and the identification of PRRSV immune escape variants is presented in Chapter 3. Alyssa B. Evans performed the cloning, sequencing, haplotyping, construction of chimeric viruses, and neutralizing antibody assays, as well as helped design experiments, and wrote and prepared the manuscript; Karin S. Dorman performed the DAPC, CMH, and all associated statistical analyses, and helped write and edit the manuscript; Sarah van Tol helped construct chimeric virus clones; Jack C.M. Dekkers provided critical analyses and review of the manuscript; and Susan Carpenter helped in the experimental design, manuscript writing and editing, and general oversight of the project. Chapter 4 describes further characterization of the mechanism of PRRSV neutralization by neutralizing antibody and the effect of immune escape on viral replication fitness. Alyssa B. Evans designed and oversaw all experiments, performed data analyses, and wrote and prepared the manuscript; Marcus Bolton performed the infectivity and replication kinetics assays; Hyelee Loyd performed neutralization mechanism assays; and Susan Carpenter oversaw the experimental design, analysis of results, and editing of the manuscript.
Chapter 5 reports the results of the synthesis, identification, and characterization of small molecule compounds with inhibitory activity against PRRSV *in vitro*. This project was done in collaboration with the George Kraus lab in the Department of Chemistry at Iowa State University. Pengfei Dong from the Kraus lab was responsible for the synthesis of all of the synthetic compounds, and George Kraus oversaw the compound synthesis. Alyssa Evans designed, performed, and analyzed biological and virological assays, wrote and prepared the manuscript; Hyelee Loyd performed binding and replication assays; Susan Carpenter helped in the experimental design, data analyses, general oversight of the project, and review of the manuscript. Chapter 6 discusses the general conclusions, implications and limitations of this dissertation work, and presents potential studies for future work.

**Literature Cited**


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CHAPTER 2: LITERATURE REVIEW

Viral Variation and its Consequence on Infection

Since their discovery in the late 1800’s and early 1900’s, viruses have been recognized as a significant cause of disease in humans, animals, and plants. High mutation rates and short generation times have made the control and prevention of many viral diseases elusive. Understanding viral variation and its effect on virus-host interactions will lead to insights into viral pathogenesis and emergence and the development of better treatment and prevention strategies.

The quasispecies structure of RNA virus populations

A common feature across RNA viruses is their existence within hosts as a complex population “cloud” of related but non-identical variants known as quasispecies (1-3). Quasispecies populations represent a “mutant cloud” of both potentially beneficial and potentially deleterious variants centered around a sequence average, or consensus sequence (3-5). While some RNA viruses undergo major genetic changes (genetic or antigenic shift) via recombination, as in human immunodeficiency virus type 1 (HIV-1; 6), or the exchange of entire genomic segments, as in influenza A virus (7) the main mechanism in the creation of mutant clouds is the accumulation of random mutations (genetic or antigenic drift) (3). This is due to the high error rate and lack of proof-reading mechanisms of viral RNA polymerases (3, 8). Viral RNA polymerase mutation rates are estimated to be between $10^{-6}$ to $10^{-4}$ substitutions per nucleotide per cell infection, or
approximately one mutation per genome in each replication cycle \(9, 10\). Despite the apparent infinite potential for variation within viral genomes, there is an inherent evolutionary trade-off for viral quasispecies. The accumulation of too many mutations can be detrimental to virus fitness because of the production of non-viable genomes, and conversely too little genetic variation decreases the reservoir of potentially beneficial variants in the mutant cloud, limiting the virus’ capability to adapt under varying circumstances and environments. Selective pressures help to shape the composition of the viral quasispecies population. Negative, or purifying, selection acts to eliminate deleterious or negative phenotypic traits from the quasispecies population, whereas positive selection acts on sets of variants with beneficial phenotypes to increase their frequency in the mutant cloud.

Viral quasispecies populations are not merely an artifact of the accumulation of variants during replication via error-prone mechanisms, but are an interactive group of variants that are beneficial to RNA virus fitness and can have complementary interactions. In fact, several studies have found that the use of high-fidelity RNA polymerase in viral replication results in decreased fitness of the viral population \(11, 12\). In a study of a poliovirus variant with a high-fidelity RNA polymerase, the authors found that the resulting homogenous virus population was less adaptable to changing environments and less virulent in mice than its wild type counterpart \(11\). However, artificially expanding the quasispecies of the high-fidelity variant via chemical mutagenesis rescued pathogenicity \(11\). In addition, the same study conducted single and co-infections of a poliovirus variant that was unable to infect the central nervous system and a wild type, neurotropic poliovirus. In the presence of wild type poliovirus, the non-neurotropic variant was capable
of infecting the central nervous system, demonstrating complementation between viral quasispecies (11). Similarly, a high-fidelity variant of chikungunya virus (CHIKV) had reduced viremia in both a mosquito vector and mouse host compared to wild type CHIKV, indicating that a decrease in quasispecies heterogeneity negatively impacted viral fitness (12).

**Antigenic variation and immune escape**

The large amount of variation in RNA viruses makes eradication and control of these pathogens extremely difficult. In mammals, a successful immune response to a virus, elicited by immunization or infection, leads to the production of B-cells and T-cells that recognize specific portions of the virus, called epitopes. A subset of these B-cells and T-cells form memory cells, and this memory component of the adaptive immune response can then be called on upon subsequent exposure to the same pathogen, which leads to a quick and effective immune response to prevent the establishment of a productive infection. Of particular importance in preventing subsequent infection by the same virus is neutralizing antibody secreted from memory B-cells (13-15). These memory B-cells circulate in low numbers throughout the body after clearance of the initial infection. Upon subsequent infection with the same virus, the memory B-cells recognize the pathogen via the binding of their B-cell receptor with the specific virus epitope, and elicit the rapid activation and production of more B-cells in order to secrete neutralizing antibody to prevent the infection from establishing in the host, or clear the infection quickly (16). This process is effective upon exposure with the same virus that expresses the same epitope that the B-cell
response recognized during the first infection, and contributes to the life-long protection generated by vaccines against smallpox virus, poliovirus, and measles virus (13).

The immune response by memory B-cells is often not effective against genetically distinct strains of the same virus due to differences or changes in the viral epitopes recognized by neutralizing antibody, a process known as antigenic variation. Antigenic variation can contribute to the spread and continual circulation of viruses via generating diversity among and between strains of the same virus. Because viruses can continually acquire new mutations and change as they replicate in hosts, each new host may be infected by a slightly different version of the virus. This can be problematic for hosts, even when they have previously mounted a successful immune response to the virus. If variation occurs in viral epitopes, the memory B-cells created from prior exposure to the virus may not recognize the new infection, and therefore cannot mount a rapid B-cell response to the virus. Antigenic variation is a major reason for the lack of effective Influenza, HIV-1, and foot-and-mouth disease virus vaccines (7). The identification of “broadly neutralizing antibodies” (bNAb) in HIV-1 and influenza A virus that are capable of cross-reactivity and preventing infection by a wide variety of genetically variant viruses within a virus family has given some hope to the idea of developing single, effective vaccines against diverse viruses; however these bNAbs have been difficult to reliably elicit via immunization (14, 17).

Antigenic variation not only occurs by the transmission of new viral variants between hosts, but also by the selection and/or replication of viral variants within individual hosts. When antigenic variation occurs in a subset of variants in a quasispecies population within a single host, the collection of variants that are resistant to the immune
response that was effective against the majority of the population are called escape mutants. These escape mutants can then replicate and establish a new, productive infection in the host and seed a new, or expanded, quasispecies population distinct from the initial population that was controlled by the host immune response. Escape variants are routinely seen within HIV-1-infected patients, which contribute to the persistence of the virus within these individuals (18).

Antigenic variation and escape from neutralizing antibody contributes to the spread of many viruses, including the important human pathogens Influenza A, HIV-1, hepatitis B virus, hepatitis C virus, as well as agriculturally important viruses such as avian and swine influenza and foot-and-mouth disease virus (19). Antigenic variation is a major cause of the continual circulation and annual epidemics of influenza virus in humans, swine, and pathogenic strains in birds. The immunity generated from previous exposure to influenza strains, via vaccine or infection, is typically not effective at controlling the next year’s epidemic strain (7, 17, 20, 21). Influenza A, a negative sense RNA virus, is particularly adept at antigenic variation and immune escape because it can generate diversity through both antigenic shift and antigenic drift. The segmented genomic structure of influenza A allows for the exchange of any of its eight gene segments between different strains during co-infection of a single cell, a type of antigenic shift known as reassortment (7). The resulting genetically mixed viruses, or reassortants, can acquire new phenotypic traits not seen in either of the parental strains. Novel reassortants have led to several influenza epidemics and pandemics, including the outbreak of highly pathogenic avian influenza in commercial chicken flocks in Canada and the United States in 2015 (22), the 2009 H1N1 pandemic in humans (23), and the currently circulating endemic strains in swine (24). However, large
changes in genetic diversity are not strictly necessary for antigenic variation. An antigenically drifted H3N2 human influenza strain during the 2014-2015 season allowed this strain to escape antibody elicited by the genetically similar H3N2 vaccine, resulting in poor performance of the vaccine (25).

Viruses with non-segmented genomes may undergo antigenic shift via recombination, but many primarily utilize antigenic drift for the production of antigenic variation, immune escape, and persistence (6,7). It is well established for HIV-1 that antigenic variation within the quasispecies population of a single patient leads to continual escape from the host’s neutralizing antibody. Patient sera are capable of neutralizing the autologous HIV-1 quasispecies population from previous times in infection, but are incapable of neutralizing concurrent virus (18, 26-28). HIV-1 employs a staggering number of strategies to evade and escape the host’s immune response, including masking of neutralizing epitopes on the envelope protein (env) through glycan shielding, and conformational and structural changes that alter antibody binding (14). The positive sense RNA virus hepatitis C virus (HCV) is capable of persisting for long periods of time within a host due, in part, to continual antigenic variation. The resulting NAb escape mutants, similar to those seen in HIV-1, utilize multiple genetic pathways of escape, leading to ineffective vaccines against HCV (29, 30). The agriculturally important foot-and-mouth disease virus, a positive sense RNA virus of cloven-hoofed livestock has also been shown to have a considerable amount of antigenic variation, and monoclonal neutralizing antibody escape variants have been described in vitro (31, 32).

Despite the enormous amount of antigenic variation within RNA viruses, cross-reactive broadly neutralizing antibodies to several RNA viruses have been identified,
notably against influenza virus and HIV-1 (14). Influenza bNAbs were identified as early as 1993 (17, 33). These bNAbs primarily target the highly conserved stem region of influenza’s hemagglutinin (HA) envelope protein (stem-reactive antibodies) and block crucial steps in fusion of the viral envelope with the host membrane (17, 34). Importantly, these bNAbs can be produced in high amounts after seasonal influenza immunization in some people (34). However, this is not a universal occurrence and few of the stem-reactive memory B cells are maintained for long periods after immunization. Better mechanisms of eliciting bNAb memory cells are needed if long-lasting immunity to influenza virus through a universal vaccine is to be achieved (17). Similarly, in HIV-1, bNAb have been identified and target multiple regions of env, including the receptor and co-receptor binding sites and the membrane-proximal external region (14, 35-37). Passive transfer studies with HIV-1 broadly neutralizing antibodies have demonstrated that in vivo protection is possible, though high serum concentrations are typically required (14, 38-40). However, bNAb were isolated from long-term HIV-infected individuals and did not protect against virus replication in these individuals, likely due to ongoing antigenic variation and immune escape (14). Therefore, while bNAb to HIV-1 may potentially have therapeutic applications, their effectiveness in preventing HIV-1 infection is unclear. Interestingly, broadly cross-reactive antibodies have also been described for the arbovirus dengue virus (DENV), but they were not neutralizing and in fact actually promoted viral replication and pathogenicity, a phenomenon known as antibody-dependent enhancement (ADE) (14, 41).

While the discovery of broadly neutralizing antibodies against influenza and HIV-1 provides hope for the development of effective, universal vaccines against these and other genetically diverse viral pathogens, the myriad strategies of antigenic variation and
immune escape employed by these viruses continue to pose substantial challenges for their control and elimination.

**Antiviral drug resistance**

When effective vaccines for viral diseases are lacking, often the best course of action is treatment of the infection with antiviral drugs or immunotherapy. Antivirals have been extremely effective in the treatment of HIV-1 and hepatitis C virus (HCV) infections. Anti-retroviral therapy (ART) has turned infection with HIV-1 in the United States from a death sentence to a manageable chronic infection (42, 43). Anti-retroviral drugs (ARDs) have been developed against virtually every step of HIV replication and are extremely effective in decreasing patient viral load and increasing lifespan (43, 44). Similar success has been found using antivirals for the treatment of HCV. For years, chronic HCV infections were typically treated via immunotherapy with interferon-alpha (IFN-α), however 20-50% of patients did not respond to the treatment due to quasispecies populations consisting of IFN-α resistant variants (45). More recently, the treatment of chronic HCV has found success through direct-acting antivirals (DAA) (45-47). These drugs were a huge breakthrough in treatment of HCV, and have reported cure rates of 90-100% (45).

However, similar to antigenic variation and immune escape, genetic variation in viruses can lead to resistance to antiviral drugs that were previously effective at controlling the infection, leading to the replication of antiviral escape variants and the emergence of drug-resistant strains. ARD resistance is a significant problem in the treatment of HIV-1 infections. Due to the high mutation rate of HIV-1 and its continual replication within its host, drug resistant escape mutants commonly arise, both in patients who are long term
ART users, and in newly infected people via the transmission of drug resistant variants (48, 49). Because of this, ART generally consists of a combination of ARDs to combat drug resistant escape mutants (50). However, multi-drug resistant escape mutants have been described, and while new ARDs are continually being developed, the continued trend of ARDs selecting for drug resistance mutants is of concern for long-term HIV treatment (48, 49). Likewise, the treatment of chronic HCV infections is hampered by the occurrence of drug resistant escape mutants. Despite the success of the direct-acting antivirals, subsets of HCV strains are resistant to DAA, and new DAA resistant escape mutants have also arisen (45, 47). While antivirals are a key component in combating persistent viral infections, the continual occurrence of drug resistant escape mutants within quasispecies populations underlies the need for the development of new antivirals, as well as alternative therapies for the treatment of persistent viral infections.

**Emergence of new viral diseases**

In addition to contributing to antigenic variation, immune escape, and drug resistance, viral quasispecies can contain subsets of the population with altered tropism and host range, which can lead to the emergence of new viral diseases through cross-species transmission events. Several high-profile viral disease outbreaks have occurred since the turn of the century, most notably the severe acute respiratory syndrome coronavirus (SARS-CoV) outbreak in 2003, and the Ebola virus (EBOV) epidemic that ravaged the West African countries of Sierra Leone, Guinea, and Liberia in 2014. Several arthropod-borne viruses (arboviruses), including chikungunya virus and most recently zika virus, have emerged and are capable of causing significant and severe disease in
humans. Additionally, the coronavirus porcine epidemic diarrhea virus (PEDV) has recently emerged in swine and caused significant economic losses to the pork industry worldwide (51). The majority of these viruses emerged via cross-species transmission facilitated by genetic changes in the virus, allowing the establishment of infection in a new host.

SARS-CoV emerged in 2002 in Guangdong province in China and infected thousands and killed hundreds of people across the globe throughout 2003 (52, 53). SARS-CoV was a novel coronavirus at the time of its discovery, but sequence analysis of SARS-CoV isolates from the outbreak with SARS-like coronaviruses (SL-CoV) from civets and bats revealed that a SL-CoV likely jumped from a bat reservoir to civets as an intermediate host, where the virus underwent additional mutations to facilitate the jump to humans (52, 54-57). The jump from civets to humans was likely mediated by mutations in the SARS-CoV receptor binding domain in the envelope spike protein, which facilitates binding to the human receptor angiotensin-converting enzyme 2 (ACE-2) (58). A recently emerged cousin to SARS-CoV, Middle East respiratory syndrome coronavirus (MERS-CoV), is believed to have similarly made the jump from bats to humans, possibly through camels as an intermediate host, (59-61). Unlike SARS-CoV, MERS-CoV to date only has limited human-to-human spread, as it apparently is not well adapted to the human host (59, 60). The closely related bat coronaviruses that MERS-CoV is believed to be derived from utilize the evolutionarily conserved dipeptidyl peptidase 4 (DPP4) receptor, and some evidence suggests that mutations in the spike protein of MERS-CoV allowed for better binding with human DPP4 and facilitated the cross-species transmission (62). Similarly, a coronavirus of swine, porcine epidemic diarrhea virus (PEDV), emerged in the United Kingdom in 1971, China in the 1980s, and the United States in 2013, likely via transmission of a mutated strain from
bats to pigs (63). Sustained pig-to-pig transmission and continual genetic variation in PEDV has led to periodic outbreaks of highly pathogenic PEDV in China and the United States (63).

The recent 2014 EBOV outbreak in West Africa that caused more than 28,000 infections and over 11,000 deaths is believed to have originated from a single zoonotic transmission event of a mutated variant from a bat to a 2-year old boy (64). The EBOV variant was readily transmissible from person-to-person, resulting in the epidemic (64). The specific mutations that facilitated this cross-species transmission event are currently not known, but 173 amino acid substitutions across all EBOV ORFs were identified between the 2014 EBOV outbreak strain and previously identified EBOV sequences (65).

Several arboviruses have recently emerged or reemerged, causing substantial disease in humans, including chikungunya virus (CHKV) and Zika virus (ZIKV) (66, 67). These viruses were first identified in the early-mid 1900’s and caused small clusters of disease in Africa and Asia, but recently have reemerged to cause increasing numbers of infections across the globe, resulting in arthritic-like disease (CHKV) and potentially microcephaly in infants and Guillain-Barré syndrome in adults (ZIKV) (66, 67). This spread is believed to be due, at least in part, to evolutionary adaptations to their mosquito vectors (CHKV) or human host (ZIKV), resulting in variants that are more easily transmissible to humans (68-72). In CHKV, a single mutation in the envelope gene has been implicated in the spread of CHKV by expanding the vector specificity from Aedes aegypti to Aedes albopictus mosquito species, resulting in increased viral load in the vector and transmission to humans (68). While research on the reemerging ZIKV is still in its infancy,
some evidence suggests that variation in codon usage in a nonstructural protein of ZIKV has resulted in variants better adapted for replication in humans (71).

Summary of viral variation

The high error rate and quasispecies population structure of RNA viruses can have many significant implications for the pathogenesis and treatment of these infections. A diverse quasispecies population contains potentially beneficial variants that can emerge under different selective pressures. This high degree of genetic variation within RNA viruses can lead to antigenic variation and immune escape, drug resistance, and the emergence of new viral diseases. Together, these consequences of viral variation pose many challenges to the prevention, treatment, and control of RNA virus pathogens.

Porcine Reproductive and Respiratory Syndrome Virus

Porcine reproductive and respiratory syndrome virus (PRRSV) is a 15kb positive-stranded RNA virus. PRRSV, which emerged simultaneously as two divergent strains in the United States (Type 2 PRRSV) and Europe (Type 1 PRRSV) in the late 1980’s causes respiratory symptoms in growing pigs and spontaneous abortions in pregnant sows (73-76). PRRSV is easily transmissible through the respiratory route, as well as sexual transmission (77). The economic losses due to PRRSV are estimated to be responsible for $670 million in losses annually for the US pork industry (78). Several vaccines have been developed against PRRSV; however their effectiveness is hampered by the high amount of virus variation and genetic diversity (79-81). Although genetic diversity of PRRSV has been
documented and characterized in great detail (82), the evolutionary and molecular mechanisms that drive the continual circulation of PRRSV variants within and between animals are not well understood. Such understanding is critical for the development of safe and effective vaccines that confer broad and long-lasting protection against PRRSV.

**PRRSV genome and virion structure**

PRRSV is an enveloped, 15kb positive strand RNA virus in the *Arteriviridae* family in the order *Nidovirales*. The genome of PRRSV consists of at least eleven open reading frames (ORFs) (83, 84). The 5' two-thirds of the genome encodes the polyproteins ORF1a and ORF1ab (produced from a -1 ribosomal frameshift) that are proteolytically processed to form at least 12 known nonstructural proteins (nsp), but up to 16 have been predicted (85, 86). The ORF1a and 1ab polyproteins are synthesized and subsequently cleaved into the individual nsps via self-cleavage of nsp1 and nsp2, with the nsp4 protease mediating the remaining known cleavages (85-87). Once cleaved, various nsps mediate virus replication and virus-host interactions, including suppression of the innate immune response (79, 88). Importantly, nsp9 is the viral RNA-dependent RNA polymerase (RdRp), and nsp2, nsp3, and nsp5 contain transmembrane domains that have been implicated in host membrane remodeling for the formation of virus replication vesicles (86, 89, 90).

The last third of the genome contains eight overlapping ORFs (ORF2-7, 2a, and 5a) that encode the virus structural proteins (91-94). ORF5 and ORF6 encode the major envelope proteins, the glycosylated GP5 and M, respectively, which interact to form a heterodimer on the virion surface that is believed important for attachment (95-98). ORF2-4 encodes the minor envelope glycoproteins GP2, GP3, and GP4, respectively, which form a
heterotrimer that interacts with the CD163 receptor (99-103). Additional minor envelope components are encoded by ORF2a and ORF5a, which are nested within ORF2 and ORF5 and encode the E protein and 5a protein, respectively (93), (94). Some evidence suggests that E may interact with the GP2/3/4 trimer and function as an ion channel (94, 102, 104, 105). Importantly, all of the envelope proteins are absolutely required for productive PRRSV infection (94, 100). The final PRRSV structural protein is the nucleocapsid (N), which is encoded by ORF7 (91, 106). N contains a nuclear localization signal, and previous studies have shown that it enters the host nucleolus, suggesting that in addition to serving as the PRRSV nucleocapsid, N may be involved in regulating host transcription (107, 108). Negative stain and cryo-electron microscopy have revealed that PRRSV has a spherical, smooth virion that is approximately 54nm in diameter (108-110). Within the interior of the virion is a hollow core composed of N dimers likely in a helical or chain-link type arrangement associated with the (+)RNA genome (108, 110).

**PRRSV replication and molecular pathogenesis**

Like all viruses, the PRRSV replication cycle consists of entry into the cell, protein and genome synthesis, and assembly and release of new virions. Entry of a virus into its host cell is typically facilitated by a series of complex interactions between the virus and the host, and many of these interactions are not known for PRRSV. To initiate the entry process, the first thing that needs to happen is an initial interaction between the virus and the host cell. This initial attachment step is often mediated through the interaction of the virus with cellular “attachment factors”, which are host cell proteins that can bind to viral proteins to bring the virion into close proximity with the cell, but do not actively promote
entry of the virus into the host cell. These initial attachment interactions are not known for PRRSV, although there is some evidence that interactions of CD169 with GP5, and heparin sulfate with M may mediate attachment (95, 97). Following attachment, PRRSV binds to its receptor, CD163, through interactions with GP2 and GP4 (102, 103). A virus receptor is a cellular protein that actively promotes virus entry into the cell upon binding to the virus, and is the primary determinant of cell tropism for a virus. Upon binding of GP2 and GP4 with cellular CD163, the PRRSV virion is taken up into the cell via endocytosis (98).

Additionally, it was recently shown that the cellular factor MYH9 binding to GP5 at a post-attachment step in entry is also a required interaction for PRRSV infection (111). Like all enveloped viruses, PRRSV must fuse its envelope with the host cell membrane, in the case of PRRSV with the endosomal membrane, in order to release its genome into the cell, however the PRRSV fusion protein that mediates this interaction is currently unknown. Upon release of the genome into the cytoplasm, PRRSV completes its replication cycle through the synthesis of viral proteins and genomes, which are assembled into new virions facilitated by GP5 and M (100), and are released from the cell via exocytosis (86).

PRRSV primarily infects cells of the macrophage lineage, with a preference for porcine alveolar macrophages (PAMs) (112). PRRSV replicates in vitro in primary cultures of PAMs and peripheral-blood monocyte derived macrophages (MDMs), however the only PRRSV permissive cell lines are those of the MA-104 lineage of African Green Monkey kidney cells, primarily a subset of highly permissive MARC-145 cells (113). For years, the host cell receptor for PRRSV remained a mystery due to conflicting results from in vitro studies. CD169 was implicated by several studies as a required receptor in PAMs but this was controversial, as MARC-145 cells do not express CD169 (97, 114-116). An additional
cellular receptor, CD163, which is expressed in PAMs and MARC-145 cells, was implicated as the PRRSV receptor through studies that showed that expression of CD163 could make non-permissive cells permissive to PRRSV (117). However, other studies found that expression of CD163 alone was not sufficient for PRRSV permissivity (115, 116). A series of experiments utilizing gene-edited pigs with knockouts of either CD169 or CD163 found that pigs without CD169 were just as susceptible to PRRSV infection as wild type pigs; however, CD163 knockout pigs were completely resistant to PRRSV infection, decisively showing that CD163, and not CD169, is a required PRRSV receptor (103, 118). The recent discovery of MYH9 as a required cellular factor for PRRSV infection may explain why some studies could not produce permissive cells through the expression of CD163 alone (111).

Attachment of the PRRSV virion to the host cell is likely mediated by interactions of the major envelope protein dimer GP5/M with cellular attachment factors. This is circumstantially supported by the fact that the GP5/M dimer is very highly expressed on the virion surface, with fewer of the minor GP2/3/4 complexes present (108, 110, 119). While CD169 is not a required PRRSV receptor, GP5 has been shown to interact with CD169 and this interaction may facilitate viral attachment (95, 97). However, this is clearly not a required interaction as CD169 knockout pigs were susceptible to PRRSV, and the permissive MARC-145 cells lack CD169 (118, 120). In addition to CD169, M has been found to interact with heparin sulfate and facilitate attachment in PAMs and MARC-145 cells (97, 98, 121). Prior to the CD163 knockout pig experiments, CD151 had been implicated as a possible PRRSV receptor, although no interaction between CD151 and any PRRSV protein has been found (120). After attachment, PRRSV binding to the receptor facilitates receptor-mediated endocytosis (122). GP2 and GP4 interact with the PRRSV receptor CD163 (102),
and this interaction likely facilitates receptor-mediated endocytosis. However, one \textit{in vitro} study found that CD163 was required for uncoating, but that CD169 facilitated endocytosis (98, 116). Therefore, while CD163 is required for productive infection, it remains unclear exactly how it facilitates PRRSV infection. However, because CD169 is not required for PRRSV infection \textit{in vivo}, it is likely that CD163 can facilitate PRRSV uptake as well. In the endosome, PRRSV undergoes pH-dependent fusion to release its genome into the host cell cytoplasm, but the PRRSV fusion protein that mediates this interaction is currently not known (98, 119).

Following fusion and uncoating, the PRRSV polyproteins ORF1a and ORF1ab are immediately translated from the positive strand RNA genome, and are proteolytically processed to generate the nsps, which assemble into a replication and transcription complex (RTC). Nsp2, nsp3, and nsp5 of the prototype arterivirus equine arteritis virus (EAV) have been shown to rearrange host endoplasmic reticulum membranes (ER) in the perinuclear region to form double-membrane vesicles associated with viral RNA replication, and it is likely that similar processes take place in PRRSV (89, 123). In addition, upon entry into the cell the nsps modulate many virus-host interactions; nsp3-8, and possibly nsp9 and nsp10, contain virulence factors, while nsp1, nsp2, nsp4, nsp5, nsp7, nsp9, nsp10 and nsp11 have been shown to mediate interactions with the innate immune response, likely facilitating a favorable environment for PRRSV replication (88, 124, 125).

Viral mRNA synthesis proceeds by the synthesis of a nested set of negative subgenomic RNAs of the 3′ ORF2-7, which are subsequently transcribed into positive stranded subgenomic RNAs, which then serve as the template for the translation of the structural proteins (90). Interestingly, these subgenomic RNAs all have the same leader
sequence from the 5’ end of the genome and their synthesis is mediated by internal transcription regulation sequences (TRS) and discontinuous RNA synthesis which joins the 5’ leader with the 3’ subgenomic regions (126, 127). In addition, full-length minus strands are generated as templates to synthesize new positive strand RNA genomes (90). Following the synthesis of new viral genomes and proteins the genomes are encapsidated by dimerized nucleocapsid proteins within a double membrane vesicle (DMV) prior to assembly (128). The envelope glycoproteins are processed in the ER and Golgi; GP5 and M form a dimer through disulphide linkage in the ER and the complex is transported to the Golgi (119). This crucial step in assembly of the virion is facilitated by GP5 and M (100). The processing of the minor envelope proteins is not well characterized for PRRSV, but in EAV GP2 and GP4 form a dimer through disulphide linkage in the ER, and GP3 forms a disulphide bond with GP4 after the virus is released from the cell (119). The minor protein E may be involved in formation of the GP2/3/4 trimer (129). After processing of the envelope proteins, the preformed nucleocapsid then buds from intracellular membranes in the Golgi to acquire their envelope (86). Through unknown mechanisms, the 5a protein is also incorporated as a very minor component of the envelope, and recent studies have also shown that nsp2 is incorporated in the virion (94, 130). Upon budding to acquire its envelope, PRRSV exits the cell via exocytosis, thus completing the virus replication cycle (86).

PRRSV infection is usually spread via the respiratory route, with the initial infection established in PAMs in the lung, causing the respiratory symptoms for which the virus is partially named (74). Infection of interstitial macrophages leads to PRRSV infection within the spleen, thymus, heart, and adrenal gland (131). Additionally, in some cases PRRSV has
been found in certain epithelial cells as well as the testes, facilitating sexual transmission of the virus (77). Establishment of infection in secondary sites such as the spleen, tonsils, thymus, and lymph nodes is frequently seen during persistent infection in some pigs, although the molecular mechanisms that facilitate PRRSV persistence are unknown (132-134).

**Immune response to PRRSV**

Immunological control of PRRSV is difficult due to the fact that PRRSV elicits a delayed and weak protective immune response (135, 136). This is the result of a number of reasons. Many of the PRRSV nonstructural proteins mediate interactions with the host, leading to suppression, or altered, innate immune response (88). It is believed that a combination of the poor innate immune response and effective epitope shielding by the virus leads to a delayed and week adaptive immune response (136). While some pigs develop persistent infection that can linger for months (132, 133), many pigs are capable of successfully clearing the virus within a month, demonstrating effective host immune control (133). However, the factors that correlate with a protective immune response to PRRSV are currently not known.

Many of the PRRSV nsps play key roles in virus-host interactions (79, 88). Nsp1, nsp2, nsp4, nsp5, nsp7, nsp9, nsp10 and nsp11 have been shown to mediate interactions with the innate immune response (79, 88, 90). Like many other viruses, some of the PRRSV nsps are involved in suppression of the innate immune response, specifically by antagonizing type 1 interferon signaling and suppression of Natural Killer cells (88, 137-140). Interference with the host immune response extends to modulation of cellular
transcription factors and regulatory networks important in induction of the adaptive immune response. The net effect of these actions is a delayed and weakened adaptive immune response.

PRRSV is recognized to have an unusual adaptive immune response, with both the humoral and cell-mediated components mounting weak and delayed responses. After infection, the pig starts to make PRRSV-specific antibodies within 7 days, although neutralizing antibodies are typically not detected until 21-28 dpi (135, 136, 141, 142). The early antibody response is believed to be associated with antibody dependent enhancement of infection (143, 144). Although there has been some debate about the protective nature of NAb due to the fact that some pigs clear virus prior to detectable NAb in serum, passive transfer experiments have established that NAb can be an important component of a protective immune response in vivo (145). Interestingly, at least one study has found neutralizing antibody as early as 8 dpi through altering the in vitro experimental conditions to accommodate slow-binding and complement-dependent neutralizing antibody, suggesting traditional virus neutralization assays may not be able to detect early PRRSV NAb (146, 147). B-cell epitopes have been detected on every structural protein and nsp2 (148). However, the only known neutralizing epitopes are on GP5, GP4, GP3, and M, but none elicit sterilizing immunity (136, 149, 150, 151). GP5 is believed to contain the major neutralizing epitope, though some evidence suggests that this may not be the case (149, 152). The GP5 neutralizing epitope is surrounded by a hypervariable region and a decoy epitope. The decoy epitope is immunodominant but not neutralizing, and is flanked by the two GP5 signal peptide cleavage sites (119, 149, 153, 154). One model of PRRSV
neutralization proposed by Lopez and Osorio suggests that variation of the GP5 cleavage sites may alter expression of the decoy epitope, and facilitate immune evasion (136).

Similarly, the cell-mediated response is also delayed, with a T-cell response typically observed around 21-28 dpi (155, 156). T-cell epitopes in several of the nsps and the structural proteins GP5, GP4, M, and N have been identified (157, 158). Like with the NAb response, the role of cell-mediated immunity in protection or clearance of PRRSV infection has been controversial. Some studies have found that a high CD8+ cytotoxic T-cell (CTL) response was actually associated with PRRSV persistence in lymphoid tissues, and little cytotoxic activity was actually observed (159, 160). Other studies reported a preferential upregulation of regulatory T-cells (Treg), which were associated with immunosuppression (88). More studies are needed to determine the role of cell-mediated immunity in a protective immune response to PRRSV. Though a considerable amount of research has gone into characterizing the pig’s immune response to PRRSV, it is still unclear what the correlates of protective immunity for PRRSV are, but it likely requires a combination of innate, humoral, and cell-mediated responses.

**PRRSV antigenic variation and immune escape**

Like all RNA viruses, PRRSV is characterized by a high mutation rate and vast genetic diversity. PRRSV emerged simultaneously in Europe and the United States as two distinct genotypes, Type 1 and Type 2, respectively (75, 80). The genesis of PRRSV variants is due primarily to a high rate of mutation and frequent recombination that occurs during virus replication (82, 161-163). The selective pressures that shape the occurrence of PRRSV variants at a population level are poorly understood, but may include selection for
phenotypic adaptations such as transmissibility, replication rate, cell tropism, and immune evasion. Indeed, evidence of positive selection has been found throughout the PRRSV genome, primarily in regions of GP5, and to a lesser extent regions of the other envelope proteins and nsp2 (153, 164-167).

As discussed above, immune selection and antigenic variation contribute to the diversity of many RNA viruses, including HIV-1 and influenza A virus; however the role of immune selection and antigenic variation in PRRSV is a matter of debate. This is due, in part, to the inherent difficulty in testing for immune selection in the PRRSV envelope proteins. There is a preponderance of overlapping reading frames in ORF2-6 making traditional statistical tests of selection based primarily on dN/dS ratios inappropriate. In addition, the delayed and/or weakened immune response to PRRSV makes immunological characterizations of selection difficult. Adding to these challenges is the limited characterization of neutralizing epitopes of PRRSV. Previous studies of neutralizing epitopes have relied on peptide scanning or phage display, which limits detection to linear epitopes. Because of the extensive protein-protein interactions and poor structural characterization of the PRRSV envelope glycoproteins, it is possible that important neutralizing conformational epitopes exist but have not been detected.

Despite these challenges, some indirect evidence for immune selection and antigenic variation has been reported, largely for the heavily studied ORF5-encoded envelope protein GP5. The GP5 ectodomain is highly variable while the endodomain is relatively conserved, suggesting selective pressures from the host’s extracellular environment, including antibodies (153, 165). Despite the large amount of variation in the GP5 ectodomain, the glycosylation pattern of GP5 is highly conserved across PRRSV isolates,
and *in vitro* studies have shown that modifications to the glycosylation sites drastically increase neutralization susceptibility of PRRSV and resulted in quick adaptation of the virus to reinstate the glycosylation epitope (165, 168, 169). A similar result was seen with GP3: the glycosylation pattern is conserved and when disrupted resulted in an increased susceptibility to neutralizing antibody (169). Mutations in the hypervariable region surrounding the neutralizing epitope of GP5 are thought to aid in immune evasion by altering the neutralizing epitope itself, or by masking the epitope through glycan shielding and/or generation of a decoy epitope (136, 153, 168). A recent *in vitro* study found selection of certain ORF5 genotypes during passage of virus under pressure from PRRSV neutralizing antiserum, resulting in the selection of virus variants that were resistant to the antiserum (170).

Other studies have analyzed viral populations within individual pigs during infection *in vivo* (162, 164). In two such studies, PRRSV ORF5 was found to exhibit a quasispecies population structure comprised of multiple variants that arose from mutations within individual pigs (162, 171). However, there was no evidence of immune selection of individual variant genotypes using either statistical analyses (162) or immunological characterization of plaque-purified virus collected at 7 days post-infection during serial *in vivo* passage (171). A limitation of these studies is that they were confined to a single time point in each pig. More recently, a study of PRRSV infection in immunocompetent and immunodeficient pigs analyzed PRRSV variation at 11 and 21 days post infection (dpi) in each pig (172). Deep sequencing of viral genotypes revealed a similar low rate of variation at 11 and 21 dpi and found no evidence of immune selection using statistical methods (172). However, due to the delayed immune response to PRRSV, it
is possible these samples were taken too early to detect immune selective pressure and antigenic variation. To date, direct evidence of antigenic variation resulting in immune escape variants during the course of an in vivo infection has not been reported.

**PRRSV vaccines and antivirals**

The high amount of genetic diversity in PRRSV directly contributes to the lack of an effective vaccine. Many studies and companies report effective protection from PRRSV infection via modified live virus (MLV) vaccines, however these vaccines are not effective against heterologous PRRSV strains and their ability to reduce transmission in non-experimental conditions is poorly characterized (81, 173, 174). Additionally, reports have shown that recombination between MLVs and field strains can lead to the occurrence of new genetic variants, although their role in PRRSV genetic diversity and their ability to transmit pig-pig are not known (175). Genetic diversity has been shown to contribute to vaccine failure against heterologous strains with less than 85% nucleotide identity, however a number of studies have shown that protection against homologous challenge is not always achieved either (81, 173, 176). MLVs still represent the best protection, even if that protection is poor. Killed vaccines have seen little success, and recombinant DNA vaccines may actually exacerbate clinical disease (177, 178).

Because of the poor performance of current PRRSV vaccines, alternative treatment strategies are needed to reduce the replication and spread of the virus within and between pigs. In the past few years, several antivirals against PRRSV have been described in the literature, however none are currently in use for treatment of PRRSV in the field. These anti-PRRSV compounds include several derived from traditional Chinese medicine,
including chlorogenic acid, scutellarin, epigallocatechin gallate palmitate, flavaspidic acid AB, *cryptoporus volvatus* extract, and atractylool (179-183). These compounds had a range of anti-PRRSV potency *in vitro* with EC$_{50}$ or IC$_{50}$ (“effective concentration” or “inhibitory concentration” that inhibits 50% of input virus) of 3.5-271μg/ml. However, it is important to note that only one of the compounds, *cryptoporus volvatus* extract, has been tested in pigs (182). The results of these studies showed that pigs treated with *cryptoporus volvatus* extract had less clinical disease and reduced virus replication compared to control pigs. However, none of the other compounds have been tested in pigs, so their *in vivo* effectiveness and safety in swine are unknown.

**Summary of porcine reproductive and respiratory syndrome virus**

The extensive genetic diversity of PRRSV and the knowledge gaps regarding the correlates of protective immunity, antigenic variation, and immune escape all contribute to the lack of an effective PRRSV vaccine. Characterization of the molecular mechanisms that underlie neutralization, protection, and potentially immune escape are desperately needed in order to develop effective vaccines to provide long-term protection. Until these knowledge gaps are filled, the development of safe and effective antiviral drugs may be the best treatment option.

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CHAPTER 3: PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS UTILIZES MULTIPLE GENETIC PATHWAYS OF IMMUNE ESCAPE DURING INFECTION IN VIVO

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Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) infection results in significant economic losses to the swine industry worldwide. Genetic diversity among PRRSV isolates contributes to the continual emergence of genetic variants and vaccine failure. In the present study, we examined genetic and antigenic variation of PRRSV using retrospective, sequential samples from five experimentally infected pigs with differing virological and immunological outcomes, including three pigs that experienced a rebound in viremia. Sequence analyses of nsp2 and ORF2-6 revealed limited genetic variation during acute PRRSV infection. However, distinct ORF2-6 haplotypes were identified in each pig, with one or more ORF2-6 haplotypes co-existing in late day virus samples. Chimeric viruses containing all or part of predominant ORF2-6 haplotypes were tested in virus neutralization assays using autologous sera. In two of the three rebounded pigs, genetic
variation in ORF2-6 resulted in antigenic variation and immune escape. Immune escape mapped to multiple envelope proteins, including GP5 or GP3, and usually, but not always, required a combination of amino acid changes. Importantly, the key amino acid changes and/or combination of changes that mediated escape varied by pig and by virus haplotype. These data demonstrate PRRSV envelope proteins are under immune selection that contributes to genetic diversity and the emergence of immune escape variants. Importantly, PRRSV utilized multiple genetic pathways of immune escape, even within an individual pig. The diverse strategies of immune escape likely contribute to difficulties in producing effective vaccines for PRRSV.

**Importance**

Major challenges remain in control and elimination of PRRSV, which continues to cost the pork industry hundreds of millions of dollars every year. Current vaccines are not effective against heterologous strains, due in large part to the high degree of genetic variation among PRRSV strains. There is limited knowledge regarding the viral targets of neutralizing antibody, and immune escape variants arising during the course of infection *in vivo* have not been identified. This study is the first to identify and characterize immune escape variants arising during the course of PRRSV infection. Knowledge of genetic changes that contribute to immune escape and recrudescence can aid in identification of neutralizing epitopes and development of vaccines effective against diverse PRRSV strains.
Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is a 15kb positive-stranded RNA virus belonging to the family Arteriviridae, which emerged simultaneously in the United States and Europe in the late 1980’s (1,2). PRRSV causes respiratory signs in growing pigs and spontaneous abortions in pregnant sows (3), with annual economic losses estimated to be $664 million in the United States alone (4). Several vaccines have been developed against PRRSV; however their effectiveness is limited by the genetic heterogeneity among field isolates as well as by the continual emergence of antigenic and phenotypic variants (5), leading to recurring infections in both vaccinated and unvaccinated herds (5-7). The selective pressures that contribute to genetic diversity in PRRSV are not well understood.

Immune selection contributes to the diversity of many RNA viruses, including HIV-1 and influenza A virus. However, the role of immune selection in PRRSV is a matter of debate as direct evidence of immune-escape variants arising during the course of an in vivo infection is lacking. This is due, in part, to the inherent difficulty in testing for immune selection in the PRRSV structural proteins. The preponderance of overlapping reading frames renders traditional statistical tests of selection based primarily on dN/dS ratios inappropriate for the envelope protein-encoding ORF2-6, and a delayed and/or weakened immune response to PRRSV makes immunological characterization of selection difficult (7-9). Despite these challenges, some indirect evidence for immune selection has been reported, largely for the ORF5-encoded envelope protein GP5. This evidence includes a high frequency of changes in the GP5 ectodomain, conservation in patterns of glycosylation,
and the presence of two hypervariable regions, one surrounding the major neutralizing epitope (10-15). An in vitro study found selection of certain ORF5 genotypes during passage of virus in the presence of PRRSV antisera, resulting in virus resistant to the antisera (16). In contrast, several studies of PRRSV evolution in vivo have found little evidence of immune selection using both statistical and immunological approaches (17-19), reviewed in (6,19). A more recent study of viral genotypes in immunocompetent and immunodeficient pigs (20) revealed a similar low rate of variation at 11 and 21 days post-infection (dpi), and found no evidence of immune selection. Due to the delayed adaptive immune response to PRRSV, however, 21dpi may have been too early to detect selective pressure from the immune response.

The neutralizing epitopes of PRRSV are not well characterized, but are most likely found in the major and/or minor viral envelope proteins, encoded by ORF2-6 (21,22). ORF5 and ORF6 encode the major envelope proteins GP5 and M, respectively, which interact to form a heterodimer on the virion surface that is believed to be important for attachment (23-26). ORF2-4 encodes the minor envelope proteins, GP2, GP3, and GP4, respectively, which form a heterotrimer that interacts with the CD163 receptor (27-31). In addition, nested within ORF2 and ORF5 are ORF2a and ORF5a, which encode the E and the 5a proteins, respectively (32,33). Both of these proteins are minor components of the virion, and there is some evidence that E interacts with the GP2/3/4 trimer and functions as an ion channel (33-35). GP5 is believed to contain the major neutralizing epitope, although some reports suggest that this may not be the case (14,36-39). Neutralizing antibodies have also been found directed against GP3 and M in Type II isolates, and against GP3, GP4 and M in Type I isolates (12,40-42). Identification of PRRSV epitopes has largely relied on peptide scanning or phage display,
which limits detection to linear epitopes. The presence of conformational epitopes, either within a single protein or formed through interactions across one or more proteins, has not been investigated.

A recent study from the PRRS Host Genetics Consortium (PHGC) reported that up to 25% of experimentally infected pigs initially controlled virus replication but experienced a rebound in viremia by 42 dpi, possibly due to escape from host immune control (43, 44). The PHGC study provides a unique sample set with which to examine the influence of immune selection on PRRSV diversity in vivo. Five pigs with differing virological and immunological outcomes were selected for detailed statistical, molecular, and immunological analyses of PRRSV variation during the course of in vivo infection. The results provide direct evidence of antigenic variation and immune escape during acute infection, and reveal multiple genetic pathways of PRRSV immune escape. Identification of specific genetic changes that contribute to immune escape can aid in identification of neutralizing epitopes and development of vaccines effective against diverse PRRSV strains.

**Materials and Methods**

**Cells, virus, and pigs**

MARC-145 cells used for virus passage and infection assays were maintained in high glucose (4500mg/L) Dulbecco’s Modified Eagle’s Medium (DMEM) (Sigma) supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine.

MARC-145 cells used for electroporation were grown in low glucose (1000 mg/L) DMEM (Gibco) supplemented with 10% FBS, 700 mg/L sodium bicarbonate, 100 U/ml penicillin,
and 100 μg/ml streptomycin.

The PHGC inoculum virus, NVSL97-7895 (GenBank accession AY545985), was kindly provided by the PHGC and passaged once in MARC-145 cells and aliquoted. Chimeric virus was generated in the pFL12 infectious molecular clone backbone, which contains the genome consensus of the NVSL97-7895 inoculum virus (45).

Five pigs experimentally infected with NVSL97-7895 were selected based on virological and immunological criteria, using viremia data and sera samples obtained from PHGC trials 1-3 (43, 44). Two pigs maintained high levels of viremia throughout 35 dpi (designated prolonged), and three pigs initially cleared the virus, but experience a rebound in viremia by 41 dpi (designated rebound). Virus neutralization assays (described below) were used to assess the immune response to the NVSL97-7895 inoculum.

**Viral RNA isolation and cloning**

Viral RNA was isolated from the NVSL97-7895 inoculum and from sera collected from experimentally infected pigs at 7 dpi and at a high-viremic late dpi (Table 3.1) using the QIAamp Viral RNA Mini Kit according to manufacturer’s instructions (Qiagen). Viral RNA was converted to cDNA via random hexamer primers using the Superscript III first strand synthesis kit according to manufacturer’s protocol (Invitrogen). Approximately 3 kb from PRRSV ORF2-6 and nsp2 were amplified using PRRSV specific primers and high fidelity platinum Taq polymerase (Invitrogen). ORF2-6 was amplified with forward primer 5’ACCAGGTACC GGCTGAATTGAAATGAAA and reverse primer 5’GGTTGAATTCGGTCAAGCATCTCCCCAAC. Nsp2 was amplified using forward primer 5’CGCCAACCGGATGAATTCCA and reverse primer 5’GAATGGCAAGACCGAGACCAG. Four
separate PCR reactions from each sample were pooled, purified with IBI’s PCR cleanup kit, TA-cloned into pGEM-T easy vectors (Promega) and transformed in Stbl2 Escherichia coli cells. Individual colonies were screened for the correct insert size, and multiple positive clones were selected for sequencing.

**Sequencing and assembly**

Multiple clones from each sample were sequenced via high throughput Sanger sequencing at the Iowa State University DNA Facility (Ames, IA). Multiple primers were used to maximize coverage across the ~3kb regions of ORF2-6 and nsp2. Both regions were sequenced with pGEM-T easy cloning site primers T7 and SP6 (Promega). In addition, ORF2-6 was sequenced with forward primers 

5’CCGGGTACCCTTCATGATTTTCAGCAATGGCTA and

5’GACGGGTACCATTGGTTTCACCTAGAATGGC and reverse primers

5’GATGGAATTCGTCTCCACTGCCC and 5’CAATTCAGAAAGATCAAAAGGTGC. In addition to T7 and SP6, nsp2 was sequenced with forward primers 5’CCTAGCAGTGTCCACCTACGCC, 5’TGATTGGGTTTTGACCTT, and 5’AGGGGTTGGTTGAGGAAATA and reverse primers 5’AACCTGACGGCTCAATTTG and 5’CCAAGTCAGCATGTCAACCCCTA. The sequences were assembled using Phred and Phrap algorithms in MacVector. ORF2-6 was then separated into the individual genes (E, GP2, GP3, GP4, GP5, ORF5a, and M), and each gene, as well as nsp2, was translated to the amino acid sequence. The nucleotide sequences for each region, nsp2 and ORF2-6, were compared via multiple sequence alignment (ClustalW) to determine average pairwise identity and generate consensus sequences.
Visualization of viral variation over time

We applied discriminant analysis of principal components (DAPC) (46) to visualize separation of sequences from different disease stages, i.e. inoculum, early (7 dpi), late. Briefly, an $n \times m$ sequence alignment of $n$ sequences at $m$ positions was converted into a $n \times 4m$ matrix $X$ of allele frequencies, where the possible alleles at each site are the four nucleotides. Deletions in the alignment were replaced with the mean allele proportions from all gap-free sequences at the position. Once the data were reformulated as matrix $X$, principal component analysis (PCA) was used to reduce the data to a lower-dimensional $n \times q$ representation, with $q < 4m$. We chose $q$ large enough so the reduced data retained at least 90% of the variation in the original data. Finally, discriminant analysis (DA) (47) was performed to find linear combinations of the $q$ coordinates that optimally separated user-defined groupings of the data.

Location and patterns of genetic variation

Due to the sequential sampling times within pigs, we were able to observe changes in single nucleotide variant (SNVs) frequencies over time across sampling days. To visualize the variation in the sequences, we used the phyclust package (http://cran.r-project.org/web/packages/phyclust/, version 0.1-15) function plotdots, grouping sequences by pig and dpi. Nucleotide changes at each position relative to the consensus nucleotide in the inoculum are shown as colored dots, making it easy to visually identify both single nucleotide variants (SNVs) that appeared with high frequency as well as coordinated changes in variant distributions across multiple pigs.
To quantify the strength of these genetic changes over time, we used the Cochran-Mantel-Haenszel (CMH) test, which is useful for identifying associations between two categorical variables while stratifying on other covariates (48). In particular, it can aggregate information across pigs to detect an association between SNV and time, while accounting for differences due to experimental trial, course of infection, timing of the late stage sampling point, and the particular environment within each pig. For these data, we stratified the data on pig, such that there is a contingency table of SNV vs. dpi for each pig. Since all pigs were inoculated with the same virus pool, we could randomly distribute the available inoculum sequences among the five pigs. As a result, we had data from three time points (day 0, day 7, and late day). For each site that segregated in these pigs, we designated the majority nucleotide in the inoculum as the major allele and all other variants are minor alleles. The result is five 2x3 contingency tables quantifying the association of variant (major or minor) with dpi (0, 7, or late). We obtained a p-value from the R function mantelhaen.test with a two-sided alternative hypothesis. The Holm correction was used to maintain the familywise error rate below 0.05 given the number of tests (total number of segregating sites in ORF2-6 and nsp2) (49). To test whether the SNVs that changed significantly in time by the CMH test were more likely to be nonsynonymous, and therefore more likely to be under selection, we used a one-sided Fisher’s exact test to compare CMH-significant SNVs with all possible changes to the consensus. Specifically, we considered all possible single nucleotide mutations to the entire inoculum consensus sequence. Mutations nonsynonymous in any reading frame were classified as nonsynonymous; otherwise, they were synonymous. Note that considering all potential mutations to the consensus includes many lethal and highly deleterious mutations that
would never be tolerated in vivo. Since such mutations are more likely to be nonsynonymous, we expect this choice of reference mutations biases against our alternative hypothesis.

**Identification of viral haplotypes**

Multiple sequence alignments of the late dpi sample from each individual pig were used to construct viral haplotypes based on the late day virus of each pig. Due to the high mutation rate of the virus, unique single nucleotide changes occurred within most virus clones. In order to remove this background variation, only sites with a minor variant frequency of ≥25% for clones within each individual pig ("variable sites") were used in haplotype construction. In addition to these variable sites, each virus sample contained dominant changes (defined as a change from the inoculum consensus present in >75% for clones within a pig), and these dominant changes were also included in the constructed virus haplotype. Because we utilized single-clone sequencing, we could identify the nucleotide present at each of the "variable sites across ORF2-6 in a single clone (representative of a single viral genome). Each unique combination of nucleotides across the variable sites within a single pig’s virus sample was designated a haplotype. The number of clones within a pig containing each unique haplotype was used to calculate the frequency of that haplotype. The haplotype within each pig present at the highest frequency was designated Haplotype A, and the haplotype present at the second highest frequency was designated Haplotype B.
Construction of chimeric virus

Chimeric viruses containing the predominant ORF2-6 haplotypes from rebound pigs were generated in the backbone of the infectious molecular clone pFL12 (45). Shuttle plasmids were constructed for subcloning of ORF2-6 haplotypes; pFL12-EcoPac contained the pFL12 sequence from an EcoRV site 89 bp downstream of the translational start site in ORF2 through a PacI site downstream of the poly(A) sequence in the pGEM-T easy vector backbone (Promega). Because some haplotypes contained variants in ORF2 upstream of the EcoRV restriction site, an additional shuttle plasmid, pFL12-AsiEco, was constructed that contained the pFL12 sequence from an AsiSI site 3184 bp upstream of the ORF2 translational start site through EcoRV in the pGEM-T easy vector backbone. The ORF2-6 rebound haplotypes were either selected from a TA-clone in our library and subcloned into the shuttle plasmids or synthesized (GeneArt, Thermo Fisher Scientific), and inserted into the pFL12 backbone and transformed in DH5α Escherichia coli cells. The resulting chimeric molecular clones were designated p3197-A, p3197-B, p1113-A, p1113-B, and p3068-A. Subclones containing either ORF2-4 or ORF5 were generated through a similar strategy, utilizing an NruI site in the FL12-EcoPac shuttle. Single amino acid mutations were synthesized and subcloned into the appropriate shuttle vector and introduced into pFL12 as described above.

Chimeric viruses (designated with a “v” prior to the haplotype name, e.g. v3197-A) were generated from the chimeric infectious clones via in vitro transcription and electroporation into MARC-145 cells, as described in (45). Briefly, plasmid DNA was linearized by digestion with AclI and viral RNA was synthesized using the T7 Ultra mMESSAGE mMACHINE in vitro transcription kit (Ambion, Life Technologies). Five μg of in
*vitro* transcripts and 5 μg naïve MARC-145 cellular RNA were added to 2x10⁶ MARC-145 cells in 400 μl DMEM containing 1.25% DMSO and electroporated at 250V and 950uF (GenePulser Xcell, Bio-Rad). Electroporated cells were plated in a single well of a 6-well plate in 5 ml DMEM supplemented with 10% FBS, antibiotics, and 1.25% DMSO. At 18 hours post transfection (hpt), media was replaced with 5 ml DMEM supplemented with 5% FBS and antibiotics. At 96 hpt, supernatants were harvested and cells were stained by immunocytochemistry to verify virus replication. Supernatants were passaged two to three times in MARC-145 cells to produce high titer chimeric virus stocks. All stocks were sequenced through ORF2-6 to confirm the presence of the correct haplotype.

**Virus neutralization assays**

Neutralizing antibody assays were performed using a focus-reduction assay adapted from Wu et al., (50). Briefly, sera was heat-inactivated, diluted, incubated for 1 hour at 37°C with 200 focus-forming units (FFU) of virus, and inoculated in duplicate or triplicate onto MARC-145 cells seeded the previous day in a 12-well plate at 3x10⁵ cells/well. Cells and virus were incubated an additional 24 hours at 37°C in 5% CO₂, then the cells were fixed in ice-cold acetone:methanol and stained for PRRSV N protein by immunocytochemistry using the monoclonal antibody SDOW17 (Rural Technology) as the primary antibody and sheep anti-mouse IgG conjugated HRP (Jackson ImmunoResearch) as the secondary antibody. Following addition of the HRP substrate, cells were rinsed with distilled water, air-dried, and foci of infected cells enumerated by light microscopy. The percent reduction in FFU compared to a virus-only control was calculated as the percent neutralization. The neutralizing antibody titer is reported as the serum dilution that neutralized 50% of virus
(ND50). The rebound serum dilution that neutralized 50% of FL12 (1:8 for 3197 42dpi and 1:4 for 1113 and 3068 35dpi) was used to determine neutralization sensitivity of the chimeric viruses to autologous sera. Assays were done in duplicate and repeated at least twice. A student’s t-test was used to compare neutralization of FL12 to each chimeric virus across all replicates, and the standard deviation of the mean was calculated from the mean of experiments.

Sera samples from PHGC pigs were kindly provided by Drs. J.K. Lunney and R.R.R. Rowland and Type II PRRSV broadly neutralizing antiserum was the gift of Harrisvaccines, Ames, IA.

**Nucleotide sequence accession numbers**

The GenBank Accession numbers for the nucleotide sequence for ORF2-6 are KX286534-KX286735 and for nsp2 are KX286736-KX286951.

**Results**

**Rebound in viremia is associated with neutralizing antibody to inoculum virus**

To aid in identifying immune escape variants, we selected five pigs with differing virological outcomes: two pigs that maintained high levels of viremia through 35 dpi, designated “prolonged”, and three pigs that initially cleared the virus, but rebounded in viral load by 35 or 42 dpi, designated “rebound” (Figures. 3.1A, 3.1B). We hypothesized that prolonged viremia was due to a weak immune response, whereas rebound in viremia was due to an initially effective immune response and subsequent selection of immune
escape variants. To determine if differences in virological outcome were associated with differences in immune response, we tested 42 dpi sera from each pig for neutralizing activity against the inoculum virus, NVSL97-7895 (Figure 3.1C). The neutralization curves observed in the prolonged and rebound pigs are consistent with previous descriptions of a weakened and delayed humoral immune response to PRRSV (7, 8). Although no sera was able to neutralize 100% of the inoculum virus, all three rebound pigs (1113, 3068, and 3197) showed moderate levels of neutralizing activity, with ND50 titers of 44, 48, and 45, respectively. In contrast, the two prolonged pigs, 1134 and 3161, had little detectable neutralizing activity against the inoculum (ND50 <4), suggesting that prolonged viremia results, at least in part, from the lack of a detectable NAb response. Although these differences in neutralizing activity against the inoculum virus are consistent with our overall hypothesis, it is not clear if the modest level of neutralizing antibody in rebound pigs was sufficient to exert selective pressure in vivo.

**Low rate of genetic variation in vivo during early stages of PRRSV infection**

The five pigs with differing virological and immunological outcomes were used to characterize virus variation during the course of PRRSV infection in vivo. By comparing viral sequences from the envelope protein-encoding ORF2-6 and nonstructural protein 2 (nsp2) at multiple time points across pigs with differing viremia profiles, we were able to perform a comparative analysis of selective pressure and viral variation during PRRSV infection. Viral RNA was isolated from the inoculum and from sera samples collected at 7 dpi and at a late dpi from each pig. Using primers to conserved regions of the PRRSV genome, approximately 3kb each of ORF2-6 and nsp2 were amplified, TA-cloned, and
individual clones were Sanger-sequenced. For each region, we sequenced up to 35 clones from each individual pig sample. In total, we analyzed greater than 200 clones per region, each representing an individual viral genome (Table 3.1).

Multiple sequence alignments of all nucleotide sequences for each gene region were generated by ClustalW to determine the amount of variation within ORF2-6 and nsp2. In both regions, the average nucleotide pairwise identity within each pig and dpi was greater than 99%, and was similar to that observed in the starting inoculum. In addition, the average pairwise identity across all sequences of ORF2-6 and nsp2 was 99.57% in both gene regions (Table 3.1). The surprisingly high level of genetic identity revealed that very little genetic variation occurred in vivo during the six weeks following experimental PRRSV infection.

**ORF2-6 is under selective pressure not found in nsp2**

Although there was an overall low level of genetic variation in vivo, it is possible that some of the observed changes were not simply random, but instead reflected selective pressure(s) present during the course of infection. Widely used tests of genetic selection based on dN/dS ratios are not appropriate for this sample set due to the preponderance of overlapping reading frames in ORF2-6 and the overall small amount of genetic variation observed in our samples. However, selection of viral variants within the population could give rise to temporal patterns of genetic variation across the viral sequences. Moreover, differences in patterns of variation between ORF2-6 and nsp2 would provide evidence of differing selective pressures in vivo.
In order to visualize patterns of variation over time in the viral sequences, we used discriminant analysis of principal components (DAPC) (46). Figure 3.2 shows how well the inoculum, 7 dpi, and late sampling days were separated in nsp2 and in ORF2-6. In nsp2, the inoculum sequences were the most easily separated group, with little separation of the 7dpi and late sequences (Figure 3.2). This pattern reveals that the majority of variation in nsp2 accumulated by 7dpi, with less variation occurring over the subsequent course of infection. A different pattern was observed in ORF2-6 (Figure 3.2). Some overlap was found between inoculum and early sequences, as well as between early and late sequences; however, the ORF2-6 inoculum and late sequences showed very little overlap. This pattern demonstrates that ORF2-6 sequences continually accumulated variation during infection within the host, which is consistent with ongoing selective pressure on the envelope proteins. Together, these results suggest that nsp2 and ORF2-6 undergo differing patterns of evolution and selective pressures during PRRSV infection in vivo.

We next used the Cochran-Mantel-Haenszel (CMH) test to detect associations between single nucleotide variants (SNV) and time during the course of infection that may contribute to the patterns seen in DAPC. While this method does not rule out genetic drift as an explanation, highly significant associations between a SNV and dpi across pigs may indicate that the SNV is under selection. All SNV sites in all five pigs (421 sites in ORF2-6; 504 sites in nsp2) were tested for association between the SNV and dpi. After controlling for multiple testing, we identified 18 SNVs in ORF2-6 and 23 SNVs in nsp2 showing significant association with dpi (Table 3.2). To aid in distinguishing genetic drift from selection, we used a one-sided Fisher’s exact test to test whether significant SNV identified by the CMH test were more likely to be nonsynonymous compared to all other possible
changes to the inoculum consensus sequence. Even when considering overlapping reading frames, a significant proportion (15/18) of the SNVs in ORF2-6 resulted in amino acid changes (p-value 0.030). In contrast, only 12 of the 23 SNVs in nsp2 were nonsynonymous, which was not a significant proportion (p-value 0.863). While we cannot rule out that some or all of these changes in allelic frequency over time are due to genetic drift, the distinct patterns of variation over time in ORF2-6 and nsp2, combined with the unusually high frequency of nonsynonymous changes in ORF2-6 but not nsp2, suggest that these two gene regions experienced different selection pressures in vivo.

**Variation in ORF2-6 is pig-specific**

The differences observed in patterns of evolution and genetic changes between ORF2-6 and nsp2 may be due to differences in immune selection pressures on the envelope vs. nonstructural proteins. The ORF2-6 encoded envelope proteins are exposed to the humoral immune response and have been shown to be targeted by neutralizing antibody (NAb) (11, 14, 37-38, 40-41, 51); therefore, they are the most likely targets of immune selection pressures from NAb. Our statistical analysis had sufficient power to detect significant changes in variation because it leveraged information across all pigs, but escape from humoral immunity may be highly pig-specific. Thus, to detect possible changes conferring immune escape, we examined variation in the envelope proteins of each of the rebound and prolonged pigs that might identify specific changes in rebound virus associated with immune escape. A multiple sequence alignment (MSA) of ORF2-6 sequences from the inoculum and all pigs and days were aligned relative to the consensus sequence of the inoculum (Figure 3.3A). Distinct patterns of SNVs were identified. Two
identical nucleotide changes, C1050T and A1553G, were dominant or nearly dominant in all pigs by 7dpi, and became fixed by the late day (Figure 3.3A, arrows). Both of these SNVs were present as minor components of the inoculum, and the increase in frequency by 7dpi and fixation by the late day is suggestive of selection of those variants for growth in vivo. In addition, several nucleotide changes arose or increased in frequency from the inoculum and became fixed by the late day only in a single pig, suggestive of selective pressures, or genetic drift, specific to the virus population within an individual pig. Numerous single nucleotide changes were observed in single clones, representative of random variation most likely due to mistakes during replication. In addition, we observed co-existence of dominant and subdominant variants in multiple or single pigs, demonstrating a quasispecies population structure of PRRSV ORF2-6 in vivo. Consistent with previous studies, we found no differences in variation within and outside known PRRSV epitopes (data not shown). The most striking observation was that virus variation was largely pig-specific and no single variant clearly distinguished virus from rebound pigs (1113, 3068, 3197) and virus from prolonged pigs (1134, 3161).

**Identification of pig-specific ORF2-6 haplotypes within late day virus populations**

The major and minor PRRSV envelope proteins interact to form oligomeric complexes on the virion surface, and it is possible that variation at a particular site in one envelope protein may affect or constrain variation at a second site in the same or different envelope protein. Because we utilized single clone sequencing, where each clone represents a single viral genome, it was possible to identify linked sites of variation across ORF2-6. Based on these linked sites of variation, we were able to identify and construct
unique viral haplotypes within the late day sample of each pig (Figure 3.3B). To reduce background variation, only sites with a minor variant frequency of ≥25% of clones within each individual pig (“variable sites”) were included in the haplotypes. Haplotypes from each sample also included the dominant changes in that sample, defined as a change from the inoculum consensus present in >75% of virus clones within a pig. Haplotypes found at the two highest frequencies within each pig were designated haplotypes A and B, respectively. Table 3.3 details the ORF2-6 virus haplotypes identified in each pig, including the SNVs and the associated amino acid change(s), the percentage of clones in the inoculum and 7dpi samples that had each SNV, and the frequency of the haplotype within the pig. Some of the SNVs included in the haplotypes were detected in the inoculum and/or 7dpi samples, while other SNVs were present in only the late dpi sample. It is not known if the SNVs appearing in late dpi samples arose de novo during the course of infection, or were present at low frequency in the inoculum. Importantly, however, none of the predominant haplotypes were observed in any of the inoculum clones, and the frequency of predominant haplotypes dramatically increased from the starting inoculum to the late dpi sample. In four of the five pigs, the late day ORF2-6 sequences were comprised of distinct haplotypes within each pig whereas in the fifth pig, rebound pig 3068, the virus sample was comprised of a largely homogenous population (Figure 3.3B). In addition to the dominant haplotypes, all samples contained minor haplotypes at lower frequencies that were composed of a combination of the dominant and subdominant haplotypes. Interestingly, while some ORF2-6 haplotype SNVs were shared across virus samples in different pigs, the haplotypes were largely pig-specific (Figure 3.3B). The only variant that was found in rebound virus haplotypes and not in prolonged virus haplotypes was a GP5 amino acid
change from alanine to valine at residue 27. However, this change was present in just three of the five rebound virus haplotypes and was detected as a minor variant in both prolonged pigs. The observation that late day virus populations in most pigs were dominated by a few distinct haplotypes suggests that co-variation across ORF2-6 may confer a selective advantage to the virus.

Rebound viremia in some pigs is due to antigenic variation and immune escape

To determine if and how genetic variation in rebound virus contributes to immune escape, the five ORF2-6 haplotypes identified in rebound pigs were inserted into an infectious molecular clone, pFL12 (Figure 3.4A), and chimeric viruses were tested for sensitivity to neutralization by autologous serum collected from the pig’s rebound day, (Figures 3.4B, C). The pFL12 infectious molecular clone (45) represents the consensus sequence of the inoculum virus, NVSL97-7895, and neutralization of FL12 was used as a reference in all neutralization assays. Due to the limited amount of pig sera, the dilution of rebound day sera that neutralized 50% of FL12 was used to evaluate rebound virus sensitivity to neutralization by autologous sera. Neutralization of FL12 was compared to each chimeric virus via a student’s t-test, and chimeric viruses that were more resistant to neutralization than FL12 were considered NAb escape variants. Chimeric viruses containing either haplotype A or haplotype B from pig 3197, designated v3197-A or v3197-B, were more resistant to neutralization by autologous sera than FL12, with average neutralization of 11% \( (p=0.0002) \) and 7% \( (p=<.001) \), respectively (Figure 3.4B). Similarly, both v1113-A and v1113-B chimeric viruses, containing haplotypes A and B from pig 1113, respectively, were more resistant to neutralization by autologous sera than FL12; v1113-A
and v1113-B had average neutralizations of 18% (p=0.009) and 29% (p=0.059), respectively (Figure 3.4C). Thus, the PRRSV ORF2-6 haplotypes dominant during rebound viremia in pigs 3197 and 1113 were resistant to neutralization by autologous sera that neutralized FL12, the inoculum consensus sequence. These results provide, for the first time, direct evidence of antigenic variation and immune escape during PRRSV infection in vivo. Interestingly, chimeric virus containing the single dominant ORF2-6 haplotype from pig 3068 (v3068-A) was neutralized at similar levels as FL12 (48%, p=0.8) (Figure 3.4D), demonstrating that selective pressure by neutralizing antibody is not the sole factor in PRRSV rebound viremia. It is possible that cell-mediated immune responses, or non-immune selective pressures, contributed to rebound in viremia in pig 3068.

**Variation in either major or minor envelope glycoproteins can mediate escape from neutralizing antibody**

In order to identify the envelope protein(s) that mediate NAb escape, we divided the rebound escape haplotypes from pigs 3197 and 1113 into their oligomeric units. This strategy preserves potentially interacting variants within the oligomeric forms of the glycoproteins. None of the escape haplotypes contained changes in ORF6 (M), so we generated chimeric viruses containing only ORF2-4 (which encodes the minor glycoprotein GP2/GP3/GP4 trimer and E) or ORF5, which encodes GP5 and 5a (Figure 3.4A). In pig 3197, immune escape mapped to ORF5 (Figure 3.4E). Both v3197-A-5 and v3197-B-5 chimeric viruses were more resistant to neutralizing autologous sera than FL12, with average neutralizations of 29% and 6% (p=0.035 and <0.001), respectively. In contrast, chimeric viruses v3197-A-2-4 and v3197-B-2-4 were neutralized at similar levels as FL12.
It was interesting to note that v3197-A-5 did not recapitulate the neutralization phenotype of v3197-A (Figure 3.4E). Although this may be due to variation in the neutralization assay, it is also possible that interactions between the major and minor glycoproteins contribute to the neutralization in the 3197-A haplotype.

In pig 1113, haplotypes A and B differed with respect to the region that mediated immune escape (Figure 3.4F). Chimeric virus v1113-A-2-4 was neutralized at 18%, (p=0.016) whereas v1113-A-5 was neutralized at levels similar to FL12. The finding that immune escape of v1113-A mapped to ORF2-4 was not surprising, as the 1113-A haplotype does not differ from FL12 in ORF5 (Table 3.3, Figure 3.5C). In contrast to v1113-A, immune escape of v1113-B was mediated through ORF5: v1113-B-2-4 chimeric virus was not neutralized at significantly lower levels than FL12 (41%, p=0.2), but v1113-B-5 chimeric virus was resistant to neutralization (19%, p=0.025) (Figure 3.4F). Together, these data suggest that both the major and minor envelope proteins can serve as targets of immune selective pressures by NAb. The finding that genetic variation in multiple envelope glycoproteins can contribute to neutralization resistance reveals that PRRSV has multiple pathways of immune escape. Moreover, the co-existence of haplotypes utilizing different immune escape pathways within an individual pig likely contributes to the difficulty of producing effective vaccines for PRRSV.

**Identification of multiple pathways of immune escape from neutralizing antibody**

To determine if we could identify specific amino acids that mediate immune escape, we compared sites of amino acid variation between the immune escape haplotypes and FL12 (Table 3.3, Figures 3.5A, C). Resistance to neutralizing antibody in both the 3197-A
and 3197-B haplotypes mapped to ORF5 (Figure 3.4E), and we observed variation at three amino acid positions in GP5 within the 3197 haplotypes (Figure 3.5A). Both 3197-A and 3197-B contained a K57E change, and both haplotypes varied at GP5 residue 32; however, 3197-A contained an N32S mutation, while 3197-B contained an N32K mutation. The N32K mutation is coincident with a Q36K change in protein 5a, which overlaps GP5. Protein 5a is a very minor component of the virion and the Q36K change is unlikely to mediate NAb escape as previous studies found that immunization with 5a does not elicit neutralizing antibodies (33, 52). In addition to the changes noted above, 3197-B GP5 also contained an A27V change. Variant viruses containing each of these single amino acid changes in the FL12 background were tested for neutralization by pig 3197 42 dpi rebound serum (Figure 3.5B). The N32S mutation resulted in a modest but significant reduction in neutralization from FL12 (Figure 3.5C, p<0.001). The 36% neutralization was similar to that observed with v3197-A-5 (29%), and not as low as the v3197-A virus (11%) (Figure 3.4E). Viruses containing the remaining single amino acid changes (A27V, N32K, and K57E) were neutralized by pig 3197 serum at levels similar to FL12 (Figure 3.5C). Thus, while GP5 N32S likely is involved in immune escape from pig 3197 NAb, it appears that multiple amino acid changes in GP5 are required for the neutralization resistance phenotype seen in v3197-A. This suggests neutralization may depend on conformational epitopes, or other structural changes to GP5 that result from interactions between these single amino acid changes.

Immune escape by the v1113-A virus mapped to changes in ORF2-4, while escape by the v1113-B virus mapped to ORF5 (Figure 3.4F). We reasoned that escape by v1113-A was mediated by unique amino acid changes in ORF2-4 that were not found in either FL12
or the 1113-B haplotype. The only amino acid change that fit these criteria was a P96S change in GP3 (Table 3.3, Figure 3.5B). This change was introduced into FL12 and tested for neutralization phenotype using pig 1113 autologous rebound serum. The GP3 P96S variant virus was somewhat more resistant to neutralization than FL12 (35%, p=0.1), but was not as resistant as the v1113-A (18%) (Figure 3.5D). Thus, escape by v1113-A may be partially mediated through GP3 P96S but, as in v3197-A and v3197-B, likely requires additional amino acid changes. The other amino acid differences between FL12 and the 1113-A haplotype were GP3 L143F and GP4 I129V (Figure 3.5B), two changes that were found in all pigs at all time points. It appears, therefore, that escape of v1113-A from autologous NAb depends on some unique combination of these three amino acid changes in GP3 and GP4. In contrast to the other chimeric viruses, immune escape of v1113-B virus mapped to a single A27V change in GP5 (Figure 3.5B, D). Interestingly, the A27V variant was found in all three rebound pigs (Table 3.3), including pig 3068. While A27V may contribute to immune escape in v3197-B, only in pig 1113 was A27V sufficient to mediate escape when present as a single amino acid change.

In summary, the mapping and mutational analyses identified immune escape variants in two rebound pigs and determined that escape can be mediated through genetic variation in multiple envelope proteins, including GP5 and GP3. Immune escape usually, but not always, required a combination of amino acid changes. Importantly, the key amino acid changes and/or combination of changes that mediated escape differed by pig and virus population.
Immune escape variants are sensitive to neutralization by broadly-neutralizing antibody

In two of the three rebound pigs, genetic variation in ORF2-6 resulted in antigenic variation and immune escape. In pig 1113, rebound viremia peaked at 35 dpi and returned to background levels by 42 dpi (Figure 3.1B). Sera collected from pig 1113 at 42 dpi neutralized v1113-A and v1113-B at levels similar to FL12 (Figure 3.6A), demonstrating that the reduction in viremia was associated with development of neutralizing antibody to rebound virus. This suggests that the immune escape variants identified in this study were not broadly neutralization-resistant, but rather were antigenic variants that arose under selection by type-specific antibody. To test this, we evaluated the sensitivity of the chimeric virus containing the escape haplotypes against Type II PRRSV broadly neutralizing antiserum (Figure 3.6b). FL12 and each of the chimeric escape viruses were all highly susceptible to neutralization by the broadly neutralizing antiserum, which neutralized each virus at ND50s >1000. A similar, episodic pattern of immune escape and subsequent control is characteristic of some other virus infections, notably equine infectious anemia virus (53).

Discussion

Genetic diversity of PRRSV results from a high rate of mutation and recombination that occurs during virus replication (reviewed in 54); however, the selective forces that drive the emergence of PRRSV variants are not well understood, and the existence of immune escape variants has been controversial (6, 8, 12, 16). The availability of retrospective
samples from pigs with known virological and immunological outcomes allowed us to directly investigate immune selection and antigenic variation during the course of PRRSV infection. Our comprehensive analyses included genetic and statistical characterization of viral variation at sequential times following infection, and empirical approaches to functionally test viral variants for immune escape. Each of the statistical analyses, including DAPC and the CMH test, revealed that ORF2-6 was under selective pressures distinct from that observed for nsp2. Even within virus samples that were greater than 99% identical, unique ORF2-6 haplotypes were present in virus populations within each pig. Chimeric viruses containing predominant haplotypes identified in rebound virus populations from two pigs were resistant to neutralization by autologous sera that neutralized the inoculum virus, indicating escape from NAb. The envelope proteins and the specific amino acids involved in neutralization differed between these two pigs, and also between virus haplotypes found within the same pig, indicating that PRRSV utilizes multiple genetic pathways for immune escape. The NAb escape variants were susceptible to neutralization by heterologous broadly neutralizing antibody, and by autologous sera collected later in infection. Together, these data provide, for the first time, direct evidence that selective pressures can result in antigenic variation and immune escape during PRRSV infection and may contribute to PRRSV diversity and persistence.

The results from this study demonstrate that PRRSV utilizes multiple pathways of escape from neutralizing antibody, which varied by pig and virus haplotype. In two OR2-6 haplotypes, immune escape mapped solely to amino acid changes in GP5, confirming that GP5 is an important target of neutralizing antibody in PRRSV-infected pigs. The only amino acid change in GP5 that was observed in all three rebound pigs was A27V, which on its own
was sufficient to mediate immune escape in pig 1113, but not pigs 3197 or 3068. A27V is located within a non-neutralizing decoy epitope in GP5 (14), as well as in one of two proposed signal peptide cleavage sites between residues 26/27 and 31/32 (55-56). Interestingly, both escape haplotypes from pig 3197 varied at the downstream cleavage site in GP5 N32S/K. It is not known whether these changes alter cleavage of the signal peptide; if so this may alter expression of the decoy epitope, and facilitate escape through a mechanism of immune evasion proposed in (8) and (19). Variation at these sites has also been associated with cross-neutralization susceptibility (57). The only haplotype to escape solely through GP2/3/4 was v1113-A from pig 1113, which contained three amino acid changes from FL12: GP3 P96S located in a previously identified B-cell epitope (58), GP3 L143F and GP4 I129V. It is interesting to note that all three changes were present as dominant variants in virus populations of other pigs (Table 3.3, Figure 3.3). GP3 L143F and GP4 I129V were present in the inoculum and throughout infection in both rebound and prolonged pigs, while GP3 P96S was observed as a transient dominant or near-dominant variant in all of the 7 dpi samples (Table 3.3, Figure 3.3). While it is not clear how these changes mediated immune escape of v1113-A in pig 1113, this pathway of escape was not favorable in any of the other pigs. With the exception of GP5 A27V in 1113-B, none of the single amino acid changes were capable of mediating escape on their own. In most cases, therefore, sensitivity or resistance to neutralization was largely context dependent: amino acid changes that mediated neutralization and escape in one virus haplotype or pig did not necessarily mediate escape in another haplotype or pig. The finding that there are multiple and complex pathways of immune escape that differ between pigs may explain some of the
confounding and contradictory evidence in the literature regarding PRRSV neutralization and neutralizing epitopes (8, 14, 39).

The use of retrospective samples from PRRSV infected pigs with different virological and immunological outcomes, combined with a virus haplotyping approach, allowed us to demonstrate for the first time that antigenic variation and immune escape can occur during early stages of PRRSV infection in vivo. The role of immune selection and escape in PRRSV has been a matter of debate (5-6, 8, 17, 59). Previous studies of PRRSV evolution in vivo have found little evidence of immune selection using both statistical and immunological approaches (17-19), reviewed in (6, 19). A limitation in most of these studies is that sequences were evaluated at only a single time point within an individual pig, which lessens the power to detect immune selection during the course of infection. In addition, some studies have relied on statistical approaches that compare variation within and outside known linear epitopes. However, these approaches likely miss variation in unidentified conformational epitopes. Indeed, our own statistical analyses did not detect significant differences in variation between epitope and non-epitope regions (data not shown). Another advantage of our approach is the use of single clone sequencing rather than next-generation sequencing (NGS) to analyze PRRSV variants. Although our approach did not distinguish variants that arose de novo from rare variants pre-existing in the inoculum, it did allow us to detect significant changes in the frequency of SNVs over the course of infection and thus identify sites that may be under selection. NGS does allow more depth of coverage and identification of rare SNVs, but the short reads make it difficult to reconstruct individual viral haplotypes. By using single clone sequencing of the entire ORF2-6 region, we were able to construct ORF2-6 haplotypes representing linked sites of
co-variation across the envelope proteins. In three of the four escape haplotypes, we observed resistance to neutralization only in the context of multiple amino acid changes across a haplotype, which would not have been possible using NGS or analyzing only the consensus sequences. Our finding of ORF2-6 immune escape variants in some rebound virus populations provides experimental support to other studies that describe indirect evidence of immune selection on the envelope protein-encoding ORFs (5, 10-12, 59).

Neutralizing antibody is not the only factor affecting clearance of PRRSV or selection of PRRSV variants; however, passive transfer experiments (60) have established that NAb can be an important component of a protective immune response in vivo. Our results indicate that modest levels of NAb can exert immune pressure and select for neutralization escape variants, and that escape from NAb can be mediated by as few as one amino acid change in PRRSV envelope proteins. Immune selection by neutralizing antibody did not occur in all pigs we analyzed, nor was it the only factor important in selection of PRRSV rebound virus. In some pigs, however, a partially effective NAb response was sufficient to exert immune pressure and select for antigenically variant virus and a transient rebound in viremia. This transient escape from NAb may contribute to the spread of PRRSV through continual infection and reinfection within and between herds.

Acknowledgements

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thanked for the PRRSV Type II broadly neutralizing antisera and Hyelee Loyd is thanked for her excellent technical assistance. We thank Dr. Fernando A. Osorio, Dr. Asit K. Pattnaik, and Dr. Byung Kwon for the pFL12 plasmid and for advice in construction of chimeric infectious clones.

**Literature Cited**


53. **Craigo JK, Montelaro RC.** 2013. Lessons in AIDS vaccine development learned from studies of equine infectious, anemia virus infection and immunity. Viruses 5:2963-2976.


### Table 3.1. Summary of sequenced clones and viral variation

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\(^a\)Number of individual clones sequenced from each sample

\(^b\)Average nucleotide pairwise identity across all clones from a sample

\(^c\)Average nucleotide pairwise identity across all clones sequenced from each gene region
Table 3.2. Summary of significant nucleotide variants and associated amino acid changes in ORF2-6 and nsp2

aSignificant SNV were identified in the CMH test, as described in Materials and Methods
bFor ORF2-6, the envelope protein(s) in which the mutation occurred
cAssociated amino acid change. Blank entries represent synonymous changes
dSignificance of the change in SNV frequency over time

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<th>Holm adj. P-value&lt;sup&gt;d&lt;/sup&gt;</th>
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Table 3.3. Predominant ORF2-6 haplotypes within late day virus samples

The nucleotide (NT) position is based on the ORF2-6 region, with the first nucleotide of the GP2 start site as the first position. The amino acid (AA) denotes the associated protein and amino acid at the NT position. If an NT change occurs in an overlapping reading frame, the associated amino acid is listed for both proteins.

Haplotypes A and B represent the haplotypes present at the highest and second highest frequencies within each pig, respectively. Haplotypes include both sites that vary between haplotypes and shared consensus changes from the inoculum. T

* Synonymous change
+ Significant in CMH
^ Change located in previously identified B-cell epitope

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| 68  | GP2| 23 |          | | 3197 | 30 | 143| 1795     | | 1884 | GP5| 57 |          | | 3068 | 50 | 143| 1523     | | 1533 | GP4| 7  |          | | 1795 | GP5| 27^|          | | 1795 | GP5| 41^ |          | | 2489 | M  | 63 |          |
| 134 | GP2| 45^|          | | 1553+| GP4| 129|          | | 1810 | GP5| 32^|          | | 1838 | GP5| 41^ |          | | 20   | GP2| 7  |          | | 1050+| GP3| 143|          | | 1523+| GP4| 119|          | | 1533+| GP4| 129|          | | 1795 | GP5| 27^|          | | 2489 | M  | 63 |          |
| 711 | GP2| 237|          | | 1795 | GP5| 30 |          | | 1811 | GP5| 32^|          | | 1811 | GP5| 36 |          | | 1838 | GP5| 41^ |          | | 20   | GP2| 7  |          | | 1050+| GP3| 143|          | | 1523+| GP4| 119|          | | 1533+| GP4| 129|          | | 1795 | GP5| 27^|          | | 2489 | M  | 63 |          |
| 1050+| GP3| 143|          | | 1426 | GP4| 86^|          | | 1795 | GP5| 27^|          | | 1810 | GP5| 32^|          | | 1838 | GP5| 41^ |          | | 20   | GP2| 7  |          | | 1050+| GP3| 143|          | | 1523+| GP4| 119|          | | 1533+| GP4| 129|          | | 1795 | GP5| 27^|          | | 2489 | M  | 63 |          |

Inoculum

| Pig | NT | AA | Inoculum | | Pig | NT | AA | Inoculum | | Pig | NT | AA | Inoculum |
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Figure 3.1. Viremia and neutralization profiles in selected pigs. **A**) Prolonged pigs are those that sustained virus loads >10^3 copies/ml sera through 35 dpi. **B**) Rebound pigs are those that initially cleared virus (<10 copies/ml sera), then experienced a greater than 100 fold-increase in viremia. **C**) Virus neutralizing activity of 42 dpi pig sera from prolonged and rebound pigs against the inoculum virus. Serum was serially diluted two-fold and tested for neutralizing activity against 2x10^2 FFU of the NVSL97-7895 inoculum using a focus-reduction assay. The mean percent neutralization at each serum dilution compared to a no-serum control is reported. Prolonged pigs are represented by solid gray lines and rebound pigs by solid black lines. Error bars represent ±1 standard deviation of the mean from at least two independent experiments.
Figure 3.2. Temporal changes in the population structure in A) nsp2 and B) ORF2-6 at sequential times after experimental infection. Plots for the discriminant analysis of principle components (DAPC) are grouped by the inoculum, 7 dpi, and late dpi. Inset plots show the F statistic, quantifying the between-group variance to the within-group variance along each of the DAPC coordinates.
Figure 3.3. ORF2-6 quasispecies structure and late day viral haplotypes. A) ORF2-6 sequence alignment of all viral clones in reference to the consensus sequence of the inoculum (top line). Sites of variation from the inoculum consensus within a clone are indicated by a colored tick. Sequences of individual clones are arranged vertically by inoculum, pig (legend on the right), and then dpi within pigs separated by dotted horizontal lines, with 7dpi sequences shaded in gray. Numbers on the left denote the number of clone sequences aligned. Colors in the plot indicate nucleotide: A=green, G=blue, C=purple, T=red, and deletion=gray. Orange ticks at the bottom of the alignment indicate sites with at least one mutation across all clone sequences from all pigs, including possible deletions. Arrows denote sites of variation common across the majority of dpi and pigs. B) Schematic of ORF2-6 haplotypes constructed from the late day virus within each pig. Haplotypes were constructed using ORF2-6 sites within each pig’s late day virus with a minor variant frequency of ≥25% (represented by small colored ticks), dominant changes >75% (represented by large colored lines), and removing all other variation within a pig’s late day virus. Nucleotide colors are consistent with A. The predominant haplotypes from within each late day pig’s virus sample are reported, with the inoculum consensus reference nucleotide displayed on the top row. The frequency of each haplotype is listed on the right side.
A. ORF1a ORF1b 2a 3 4 5a 6 7

FL12 ORF2-6 chimera ORF2-4 chimera ORF5 chimera

B. 3197 42dpi serum
C. 1113 35dpi serum
D. 3068 35dpi serum

E. 3197 42dpi serum
F. 1113 35dpi serum
**Figure 3.4.** Neutralization phenotype of rebound haplotypes against autologous rebound sera. **A)** Schematic of chimeric viruses containing rebound haplotypes. The PRRSV genomic structure is depicted at the top, the NVSL97-7895 consensus sequence of the FL12 infectious molecular clone is shaded gray and the black boxes denote the regions of FL12 that were replaced with rebound haplotype variants. **B and E)** Neutralizing activity of pig 3197 42 dpi serum diluted 1:8 against FL12 and chimeric viruses containing 3197-A and 3197-B haplotype variants. **C and F)** Neutralizing activity of pig 1113 35 dpi serum diluted 1:4 against FL12 and chimeric viruses containing 1113-A and 1113-B haplotype variants. **D)** Neutralizing activity of pig 3068 35 dpi serum diluted 1:4 against FL12 and chimeric virus containing the single haplotype 3068-A haplotype. Assays were performed in duplicate and the mean of at least two independent experiments is reported, and error bars represent ±1 standard deviation of the mean. Asterisks indicate significant difference (p<0.05) in neutralization compared to FL12 by a student’s t-test.
Figure 3.5 The effect of single amino acid variants from ORF2-6 rebound haplotypes on resistance to autologous neutralizing serum. Sites of amino acid variation between the inoculum/FL12 consensus and the A) 3197 haplotypes and C) 1113 haplotypes. Amino acids that differ from the inoculum/FL12 consensus are in bold. The single amino acid variants were introduced into FL12 and the resulting chimeric viruses were tested for resistance to autologous neutralizing serum for B) pig 3197 42 dpi serum at 1:8; and D) pig 1113 35 dpi serum at 1:4. The mean of at least two independent experiments is reported, and error bars represent ±1 standard deviation of the mean. Asterisks indicate a significant difference (p<0.05) in neutralization compared to FL12 by a student’s t-test. ^Denotes that this virus is the same as v1113-B-5.
Figure 3.6. Immune escape variants are sensitive to neutralization by broadly neutralizing sera. **A)** FL12 and v1113-A and v1113-B escape viruses were screened for neutralization sensitivity to 1113 42 dpi serum. **B)** FL12 and the escape haplotypes from pigs 3197 and 1113 were tested for neutralizing sensitivity against broadly neutralizing PRRSV anti-sera. The mean of at least two independent experiments is reported, and error bars represent ±1 standard deviation of the mean.
CHAPTER 4: CHARACTERIZATION OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS NEUTRALIZATION AND REPLICATION PHENOTYPES OF NEUTRALIZING ANTIBODY ESCAPE VARIANTS

A paper to be submitted for publication

Alyssa B. Evansa, Marcus Boltona, Hyelee Loyda, and Susan Carpentera

Department of Animal Science, Iowa State University, Ames, Iowa, USAa

Abstract

We previously identified neutralizing antibody (NAb) escape variants that arose during the course of experimental infection with porcine reproductive and respiratory syndrome virus (PRRSV). Escape variants arose in pigs that experienced rebound viremia during a 42 day infection period. The escape variants were resistant to autologous rebound day serum that neutralized the inoculum virus, however the step in replication targeted by NAb was not known. To determine which step(s) in PRRSV replication NAb targeted, we performed virus-binding and replication assays in vitro in the presence and absence of pooled PRRSV anti-sera. A post-attachment, pre-genome synthesis step was revealed as the primary target of PRRSV NAb. We previously showed that NAb escape was mediated through mutation in GP5 and/or the GP2/GP3/GP4 trimer. Because all envelope proteins are required for PRRSV infection, the mutations that mediated immune escape may result in a reduction in replication fitness of the virus. In order to evaluate replication fitness of the immune escape variants, we performed a series of infectivity and replication kinetics
assays using the escape variants and the parental FL12 virus. Our results showed that one of the four escape variants had reduced infectivity compared to FL12, but none of the escape variants had markedly reduced growth rates in MARC-145 cells. These results suggest that the NAb that selected for NAb escape variants targeted regions of the envelope proteins not crucial for virus entry, and therefore escape mutations did not alter the replication phenotype in vitro.

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) continues to pose an economic burden on the worldwide pork industry due to significant economic losses from the disease (1). PRRSV causes stillbirths and abortions in pregnant sows and respiratory disease in growing pigs. Despite its prevalence for nearly thirty years (2), PRRSV is little better controlled today than it was when it first emerged simultaneously in Europe and the United States in the early 1990s (1, 2). This is due in large part to the lack of success of current vaccine strategies. Even the most effective PRRSV vaccines, modified live virus (MLV) vaccines, are limited in their effectiveness and are generally thought to be protective only against infection by homologous strains, and reversion of vaccine strains to pathogenic versions is a concern (3-5). Despite the general acceptance that MLV vaccines elicit protection against homologous challenge, we recently identified PRRSV neutralizing antibody (NAb) escape variants within virus samples with >99% nucleotide identity from experimentally infected pigs, demonstrating that even small changes in the virus can alter the virus’ susceptibility to neutralizing antibody. This finding suggests that escape from
neutralizing antibody may contribute to the continual occurrence and circulation of PRRSV within and between both vaccinated and unvaccinated herds.

We previously identified PRRSV neutralizing antibody escape variants that arose during the course of *in vivo* infection that were resistant to concurrent, autologous serum, but not heterologous serum. Additionally, we found that PRRSV neutralization targeted early steps of viral replication (prior to protein synthesis), and escape mapped to multiple envelope proteins, including the major protein GP5 and the minor GP2/GP3/GP4 trimer. Others have also identified neutralizing antibody targeted to GP5, GP3, and GP4 (6-9), suggesting multiple envelope proteins are required for viral entry. However, despite identifying some of the targets of PRRSV neutralizing antibody, it remains unclear which steps in PRRSV replication each envelope protein is involved in, and therefore which step(s) NAb blocks. Due to the complex nature of the PRRSV envelope protein-protein interactions and conflicting results of *in vitro* experiments, the exact roles of the PRRSV envelope proteins during entry are unclear.

For years, the host cell receptor for PRRSV remained a mystery; CD169 was implicated by several studies as a required receptor in the PRRSV natural host cell, porcine alveolar macrophages (PAMs), but this was controversial as the only PRRSV permissive cell line, MARC-145 African Green Monkey kidney cells, do not express CD169 (10-13). An additional cellular receptor, CD163, which is expressed in PAMs and MARC-145 cells, was also implicated as the PRRSV receptor through studies that showed that expression of CD163 could make non-permissive cells permissive to PRRSV (14). Due to the lack of CD169 on PRRSV-permissive MARC-145 cells and the presence of CD163 in all permissive cells, it was hypothesized that CD169 was an attachment factor and CD163 was a required
PRRSV receptor. This proposed mechanism of entry was circumstantially supported by the fact that CD169 binds to GP5, which is the most abundant PRRSV envelope protein and therefore likely mediates the initial attachment of the virion with the host cell, while CD163 binds the minor GP2 and GP4 envelope proteins, which are expressed sparsely on the virion, potentially protecting the receptor binding site by limiting its expression (15-17). Recently, a series of experiments utilizing gene-edited pigs with knockouts of either CD169 or CD163 found that pigs without CD169 were just as susceptible to PRRSV infection as wild type pigs; however, CD163 knockout pigs were completely resistant to PRRSV infection, decisively showing that CD163 is a required PRRSV receptor (18, 19). It has also recently been shown that GP5 interacts at a post-attachment step of entry with cellular factor MYH9, and this interaction is required for PRRSV entry (20). Furthermore, because CD169 is not a required attachment factor, other attachment factors may be necessary for PRRSV infection, possibly via interactions of GP5 with a different cellular factor, or GP5’s binding partner, M, which has been found to interact with heparin sulfate and facilitate attachment in PAMs and MARC-145 cells (12, 21, 22).

Previous studies in other viruses have shown that neutralizing antibody often targets epitopes in functionally important regions of the virus envelope proteins (23-28). This prevents the virus from completing its replication cycle, often targeting the critical steps of entry and fusion, resulting in neutralization of the infection. Due to the high error rate of RNA viruses, in some cases variation in the neutralizing epitopes occur preventing their recognition by host neutralizing antibody, which can result in immune escape. However, because mutations that mediate escape often occur in functionally important regions of the virus, escape mutants often have decreased replication fitness compared to
the parental, wild type virus. This cost of immune escape has been well characterized in human immunodeficiency virus type 1 (HIV-1). The discovery of broadly neutralizing antibodies (bNAb) capable of neutralizing a wide variety of genetically variant HIV-1 strains was a breakthrough for the field, and unsurprisingly these antibodies target crucial regions of the HIV-1 envelope protein (env), including the receptor binding site and the membrane-proximal external region required for fusion (23-26). However, viral variants capable of escape from bNAb that targets the CD4 binding site of env are commonly seen in patients chronically infected with HIV-1, and these escape variants are usually associated with inhibited CD4-mediated viral entry and replication, demonstrating a decrease in function of the escape mutations in the CD4-binding site (27, 28).

In the current study, we used pooled serum from the PRRS Host Genetics Consortium that was capable of neutralizing the inoculum virus as well as all of our identified escape mutants to identify which step(s) of PRRSV replication are targeted by neutralizing antibody. Due to our discovery that neutralizing antibody from individual pigs can target both the major and minor envelope proteins, the pooled serum from 200 pigs should contain a broad range of neutralizing antibodies targeting multiple PRRSV envelope proteins. We hypothesized that this pooled neutralizing sera would block attachment and uptake, due to NAb targeted against GP5 and GP2/3/4, respectively, due to their proposed roles in PRRSV entry. We also characterized the replication phenotype of the escape variants via infectivity and replication kinetics assays to determine whether the NAb escape came at a fitness cost, which would suggest the mutations that conferred escape were within functionally important regions of the envelope proteins. Our results indicated that the pooled neutralizing antibody targeted a post-attachment step of the replication
cycle, and that NAb escape was not associated with a decrease in infectivity in MARC-145 cells, however individual viruses did vary in replication fitness. Together, these results show that PRRSV NAb targets a post-attachment step of entry, lending further support to previous findings that both the GP5/M dimer and the GP2/3/4 trimer play crucial roles during post-attachment steps of entry, and the escape variants may be the result of the virus “sampling” the most replicative favorable variants for immune escape.

Materials and Methods

Cells and viruses

MARC-145 cells were maintained in high glucose (4500mg/L) Dulbecco’s Modified Eagle’s Medium (DMEM) (Sigma) supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Primary porcine alveolar macrophages (PAMs) were the kind gift of Harrisvaccines and were cultured in RPMI 1640 (ATCC Modified – Gibco by Life Technologies) supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.5μg/ml Fungizone (Gibco by Life Technologies).

The PRRS Host Genetics Consortium inoculum virus, NVSL97-7895 (GenBank accession AY545985), was used in neutralization mechanism assays. The NVSL97-7895 virus was provided by the PHGC, and was passaged once in MARC-145 cells. The immune escape and non-escape chimeric viruses were constructed from the infectious cDNA clone pFL12 containing the NVSL97-7895 backbone, kindly provided by Dr. Fernando A. Osorio and Dr. Asit K. Pattnaik (University of Nebraska-Lincoln), as described previously in (29) and Chapter 3. Briefly, four ORF2-6 haplotypes that mediated escape from autologous NAb
(“escape viruses”) and three haplotypes that did not escape autologous NAb (“non-escape viruses”) were inserted into the pFL12 infectious molecular clone backbone via subcloning. FL12 and chimeric viruses were produced via in vitro transcription and electroporation of in vitro transcript products into MARC-145 cells, resulting in FL12 backbone virus, immune escape chimeric viruses and non-escape chimeric viruses. In addition, three of the escape viruses and one non-escape virus were separated into ORF2-4 and ORF5-6 regions and inserted into the FL12 backbone. The fourth escape virus, 1113-A, was not separated as it only contained mutations in ORF2-4. The virus constructs are summarized in Table 4.1.Viruses were passaged two to three times in MARC-145 cells to obtain high-titer stocks, with the exception of v1113-B and v1113-B-2-4, which did not grow in MARC-145 cells following electroporation and thus were passaged once in PAMs to produce a high enough titer to be infectious in MARC-145 cells. All viruses were harvested by collecting supernatants from infected MARC-145 cells and clarifying the supernatants via centrifugation at 1000xg for ten minutes. All viruses were aliquoted and stored at -80°C.

**Virus neutralization assays**

Neutralizing antibody assays were performed using a focus-reduction assay adapted from (30). Briefly, pooled sera obtained from the PRRS Host Genetics Consortium (PHGC) trial two was heat-inactivated, diluted 1:8 in media, incubated for 1 hour at 37°C with 200 focus-forming units (FFU) of the wild-type FL12 or chimeric viruses and inoculated in duplicate or triplicate onto MARC-145 cells seeded the previous day in a 12-well plate at 3x10⁵ cells/well. Cells and virus were incubated an additional 24 hours at 37° in 5% CO₂, then the cells were fixed in ice-cold acetone:methanol and stained for PRRSV N protein by
immunocytochemistry using the monoclonal antibody SDOW17 (Rural Technology) as the primary antibody and sheep anti-mouse IgG conjugated HRP (Jackson ImmunoResearch) as the secondary antibody. The percent reduction in FFU compared to a virus-only control was calculated as the percent neutralization. Assays were done in duplicate and repeated at least twice. A student’s t-test was used to compare neutralization of FL12 to each chimeric virus across all replicates, and the standard deviation of the mean was calculated from the replicates in experiments.

**Binding and PRRSV genome replication assays**

MARC-145 cells were plated at 2x10^5 cells per well in 12-well plates 24 hours prior to treatment. 10^5 FFU of PRRSV FL12 virus was incubated at 37° for one hour in the presence or absence of undiluted PHGC pooled serum, and then MARC-145 cells infected with the virus±serum mixture in duplicate. For binding assays, cells and virus were incubated at 4° for one hour to facilitate virion attachment to, but not uptake into, the cells. After incubation, supernatants were removed and cells were washed six times with serum free media to remove unattached virions. The remaining attached virions were eluted from the cells with trypsin and viral RNA isolated via QIAamp Viral RNA Mini Kit according to manufacturer’s instructions (Qiagen). RT-qPCR using probes specific for PRRSV genomic RNA (RNA) was performed on the eluates to determine the number of attached virions. For entry and replication assays, after the 4° incubation and wash, cells were moved to 37° to facilitate uptake of attached virions into the cells for genome replication assays. Cells were harvested at 0, 4, 8, 12, and 24 hpi, total RNA isolated using Qiamp RNeasy mini kit (Qiagen), and RT-qPCR for PRRSV RNA performed. For binding and replication assays, the
mean copy number of duplicate RNA samples are reported, and standard deviations were calculated using replicate values. A student’s t-test was used to analyze genome replication at each time point between the virus only and the serum plus virus cells.

**Particle to infectivity ratio assays**

Viral RNA was isolated from each of the FL12 and chimeric virus stocks using the QIAamp Viral RNA Mini Kit according to manufacturer’s instructions (Qiagen), aliquoted, and stored at -80°C. Viral RNA was quantified via PRRSV ORF7-specific reverse transcriptase quantitative PCR (RT-qPCR) using the VetMAX NA and EU PRRSV Kit (Applied Biosystems). RT-qPCR was performed in duplicate using viral RNA from two separate virus stock aliquots and averaged to determine the virus copy number per ml of the virus stocks. Because virus was harvested from supernatants, which contain cell-free virus, each viral RNA copy is equivalent to one virus particle.

To determine the particle to infectivity ratios of each virus, MARC-145 cells were seeded at 3x10^5 cells per well in 12-well plates 24 hours prior to infection. MARC-145 cells were infected with equivalent serial dilutions of FL12 and the immune escape variants from 10^7-10^5 virus copies per well. Cells were incubated at 37°C for 24 hours and cells were fixed in acetone:methanol and immunocytochemistry to detect the PRRSV nucleocapsid (N) protein was performed using the monoclonal antibody SDOW17 (Rural Technology) and sheep anti-mouse IgG conjugated HRP (Jackson ImmunoResearch) as the primary and secondary antibodies, respectively. After addition of the HRP substrate, foci of infected cells were enumerated by light microscopy. Each foci, or focus-forming unit (FFU) corresponds to a single infectious unit, and infectivity of the PRRSV variants was
determined by calculating the number of virus copies (virus particles) required for one FFU.

**Replication kinetics assays**

The kinetics of virion production for FL12 and the immune escape variants were evaluated in MARC-145 cells. MARC-145 cells were plated at $1.4 \times 10^5$ cells per well in 24-well plates the previous day and infected with 100 FFU of FL12 or the escape viruses per well (MOI $\sim 0.001$) in duplicate. Wells were washed once at 1 hpi and replenished with media. Supernatants were harvested at 12, 24, 36, 48, and 72 hpi and supernatants containing cell-free virions were harvested and media was replenished in the wells. Supernatants were clarified by centrifugation at 1000xg for 10 minutes, aliquoted, and stored at -80°C. Viral RNA was isolated from three aliquots from duplicate wells using the QIAamp Viral RNA Mini Kit according to manufacturer’s instructions (Qiagen), and qPCR was performed in duplicate for all viruses at all time points using the VetMAX NA and EU PRRSV Kit (Applied Biosystems) following manufacturer’s protocol. Virus copies per ml supernatant were calculated based on the standard curve from 10-fold serial dilutions of PRRSV RNA standards. Additionally, to determine the rate of infectious virion production, two aliquots of supernatants were titered in duplicate to determine the amount of infectious virus produced at each time point. Virion and FFU production rates were calculated in PRSIM (GraphPad) as previously described in (31). Briefly, growth rates were calculated for the three experimental replicates for the five viruses from 12-72 hpi using a linear (first order) function on log-transformed virus copy numbers. A Kruskal-Wallis test of equal slopes one-way ANOVA was performed to analyze differences in slope across the
virus variants. Follow-up comparisons between viruses were performed via Dunn’s multiple test comparison.

Results

Immune escape and non-escape variants susceptible to neutralization by pooled serum

We previously identified viral variants, containing specific ORF2-6 haplotypes, that arose during the course of infection that either escaped or did not escape autologous neutralizing sera (Table 4.1). To evaluate the neutralizing activity of PHGC pooled serum, we performed neutralization assays against the escape, non-escape, and the parental FL12 (containing the NVSL97-7895 inoculum backbone) viruses. Serum was diluted 1:8 and incubated with 200 FFU of each virus at 37°C for one hour, then inoculated on MARC-145 cells. At 24 hpi, foci were counted and the percent reduction of serum-treated samples was compared to virus-only control wells. All of the viruses were susceptible to neutralization by the pooled serum (Figure 4.1). Interestingly, escape viruses 3197-A, 3197-B, and 1113-B did appear to have a slight reduction in neutralization sensitivity compared to FL12 (Figure 4.1), however none were statistically significant (p-values=0.64, 0.08, 0.06, respectively). These results indicate the pooled serum contains a sufficient mixture of neutralizing antibodies to neutralize diverse immune escape and non-escape virus variants.
Neutralizing antibody blocks a post-attachment, pre-genome synthesis step in PRRSV replication

To determine which step or steps in PRRSV replication NAb targets, we used the PHGC pooled pig serum, which contained all of the antibodies produced from nearly 200 pigs after experimental infection with PRRSV strain NVSL97-7895. Assays were designed to quantify the amount of virus at each step of the replication cycle, and compare virus only samples with virus plus pooled sera samples. To determine if the pooled NAb blocked attachment, we carried out virus binding assays at 4°C. These experimental conditions facilitate attachment of virions to the cell, but are not kinetically favorable to allow uptake of the virus into the cell. Virus only and virus plus serum samples were incubated at 37°C for one hour, and inoculated onto MARC-145 cells and incubated at 4°C for one hour. After incubation, cells were extensively washed with serum free media to remove unattached virions. To assess attachment, attached virions were eluted from the cells with STV, viral RNA isolated, and PRRSV genomic RNA (RNA)-specific RT-qPCR was performed. Virus copy number of the virus only and serum-treated samples was quantified. Results are presented as both the absolute copy number in each treatment (Figure 4.2A, Elution bars) and as the corresponding log_{10} reduction (Figure 4.2B, Elution bars). The copy numbers of virus only and serum-treated samples were compared via a t-test, and no significant difference was found between virus copy number in the presence and absence of NAb (p-value=0.087), indicating that NAb does not block attachment of virions to the cell.

To further characterize inhibition by NAb at post-attachment steps, we performed replication assays. To do this, after the binding assay and washes, rather than eluting attached virions, cells were moved to 37°C to facilitate entry of the virus into the cell. Cells
were harvested at 0, 4, 8, 12, and 24 hours post 4° incubation (hpi), total RNA was isolated, and PRRSV RNA-specific RT-qPCR performed to quantify virus copies in the cells. Copy numbers and the associated log_{10} reduction are reported in Figure 4.2A and B. The 0 hpi cells were harvested prior to the 37° incubation in order to measure virus attachment to the cell, and therefore have a similar ratio of virions as the elutions. However, while there was no significant difference in virus copy number between the virus only and virus plus pooled serum elutions, there was a statistical difference between copy number harvested from the 0 hpi cells (p=0.0001). This difference between the 0 hpi cells of virus only and serum-treated cells represented a reduction of less than half log_{10} virus copies. The discrepancy between the elutions and 0 hpi cell RNA copy numbers may be due to the inherently greater experimental error in harvesting cells versus harvesting supernatants. However, because of these conflicting results, it remains unclear whether NAb blocks attachment in a biologically significant manner.

To determine if the pooled serum blocks PRRSV at post-attachment steps in viral replication, PRRSV RNA from the 4, 8, 12, and 24 hpi cells was quantified (Figure 4.2). In the virus-only cells, there was a reduction in RNA between the 0 and 4 hpi time points, indicative of the eclipse phase, where little or no genome replication occurs, followed by a large increase in RNA by 8 hpi (Figure 4.2). This suggests that the virus undergoes early steps in replication, such as uptake, fusion, and uncoating up to 4 hpi, and rapid genome replication begins between 4 and 8 hpi. The pooled serum significantly reduced the amount of RNA detected in cells at 4, 8, 12, and 24 hpi compared to the virus-only wells (p-values=0.0001, 0.0002, 0.0001, and <0.0001, respectively), and the corresponding log_{10} reductions are reported in Figure 4.2B. The significant reduction in PRRSV RNA from the
virus-only samples at 4 hpi that is maintained up to 8 hpi, followed by delayed RNA replication at 12 and 24 hpi, combined with the lack of evidence of blocked attachment, indicates that the NAb primarily targeted a post-attachment step of PRRSV entry prior to genome replication.

Neutralizing antibody escape not associated with decreased infectivity

Replication fitness can be encompassed by two main components; 1) the infectivity of the virus, or how well the virus can enter cells and begin replication, and 2) the replication kinetics of the virus, or how well the virus produces new progeny virus. To determine if NAb escape is associated with a decrease in infectivity, we determined the particle-to-infectivity (P:I) ratios of the backbone virus, FL12, the escape viruses, and the non-escape viruses. The P:I ratios represent the number of virus particles required to form one focus-forming unit (FFU), which is equivalent to one infectious unit. To do this, we first determined the numbers of viral copies/ml for each of the FL12 backbone and the immune escape chimeric viruses via RT-qPCR. The virus stocks consist of clarified supernatants from infected MARC-145 cells, and thus contain only cell-free virus, so that each viral copy identified from RT-qPCR is equivalent to the genome of a single virus particle. MARC-145 cells were then infected with equivalent copies of the viruses in serial dilutions, immunocytochemistry performed at 24 hours post infection (hpi), and individual foci counted.

Somewhat surprisingly, all of the viruses required >10⁴ virus copies to result in a single infectious unit. The FL12 backbone virus had a P:I ratio of 2.87x10⁴ viral copies per FFU (Figure 4.3A). Of the four escape viruses, only v1113-B had a significantly higher P:I
ratio of $6.58 \times 10^4$ compared to FL12 (p-value=0.006; Figure 4.3A). The decrease in infectivity of this variant was expected as it did not grow well in MARC-145 cells during its production from the infectious clone. None of the other escape viruses, or the non-escape viruses, had significantly different P:I ratios compared to FL12 (Figure 4.3A).

It is possible that mutations that mediated escape caused a reduction in infectivity, but were compensated by mutations in other regions of the haplotype. To test this, the escape viruses were broken up into oligomeric units: GP2/3/4 (ORF2-4) or GP5 (ORF5) and/or M (ORF6) (Table 4.1). The P:I ratios of the chimeric viruses containing the parsed haplotypes were determined (Figure 4.3B). All of the partial haplotype chimeric viruses had reduced infectivity compared to FL12, except for viruses from the 3197-A haplotype, regardless of which envelope protein(s) mediated escape. The only escape virus that had a reduced infectivity, 1113-B, escaped NAb through mutations in GP5 (Table 4.1). However, chimeric viruses containing just the GP2/3/4 and GP5 mutations both had significantly higher P:I ratios than FL12, indicating that mutations in both regions of the haplotype contributed to the reduced infectivity. Interestingly, in escape virus 3197-B and non-escape virus 3068-A, which did not have significantly different P:I ratios compared to FL12, the viruses containing just the mutations in GP2/3/4 or GP5/M from these haplotypes had significantly reduced infectivity (Figure 4.3B), suggesting that mutations within these ORF2-6 haplotypes may complement to increase infectivity of the individual regions.

Escape of the 3197-B virus was mediated through mutations in GP5, however both the 3197-B-2-4 and 3197-B-5 had significantly higher P:I ratios compared to FL12 (p-values <0.0001 and 0.001, respectively). The 3197-B virus had a P:I ratio of $4.74 \times 10^4$, which was actually higher than the 3197-B-5 chimeric virus ($3.92 \times 10^4$) but not significantly different
from FL12 due to a large standard deviation (Figure 4.3A and B). Therefore, it is possible that escape virus 3197-B actually has a reduction in infectivity. The non-escape virus 3068-A has a slightly lower P:I than FL12 of 2.34x10^4, however viruses containing mutations from only GP2/3/4 (3068-A-2-4) or GP5/M (3068-A-5-6) had significantly higher P:I ratios of 4.34x10^4 and 1.14x10^5 (p-values=0.014 and <0.0001, respectively), indicating that mutations in the two envelope protein regions may complement to increase infectivity. Taken together, these results suggest that immune escape from autologous serum is not directly associated with a decrease in infectivity, and infectivity phenotypes can be affected by mutations in both the GP2/3/4 trimer and GP5/M dimer.

**FL12 and escape variants have similar rates of virion production and infectious unit production over time**

The P:I ratios assess how well the viruses are able to enter cells and begin replication, however they are not the sole determinant of replication fitness. In order to determine if the escape viruses had similar growth rates compared to FL12, replication kinetics assays characterizing the rate of total virion production and infectious unit production over time were performed in MARC-145 cells. Due to slight difference in infectivity previously described, cells were infected with equivalent infectious units, 100 FFU per well, of each of the viruses. At 12, 24, 36, 48, and 72 hpi supernatants were harvested, clarified, virion production determined by RT-qPCR and infectious unit production determined by titering supernatants on MARC-145 cells to determine their FFU/ml. Virion production and infectious unit production are reported in Figures 4.4A and 4.4B, respectively. Virus growth rates were analyzed by comparing slopes of each virus’
virion and FFU production rates over time as described in (31). The Kruskal-Wallis test found significant differences between slopes of the virion production of the viruses (p-value=0.001; Figure 4.4A), however follow-up analyses via Dunn’s multiple comparison test revealed that none of the escape viruses had significantly different growth rates compared to FL12 (p-values= 0.823 for 3197-A, 0.475 for 3197-B, and >0.999 for 1113-A and 1113-B). Analysis of the rate of production of infectious units revealed no statistical difference between FFU production rates of any of the viruses (p-value=0.079; Figure 4.4B). While there were no significant differences in rates of virion or infectious unit production, when plotted, copy number and infectious units had similar trends, where the 3197-A and 3197-B viruses appeared to have both the highest virion production and FFU production up until 48 hpi. It is interesting to note that the only escape virus previously found to have a reduction in infectivity, 1113-B, did appear to have a reduction in total number of virions and infectious units produced compared to the other viruses (Figure 4.4A and B). While its growth rate was not significantly different from FL12, its reduction in infectivity may account for the apparent reduction in total virions and FFU produced. These results suggest that regardless of infectivity, all of the escape variants produce virions at similar rates to FL12, and that the immune escape variants do not have a substantial reduction in replication fitness due to their escape phenotype.

**Discussion**

Virus escape from the host’s immune response is often accompanied by a decrease in infectivity and/or replication fitness of the immune escape variant due to changes in
epitopes in functionally important regions of the viral envelope proteins. We previously identified PRRSV immune escape variants within pigs that arose during the course of in vivo infection. Neutralization escape mapped to mutations occurring in multiple envelope proteins, however the mechanism of neutralization and its effect on the escape virus' replication fitness was not known. In the present study we used pooled anti-PRRSV sera from a trial of the PHGC to identify the step(s) in the virus replication cycle targeted by NAb, and identified post-attachment, early stages of virus entry as the primary target of PRRSV NAb. Infectivity and replication kinetics assays using our previously identified immune escape viruses, as well as non-escape viruses, revealed that NAb escape was not associated with a loss of replication fitness. One escape virus, 1113-B, had the lowest infectivity of any of the viruses tested, and consistently had lower total virion and FFU production than the other viruses, indicating that this virus had a decrease in replication fitness, though none of the differences in kinetics were statistically significant. A loss of fitness was not observed in any of the other escape or non-escape viruses assayed. However, chimeric viruses containing the parsed envelope oligomeric regions (GP2/3/4 mutations or GP5/M mutations) from two viruses, escape virus 3197-B and non-escape virus 3068-A, had significant reductions in infectivity compared to the parental FL12 virus, indicating mutations in these viruses may complement each other to recover infectivity. Taken together, these results suggest that both the GP2/3/4 trimer and GP5/M dimer are involved in early PRRSV entry, but that mutations involved in the escape from NAb may not have occurred in critical envelope protein domains required for entry.

The role of neutralizing antibody has been widely debated in the PRRSV literature, largely due to the fact that viremia can begin to clear before NAb is detected in pig sera (8,
However, passive transfer studies have established that neutralizing antibody from PRRSV anti-sera is protective against homologous infection (8). Previously, we identified neutralizing pig sera targeted against both GP5 and the GP2/3/4 trimer, consistent with previous studies that identified NAb targeted to GP5, GP3, and GP4 (6-9). Attachment of virions to the cell may be facilitated by interactions between the major envelope proteins GP5 and/or M with the host cell, while entry of the virus into the cell is likely mediated through interactions of GP2 and GP4 with cellular CD163 and GP5 with cellular factor MYH9 (16, 17, 19, 20, 35). Therefore, it is reasonable that NAb targeting GP5 would block entry of the virus into cells, and possibly attachment of virions to the cell, and those targeting GP2/3/4 would disrupt post-attachment steps in entry. However, one in vitro study has shown that entry of PRRSV into cells can occur independently of GP5, suggesting that GP5 is not involved in early steps of the PRRSV replication cycle, directly conflicting with more recent results indicating GP5 binding to MYH9 is crucial for PRRSV entry (20, 36). In order to elucidate the steps of PRRSV replication targeted by a broad range of NAb, pooled anti-PRRSV serum was used in virus neutralization assays. Virus attachment assays revealed that the pooled serum reduced attachment, however this was a reduction of less than half a log and there was a discrepancy between the evaluation of attached virus from eluates and cells, overall suggesting that the reduction in attachment was minimal (Figure 4.2A, B). There was a significant and sustained reduction of PRRSV RNA synthesis at all other time points of infection, indicating that the pooled serum primarily targeted a post-attachment step in PRRSV entry. This result was somewhat surprising, as the pooled serum contains a large collection of polyclonal antibodies targeting all of the envelope proteins, and with the proposed role of GP5 in attachment, we would expect to see a greater
reduction in attachment. However, the results are consistent with NAb directed against the GP2/3/4 trimer to block CD163 binding, therefore preventing uptake and uncoating, or with NAb targeting GP5 to prevent MYH9 binding (13, 20, 36). It is also possible that the NAb does not directly interfere with virion attachment to the cell or its receptors, but rather acts through steric hindrance to block entry.

To determine the effect of escape on replication fitness, a series of infectivity and replication kinetics assays were performed using the previously identified NAb escape variants and FL12. None of the escape variant viruses had significantly different growth rates compared to FL12, in either virion production rates or infectious unit production rates (Figures 4.4A and B). The 1113-B virus had a significant reduction in infectivity, and consistently had lower virion and FFU production compared to FL12 although there was no statistically significant difference in growth rates. Furthermore, 1113-B did not grow well in MARC-145 cells during the initial production of the virus and had to be passaged in PAMs once to obtain a high enough titer to be infectious in MARC-145 cells, further supporting a decrease in infectivity. The reduction in infectivity was only 2-fold compared to FL12, which may explain why the virus had reduced total virion and FFU production, but not statistically significantly reduced replication kinetics. Likely, 1113-B produced similar proportions of virions during a single round of virus replication as the other escape variants and FL12, but because of its modest reduction in infectivity, fewer cells were successfully infected in subsequent rounds of infection, leading to the modest reduction in virion production and infectious unit production (Figures 4.4A and B). Interestingly, viruses 1113-B-2-4 and 1113-B-5, containing just the mutations from GP2/3/4 or GP5 of 1113-B, respectively, both had significantly reduced infectivity (Figure 4.3B). This result
was surprising as during production of the chimeric viruses, only 1113-B and 1113-B-2-4 grew poorly in MARC-145 cells, whereas 1113-B-5 grew to similar titers at similar rates as the other chimeric viruses. These results indicate that mutations in both GP2/3/4 and GP5 of this virus did contribute to a reduction in replication fitness.

The overall finding that the escape variants did not have significant reductions in replication fitness suggests that the mutations that mediated escape did not occur in critical functional regions of the virus, or that compensatory changes in other envelope proteins mitigated the effects. Three out of the four escape viruses, 3197-A, 3197-B, and 1113-B, mediated escape through mutations in GP5, and the GP5 mutations from two of these viruses, 3197-B and 1113-B, had reduced infectivity, however so did their GP2/3/4 counterparts, indicating it was not just the mutations that were directly involved in immune escape that could alter infectivity. The only escape virus that mediated escape through mutations in GP2/3/4, 1113-A, had a slight reduction in infectivity compared to FL12, but this was not statistically significant. Because the GP2/3/4 trimer is involved in entry of PRRSV into the cell (35), it was expected that this escape variant would have a decreased infectivity due to mutations occurring in functionally important regions of the virus, however this was not the case. These results suggest that neutralization and escape of these PRRSV variants is primarily mediated through the binding of neutralizing antibody to regions of the envelope proteins that are not critical domains, but block the ability of functionally important regions, such as the receptor binding sites, from carrying out the necessary steps of virus entry.

The finding that neutralization and escape targets steps in entry by binding non-functionally critical regions of the virus may explain the weak escape phenotype of these
viruses. Even within a pig, the escape was transient and not effective against broadly neutralizing PRRSV anti-sera or the PHGC pooled sera (Figure 1; see Chapter 3). It may be the case that these immune escape variants arise because they are the most replication competent NAb-resistant variants, capable of a short burst of productive infection via quick replication, but easily neutralized after a short time by a secondary antibody response, as is commonly seen in HIV-1 infected patients (37). Thus, while the escape by these PRRSV NAb escape variants was short-lived, the finding that they do not result in a substantial reduction in replication phenotype suggests that these escape viruses can be transmitted between animals and may contribute to the continual circulation of PRRSV.

**Literature Cited**


Table 4.1 Summary of chimeric viruses.

^A denotes the haplotype present at the highest frequency in the pig’s virus sample; B denotes the haplotype with the second highest frequency.

*Denotes haplotype with no mutations in ORF5.

<table>
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<tr>
<th>Virus From PHGC Pig</th>
<th>Viremia Outcome</th>
<th>ORF2-6 Haplotype^</th>
<th>Chimeric Virus</th>
<th>Portion of Haplotype</th>
<th>Escape?</th>
<th>Protein(s) involved in Escape</th>
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<td>Rebound</td>
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<td>ORF2-6</td>
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<tr>
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<td>3161-A</td>
<td>ORF2-6</td>
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</tbody>
</table>
Figure 4.1 Neutralizing activity of PHGC pooled serum versus FL12, escape, and non-escape viruses. Serum was diluted 1:8 and incubated with $2 \times 10^2$ FFU of the FL12 infectious clone backbone virus, the rebound pig escape viruses (bars shaded with diagonal lines), and non-escape viruses (solid bars). Percent neutralization represents the reduction of FFU compared to virus-only controls. Means of two separate experiments are shown, and the error bars represent ± one standard deviation of the means.
Figure 4.2 Attachment and PRRSV RNA replication assays ± pooled serum. **A)** Average RNA copy number per well as determined by RT-qPCR. Elution represents the copy number present in elutions of attached virions after incubation at 4°. For RNA replication assays, cells were harvested at 0, 4, 8, 12, and 24 hpi, RNA isolated and RNA copy per well determined. Asterisks represent statistically significant differences between virus-only and serum-treated cells as determined by a student’s t-test. **B)** The log(10) reduction in RNA in serum-treated samples compared to the virus-only samples. The means from two experimental replicates are reported, and error bars represent ± standard deviations of the means.
Figure 4.3 Particle to infectivity ratios for FL12 and the NAb escape and non-escape variants A) P:I ratios of the escape (bars shaded with black diagonal lines) and non-escape (solid bars). B) P:I ratios of the GP2/3/4 portion or GP5/M portion of selected viruses The portions of the haplotypes previously determined to mediate escape are shaded with white diagonal lines. The average virus copy required to form a single infectious unit is plotted for each virus construct. Error bars represent ± standard deviations of the means, and asterisks denote viruses with statistically significantly different (p-value >0.05) P:I ratios compared to FL12 via t-test.
Figure 4.4 Replication kinetics assays measuring **A)** total virion production; and **B)** infectious virion production as measured by FFU of FL12 and the NAb escape variants. MARC-145 cells were infected with 1x10^2 FFU of each virus and supernatants harvested at 12, 24, 36, 48, and 72 hpi and virion production and FFU production were measured at each time point. Growth rates were compared to FL12 by analyzing differences in slopes. None of the escape viruses had statistically significant differences in virion production or FFU production rates compared to FL12.
CHAPTER 5: IDENTIFICATION AND CHARACTERIZATION OF SMALL MOLECULE INHIBITORS OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS

A paper to be submitted to Antiviral Research

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Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) is poorly controlled by the currently available vaccines. Because of this, outbreaks often spread quickly between farms in both vaccinated and unvaccinated herds. Alternative control strategies are needed to help prevent the continual circulation of the virus. Antivirals have proven to be an extremely successful treatment strategy for several human viral diseases, and research efforts have recently begun to identify antivirals for use in treatment of livestock diseases. Previously, the natural compound atracylodinol was reported to have anti-PRRSV activity \textit{in vitro}. In the current study, we synthesized atracylodinol and fourteen analogs to characterize their anti-PRRSV activity \textit{in vitro}. Seven of the analogs had potent inhibitory activity against $5\text{-log}_{10}$ infectious units of PRRSV at low $\mu$M concentrations. Atracylodinol and analog compound 19 were shown to inhibit PRRSV primarily at a post-attachment step during PRRSV entry. These results provide evidence that the atracylodinol analogs are promising antiviral candidates for trials in pigs.
Introduction

Porcine reproductive and respiratory syndrome (PRRSV) is the etiological agent of PRRS, an economically significant disease of swine worldwide. PRRSV causes reproductive losses from abortions and stillbirths in pregnant sows, and respiratory disease in growing pigs, resulting in an estimated $670 million in losses to the US pork industry annually (1-3). Currently, PRRSV is poorly controlled by the available vaccines and containment strategies employed by the swine industry. Vaccines are not effective against heterologous strains of PRRSV due to the large amount of genetic diversity in the virus (4-6). In addition, vaccination can be a costly and timely endeavor because each pig must be inoculated individually. After pigs have been infected with PRRSV, the virus is capable of persisting within some animals for months through unknown mechanisms, potentially leading to the occurrence of additional outbreaks within herds (7-9). Because of the lack of an effective vaccine, prevention of PRRSV currently primarily relies on biosecurity and containment measures. However, due to the high transmissibility of the virus, outbreaks continually occur in herds, and the virus can quickly spread from one barn and/or farm to others. Because of these reasons, antiviral drugs could be a useful tool for the control and containment of PRRSV outbreaks within herds. Antivirals could be added to feed in order to treat many animals at once, and treatment would decrease the amount of circulating virus in order to promote quicker recovery of animals, as well as prevention of the spread of the virus to additional barns and/or farms. Despite the need for effective PRRSV treatments, there are currently no antiviral drugs or therapeutics approved for the treatment of PRRSV infection.
The treatment of viral infections via antiviral compounds has proven extremely successful for several human diseases. Antivirals have found the greatest success for treatment of infections for which there are no effective vaccines, including HIV-1 and hepatitis C virus (HCV), and for influenza virus, for which the annual vaccines are not always reliable. For HIV-1, anti-retroviral therapy has turned infection with HIV-1 in the United States from a death sentence to a manageable chronic infection (10, 11). Synthetic anti-retroviral drugs have been developed against virtually every step of HIV replication and are extremely effective in decreasing patient viral load and increasing lifespan (11, 12). More recently, the treatment of chronic HCV has found success through synthesized direct-acting antivirals (DAA) (13-15). These treatments were a huge breakthrough in treatment of HCV and a vast improvement over the previous treatment strategy via immunological therapy with interferon-alpha. The synthetic, direct-acting antivirals have reported up to 90-100% cure rates for HCV infected patients (13). In addition, the synthetic drug oseltamivir (Tamiflu) has been successfully implemented for the treatment of influenza virus infections at early stages of infection (16, 17).

In the past few years, several antivirals against PRRSV have been described in the literature. The reported anti-PRRSV compounds were primarily derived from mushrooms, herbs and plants used in traditional Chinese medicine, which have previously been shown to have antiviral activity against a wide variety of viruses (18). The natural compounds with reported anti-PRRSV activity include chlorogenic acid, scutellarin, epigallocatechin gallate palmitate, flavaspidic acid AB, cryptoporus volvatus extract, sodium tanshinone IIA sulfonate, and atracylolidinol (19-25). These compounds have all been reported to be strong inhibitors of PRRSV with variable ranges of potency and cytotoxicity in vitro. The
effective concentration, or inhibitory concentration, that inhibits 50% of virus-induced cytotoxicity, or 50% of input virus (EC$_{50}$ or IC$_{50}$), respectively, for the various compounds have been reported to be in the range of 3.5-360 μg/ml and <1-40 μM, Currently only one of these antivirals, cryptoporus volvatus extract, has been tested in pigs, and was found to decrease clinical symptoms of PRRSV and slowed replication of the virus in treated pigs compared to the control group (22). It is important to note that the cryptoporus volvatus extract-treated pigs received twice daily intramuscular injections of the extract for eight days, which is not a practical application for the field. However, these results demonstrate the potential for the development of antivirals for treatment of PRRSV infection. Many of these natural compounds are difficult and costly to extract in high quantities, thus in order to feasibly produce large amounts of anti-PRRSV compounds, potent anti-PRRSV compounds that can be easily and cost-effectively synthesized are needed.

The goal of the present study was to synthesize and evaluate anti-PRRSV compounds with the potential for large-scale production. We selected the natural compound atracylodinol, which has been identified as having potent anti-PRRSV properties in (24), as our target molecule for synthesis. This compound is naturally derived from the rhizomes of the plant Atractylodes lancea, but the isolation procedure is expensive and produces low yields (26). Therefore, our strategy included designing an efficient synthesis route to produce large amounts of atracylodinol and/or atracylodinol analogs and assess their anti-PRRSV activity. In total, we screened fifteen synthesized compounds for anti-PRRSV activities and identified eight with potent anti-PRRSV activity in vitro with EC$_{50}$ ranging from 4.19 to 29.82 μM.
Materials and Methods

Synthesis of 1-(E)-Atractylopin and analogs

The synthesis strategy is summarized in Figure 5.1 and detailed in (26). The original compound identified by (24), 1-(E)-Atractylopin (compound 1), was synthesized in seven steps from the commercially available 3-(2-furyl)acrolein (compound 4) and cis-2-butene-1,4-diol (compound 5), and via the intermediate (E)-2-(But-1-en-3-yn-1-yl)furan (compound 2), with a total yield for compound 1 of 11% (Figure 5.1A). Because of the low yield, an additional synthetic route was developed via the production of analog ester (compound 13), which was synthesized in five steps from commercially available materials with 35% yield (Figure 5.1B), and could further be converted to compound 1 by an additional step. Due to the difficult reaction conditions, however, this was not a feasible reaction for large-scale production, and so additional analogs were synthesized. The analog (1E,7E)-1,8-Di(furan-2-yl)octa-1,7-dien-3,5-diyne (compound 16) was synthesized (Figure 5.1C) from compound 2 in three steps from commercially available materials with 36% yield. Analogs 5-(((1E,7E)-8-(Furan-2-yl)octa-1,7-dien-3,5-diyn-1-yl)furan-2-carbaldehyde (compound 17), (5-(((1E,7E)-8-(Furan-2-yl)octa-1,7-dien-3,5-diyn-1-yl)furan-2-yl)methanol (compound 18), and 1,1’-(((1E,7E)-Octa-1,7-dien-3,5-diyn-1,8-diyl)bis(furan-5,2-diyl))bis(N,N-dimethylmethanamine) (compound 19) were synthesized and produced yield of 33, 98, and 60%, respectively (Figure 5.1C). Compounds 17 and 18 were synthesized in four and five steps, respectively, but required unfavorable coupling and formulation steps. Compound 19 was synthesized in four steps from commercially available materials, and only went through one unfavorable coupling step (Figure 5.1C).
Additional analogs 1-(5-Methylfuran-2-yl)but-3-yn-1-ol (compound 21) and (1E,7E)-1,8-Bis(5-methylfuran-2-yl)octa-1,7-dien-3,5-diyne (compound 23) were synthesized with yields of 53 and 52%, respectively (Figure 5.1D); and Ethyl (E)-7-(benzo[d][1,3]dioxol-5-yl)hepta-2-en-4,6-diynoate (compound 26) and 1,4-Bis(benzo[d][1,3]dioxol-5-yl)buta-1,3-diyne (compound 27) were synthesized in three steps with 25% yields (Figure 5.1E). Finally, small molecule analogs of compounds 2 and 12 were made: compound 28, Ethyl (E)-4-(4-(furan-2-yl)but-3-en-1-yn-1-yl)benzoate (compound 29), and compound 30 which could all be synthesized in less than four steps (Figure 5.1F). All synthesized compounds were analyzed by $^1$H NMR to verify their composition and structure. Because some compounds were not readily soluble in water, all compounds were solubilized in DMSO at a concentration of 1 mg/ml, and further dilutions for the anti-viral assays were made in culture media.

**Cells and virus**

MARC-145 cells were used for anti-viral assays and were maintained in high glucose (4500mg/L) Dulbecco’s Modified Eagle’s Medium (DMEM) (Sigma) supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. The PRRSV strain NVSL97-7895 (GenBank accession AY545985), was passaged once in MARC-145 cells, and titered to determine the focus-forming units (FFU) per ml.

**Screening compounds for anti-PRRSV activity**

Compounds were initially screened for anti-PRRSV activity using a focus-reduction assay adapted from (27). Briefly, MARC-145 cells were seeded at 3x10^5 cells/well in a 12-
well plate 24 hours prior to the anti-viral assay, and media changed to 1 ml/well directly before infection. For each compound, and a DMSO control, 35 μg (35 μl) was added to 700 focus-forming units (FFU) of PRRSV in a volume of 1.2 ml (incubation volume), for an incubation concentration of 29.17 μg/ml of the compound. In addition, a virus-only sample with 700 FFU PRRSV in 1.2 ml media was used as a control. The virus-compound mixtures and virus-only control were incubated at 37° for one hour. Samples were brought to a total volume of 3.5 ml and 1 ml was inoculated per well in triplicate, resulting in each well containing 10μg compound and 200 FFU in a well volume of 2 ml for a final well concentration of 5 μg/ml of compound. The plates were incubated at 37° supplemented with 5% CO₂. At 24 hours post infection, cells were fixed in ice-cold methanol:acetone and immunocytochemistry performed using the PRRSV N protein specific monoclonal antibody SDOW17 (Rural Technology) as the primary antibody and sheep anti-mouse IgG conjugated to HRP (Jackson ImmunoResearch) as the secondary antibody. Following addition of the HRP substrate, cells were rinsed with distilled water, air-dried, and foci of infected cells were enumerated by light microscopy and individual foci counted. Experiments were performed twice in triplicate, and percent virus inhibition was calculated compared to virus-only control wells. Standard deviations were calculated using the means of separate experiments. Compounds that inhibited more than 90% of FFU compared to virus-only control wells were selected for further characterization.

**PRRSV inhibition assays**

The compounds with anti-PRRSV activity at an incubation concentration of 29.17 μg/ml (10 μg/well) were tested for PRRSV inhibition activity at serial dilutions of the
compound via focus reduction assays in order to determine the concentration of compound capable of inhibiting 50% of input virus (IC\textsubscript{50}). Again, MARC-145 cells were seeded at 3x10\textsuperscript{5} cells/well in a 12-well plate 24 hours prior to the anti-PRRSV assays. Compounds were serially diluted 3-fold, added to 500 FFU virus in 400ul, and incubated at 37° for one hour, along with a virus-only control sample. Compound incubation concentrations ranged from 82.5 μg/ml to 1.0 μg/ml. After incubation, samples were brought to a total volume of 2.5 ml, and 1 ml per well was plated in duplicate. Final well concentrations of the compounds ranged from 6.6 μg/ml to 0.08 μg/ml vs. 200 FFU of virus per well. At 24 hours post infection, PRRSV inhibition was assayed as described above. The IC\textsubscript{50} was calculated for each compound as the dilution that inhibited 50% of input virus in GraphPad Prism 7, using normalized inhibition dose response linear regression. The means and 95% confidence intervals for each compound’s IC50 from duplicate experiments was reported.

**Potency assays**

To determine if the compounds were effective against large quantities of virus, potency assays were performed via focus reduction assays as described above. Potency assays were performed at incubation concentrations of 31.25 μg/ml against \~2.5x10\textsuperscript{2}, \~2.5x10\textsuperscript{3}, \~2.5x10\textsuperscript{4}, and \~2.5x10\textsuperscript{5} FFU of virus in 800 μl, and final well concentrations of 5 μg/ml vs. 10\textsuperscript{2}, 10\textsuperscript{3}, 10\textsuperscript{4}, and 10\textsuperscript{5} FFU of virus in 2 ml. PRRSV inhibition was assayed as described above. All assays were performed in duplicate and repeated twice, the mean of duplicate experiments was reported, and the standard deviations were calculated using the means of repeated experiments. To compare PRRSV inhibition activity of a compound
across the four virus treatment groups, one-way analysis of variance (ANOVA) was performed using GraphPad Prism 7 software.

**Cytotoxicity assays**

Cytotoxicities of the anti-PRRSV compounds were determined using the TACS XTT cell viability assay (Trevigen). At 24 hours prior to treatment, MARC-145 cells were seeded at 2.7x10⁴ cells per well in a 96-well plate. At the time of treatment, media was removed and cells were treated with 100 μl media, or 2-fold serial dilutions of the compounds (or the equivalent volume of DMSO) from 200 μg/ml to 6.25 μg/ml (or 20% to 0.625% DMSO) in triplicate. At 24 hours post treatment, media was changed to 100 μl per well. The XTT reagent was prepared according to manufacturer's instructions, and 50 μl added per well of the 96-well plate. Plates were incubated at 37° for two hours and the absorbance was read at a wavelength of 450 nm on an ELISA microplate reader (BioTek ELx800). Cytotoxicity was calculated as the percent reduction in absorbance of treated wells compared to cell-only control wells, and the mean of replicates was reported. The 50% cytotoxic concentration (CC₅₀) was calculated as the compound concentration that reduced the absorbance by 50% in GraphPad Prism 7, using normalized inhibition dose response linear regression. The means and 95% confidence intervals for each compound's CC₅₀ from duplicate experiments was reported

**Time of addition assays**

Anti-PRRSV activity of compound 19 at sequential times post infection was tested. MARC-145 cells seeded at 3x10⁵ cells/well in a 12-well plate 24 hours prior were infected
with 200 FFU virus, and treated with the compound 19 at 0 (concurrent), 1, 3, or 6 hours post infection (hpi). At the time of infection, media in the plates was changed to 1 ml per well. For the 0 hpi treatment, 25 μg of the compound was added to 500 FFU of PRRSV in a volume of 2.5 ml (at 10 μg/ml), and 1 ml was plated per well for a final well concentration of 5 μg/ml vs. 200 FFU of PRRSV. For the virus-only and the 1, 3, and 6 hpi treatments, each well was infected with 200 FFU of virus, for a total well volume of 2 ml. At each treatment time point, the media was changed to 1 ml/well, and 1 ml of compound at 10 μg/ml was added per well, for a final well volume of 5 μg/ml vs. 200 FFU of PRRSV. Assays were performed in duplicate and repeated twice, and virus inhibition was determined as described above. The mean of duplicate experiments was reported, and standard deviations were calculated using the means of repeated experiments. To compare PRRSV inhibition activity of the compound across the four treatment groups, one-way analysis of variance (ANOVA) was performed in Prism 7 software (GraphPad).

**Binding and PRRSV genome replication assays**

MARC-145 cells were plated at 2x10^5 cells per well in 12-well plates 24 hours prior to treatment. Cells were infected with 10^5 FFU virus per well in the presence of DMSO (virus-only control) or 10 μg of compound 19 per well (5 μg/ml well concentration). For binding assays, cells were incubated at 4° for one hour to facilitate virion attachment to, but not uptake into, the cells. After incubation, supernatants were removed and cells were washed six times with serum free media to remove unattached virions. The remaining attached virions were either eluted from the cells with STV or cells were harvested and total RNA isolated using Qiamp RNeasy RNA isolation kit (Qiagen). RT-qPCR using probes
specific for PRRSV genomic RNA (RNA) was performed on the eluates and cellular RNA to determine the number of attached virions. Genome replication assays were performed by moving cells to 37° after binding assays in order to facilitate entry of the attached virions. Cells were harvested at 4, 8, 12, and 24 hpi, total RNA isolated, and RT-qPCR for PRRSV RNA performed. Mean copy numbers of duplicate RNA samples are reported, and standard deviations calculated from the replicates. A student’s t-test was used to analyze genome replication at each time point across virus-only and compound-treated cells in Prism (GraphPad).

Results

Identification of small molecule compounds with anti-PRRSV activity

Compound 1 (atractyldinol) had previously been reported to exhibit anti-PRRSV activity in vitro (24), but anti-PRRSV activity of the synthesized atractyldinol analogs was not known. To compare anti-PRRSV activity of compound 1 and its analogs, we initially screened 10 μg of each compound for inhibitory activity of 200 FFU virus, and calculated PRRSV inhibition as a percent reduction in FFU compared to virus-only control wells. Consistent with previous findings, compound 1 inhibited 100% of input virus. The analogs, compounds 2, 13, 16, 17, 18, 19, and 23, were also capable of inhibiting PRRSV at 93% or greater (Figure 5.1, Figure 5.2). Although none of the analogs reached the full 100% inhibition of compound 1, compound 19 inhibited >99.9%, compounds 13, 23, and 18 inhibited >99% of the input virus, and compounds 17, 16, and 2 inhibited >93% of the input virus (Figure 5.2) We also identified analogs with low inhibitory effects on PRRSV,
including compounds 29, 26, 30, 12, 21, 27, and 28, which inhibited ≤66% of the input virus (Figure 5.2). The DMSO control had no inhibitory effect on PRRSV, thus the anti-viral effects were due to the compounds, not the DMSO that they were solubilized in. The eight compounds with >90% PRRSV inhibitory activity were selected for further characterization of anti-PRRSV activity.

**Determining inhibitory concentrations of the anti-PRRSV compounds**

To determine the inhibitory concentrations of the eight compounds, we used 3-fold serial dilutions ranging from 82.5-1.0 μg/ml incubated with 500 FFU PRRSV, and plated at final well concentrations of 6.6 μg/ml to 0.08 μg/ml vs. 200 FFU of virus. The percent virus inhibited at each concentration was calculated compared to virus-only control wells, and results are shown in Figure 5.3. The incubation concentration of the compounds that inhibited 50% of input virus (IC₅₀), the corresponding molarity, and 95% confidence intervals were calculated for each of the eight compounds (Table 5.1). The IC₅₀ ranged from 1.10 to 5.91 μg/ml, or 4.19 to 29.82 μM. The maximum PRRSV inhibition activity at the highest compound concentration was also reported (Table 5.1). Two of the compounds, 1 and 2, had IC₅₀ greater than 10 μM, three compounds (16, 13, and 17) had IC₅₀ between 5 and 10 μM, and three (compounds 19, 18, and 23) had IC₅₀ <5 μM (Table 5.1).

Interestingly, while compound 1 had the highest anti-PRRSV activity in the initial anti-PRRSV assay, it had the highest IC₅₀ of the anti-PRRSV compounds at 5.91 μg/ml (29.82 μM, 95% CI: 23.51, 37.69 μM). This is a similar IC₅₀ to the 39.4 μM reported for this compound in (24). The other compound to have an IC₅₀ above 10 uM, compound 2, was also the least effective of the eight anti-PRRSV compounds, with a maximum PRRSV
inhibition of 93.4%. The three compounds with IC$_{50}$s <5 µM, compounds 19, 18, and 23, reached maximum PRRSV inhibition of 100, 100, and 99.1%, respectively. These results demonstrate that all eight of the anti-PRRSV compounds inhibited PRRSV in a dose-dependent manner, and were effective at concentrations similar to, or lower, than reported in the literature for other anti-PRRSV compounds (19, 20, 22, 25).

Effectiveness of compounds is maintained against $10^5$ infectious units of PRRSV

In addition to testing the effective concentration of the compounds, we also determined their potency in vitro. To do this, we again used 10 µg of each compound per well, as all of the compounds reached their maximum PRRSV inhibition level at this amount. Anti-PRRSV activity of the compounds was tested against 10-fold dilutions of the virus, from $10^5$ to $10^2$ FFU, and percent virus inhibition was calculated compared to the virus-only control wells, and results are summarized in Figure 5.4 and Table 5.2. Remarkably, all of the compounds maintained similar PRRSV inhibition levels regardless of the amount of virus added. Compounds 1 and 19 were the most striking, inhibiting 100 and 99.93% of input PRRSV up to $10^5$ FFU, respectively (Figure 5.4). One-way ANOVA was used to test whether there were differences in effectiveness across virus treatment groups for each compound, but none of the compounds were found to have statistically significant differences in inhibition at $10^2$ to $10^5$ FFU virus (Table 5.2). These results indicate that the eight compounds maintained their anti-PRRSV properties up to $10^5$ FFU virus in vitro.
Assessing cytotoxicity of the anti-PRRSV compounds

Cytotoxicity of the compounds was calculated via an XTT assay. Cells were treated with growth media, serial dilutions of the compounds from 200 μg/ml to 6.25 μg/ml, or an equivalent volume of DMSO. Cytotoxicity was measured as the reduction in absorbance of treated wells as compared to the cell-only well. The concentration of compound that reduced absorbance by 50% (CC50) was calculated for each compound and DMSO, and reported in Table 5.1. Surprisingly, compound 1 was the most cytotoxic of all eight of the anti-PRRSV compounds, and its CC50 could not be calculated due to the fact that the high concentrations used for the cytotoxicity assays killed not only the treated cells, but also the cells in the untreated control wells. This suggests that the anti-PRRSV activity of compound 1 may be due to processes that also inhibit cellular processes. All of the anti-PRRSV analogs were much less cytotoxic, with CC50 ranging from 107.88 to 448.20 μM or 37.59 to 91.78 μg/ml (Table 5.1). Compound 19 had the lowest CC50 of the analogs at 107.88 μM (37.59 μg/ml), however this was still well above the compound’s IC50 of 4.82 μM (1.68 μg/ml). Importantly, all of the compound’s CC50 were well above the 5.0 μg/ml concentration used in the inhibition and potency assays. The least potent anti-PRRSV analog, compound 2, had the highest CC50 molarity at 448.20 μM, and the other analogs had CC50 of 301 to 392 μM. Except for compound 1, all of the anti-PRRSV compounds had a higher CC50 than IC50, suggesting that they may be good candidates for safe and effective treatment of animals.

Compound 19 inhibits PRRSV when added post-infection

Based on the above results, compound 19 was identified as the most suitable for large-scale production because this compound was one of the most potent anti-PRRSV
analogs, was much less cytotoxic than compound 1, and was easily synthesized. Therefore, compound 19 was used in a series of mechanism of action studies. To determine the time during infection compound 19 was effective, we performed time of addition assays to determine if compound 19 could inhibit PRRSV when cells were treated at times post-infection. MARC-145 cells were infected with 200 FFU per well, and cells were treated with 10 ug compounds at a concentration of 5 ug/ml at 0, 1, 3, and 6 hpi. Percent virus inhibition was calculated compared to virus only control wells. Compound 19 inhibited 99.7% of PRRSV when cells were treated concurrently with infection, which was not significantly different from the 99.9% inhibition observed when virus was pre-treated with compound 19 (p-value=0.17). However, the anti-PRRSV activity of compound 19 decreased when cells were treated post-infection, and there was a statistically significant difference between treatment groups as determine by one-way ANOVA (p-value <0.0001, Figure 5.5A). Further analysis by Tukey multiple test comparison revealed no statistically significant reduction in PRRSV inhibition between 0 hpi and 1 hpi treatment groups (p-value=0.72), but there were statistically significant differences between 1 and 3 hpi, and between 3 and 6 hpi (p-values=0.001 and <0.001, respectively). However, even with this decrease in inhibition, when cells were treated with compound 19 at 6 hpi, the compound was still able to inhibit 79.7±4.3% of the input virus. These results suggest that compound 19 inhibits PRRSV during late stages of entry or early stages of the virus replication cycle. Because little to no difference in inhibition was seen for compound 19 between pre-treatment, treatment at 0 hpi, or treatment at 1 hpi, compound 19 likely does not act directly on cell-free virions to “kill” the virus, but rather targets subsequent step(s) during the virus replication cycle.
Compound 19 targets attachment and a post-entry step of PRRSV replication

Results from the time of addition assays indicated that compound 19 was not acting directly on virions, but rather blocked steps of PRRSV replication. In order to directly test if compound 19 blocked virus attachment to the cell, we performed virus-binding assays. Binding assays were performed by inoculating cells with $10^5$ FFU PRRSV at $4^\circ$ for one hour, in the presence or absence of compound 19, to facilitate attachment of virions to the cell but not uptake of the virus into the cells. After incubation, cells were extensively washed to remove unattached virus. To determine the number of attached virions, the remaining virus attached to the cells was eluted with STV, viral RNA isolated, and PRRSV genomic RNA (RNA) was quantified via RT-qPCR. The average copy number of virus-only and compound 19-treated cells are reported in Figure 5.5B (“Elution” bars). In the eluted samples, compound 19 had a 0.8 log$_{10}$ reduction in attached virions (Figure 5.5C), which was significantly different from the virus-only elution (p-value=0.0004; Figures 5.5B). This suggests that compound 19 may act, at least in part, by inhibiting PRRSV attachment to the cell.

To determine additional step(s) in PRRSV replication that may be targeted by compound 19, PRRSV RNA replication assays were performed. After the binding assays, cells were moved to $37^\circ$ to facilitate uptake of attached virions into the cell, and cells were harvested at 0, 4, 8, 12, and 24 hours post $4^\circ$ incubation (hpi), total RNA isolated, and PRRSV RNA quantified by RT-qPCR. Consistent with the elution results, there was a statistically significant difference in attached virus copy number between virus only cells and compound-treated cells at 0 hpi cells (p-value=0.026), again suggesting that compound 19 inhibited PRRSV attachment to the cell. However, combining attachment results from
the elution and 0hpi cells, compound 19 reduced the attached virus copy number by less than one log_{10} (equivalent to ~40% reduction), thus it is unclear how biologically significant this reduction is. In addition, our previous assays showed that compound 19 inhibited >99.9% of PRRSV FFU, therefore the compound likely targets additional steps in the replication cycle.

PRRSV RNA quantification of cells harvested at 4, 8, 12, and 24 hpi revealed that the virus-only wells had a slight reduction in PRRSV RNA copy number from 0 to 4 hpi, indicative of the eclipse phase, suggesting the virus was still undergoing early phases of entry and replication, such as uptake, fusion, uncoating, and translation of early nonstructural proteins, prior to the 4 hpi time point. This was followed by rampant genome replication by 8 hpi (Figure 5.5B). The compound-treated cells had significantly less PRRSV RNA at 8, 12, and 24 hpi compared to the virus-only cells (p-values=0.003, 0.001, and 0.002, respectively). While the compound-treated cells had less PRRSV RNA compared to the virus-only cells at 4 hpi, this difference was not statistically significant (p-value=0.063). Interestingly, the log_{10} reduction in copy number in compound-treated cells was similar between 0 and 4 hpi (0.49 and 0.61, respectively), and then drastically increased at 8 hpi (3.02) (Figure 5.5C). However, the total RNA copy number remained relatively similar between 0, 4, and 8 hpi, with a slightly declining trend (8.65x10^{3}, 4.29x10^{3}, and 2.71x10^{3}, respectively). In the virus-only cells, rampant genome replication was observed between 4 and 8 hpi, therefore the lack of an increase in RNA in the compound-treated cells between 4 and 8 hpi suggests that the compound directly inhibited PRRSV genome replication at a post-entry step (Figure 5.5C). These findings are consistent with the results from the time
of addition assays, in which compound 19 had a reduction, but not a complete loss, of inhibition activity when cells were treated with compound 19 up to 6 hpi.

**Discussion**

The natural compound atracylodinol was previously reported to have anti-viral activity against PRRSV *in vitro* (24). We successfully synthesized atracylodinol and seven other analogs with potent PRRSV inhibition activity *in vitro* at low μM concentrations. Eight compounds inhibited >90% of PRRSV up to $10^5$ infectious units, with compound 1 (atracylodinol) and analog compound 19 capable of inhibiting 100 and 99.9% of PRRSV, respectively, using just 10 μg of the compounds per well. Although compound 1 had high cytotoxicity, the seven other analogs were much less cytotoxic, and due to their potent anti-PRRSV activity, these seven analogs have the potential to be effective *in vivo* treatments for PRRSV infection. However, due to the difficulty in synthesis of some of the compounds, large-scale production is not practical for most of the compounds. Compound 19 is the most feasible for large-scale production because it can be synthesized from commercially available materials in only four steps. Compound 19 was also the most effective of the atracylodinol analogs we synthesized, and was less cytotoxic than atracylodinol; 10μg inhibited >99.9% of PRRSV *in vitro* up to $10^5$ FFU, and had an IC$_{50}$ of 1.68 μg/ml (4.82 μM) and a CC$_{50}$ of 37.59 μg/ml (107.88 μM), making it an excellent candidate for *in vivo* trials.

Previous studies have identified several anti-PRRSV compounds *in vitro*, primarily comprised of natural products derived from plants used in traditional Chinese medicine (19-25). Due to the variation in protocols to assay virus inhibition, use of whole extracts
versus purified compounds, and discrepancies in reporting EC$_{50}$/IC$_{50}$ in µg/ml vs. µM, it is difficult to make direct comparisons of antivirals across studies. However, most of the previously described anti-PRRSV compounds inhibited PRRSV replication with IC$_{50}$ in the range of 3.5 to 360 µg/ml and <1 to 98 µM, and when reported, CC$_{50}$ in the range of 76 µg/ml to 147 mg/ml, and 94 to 431 µM, with varying degrees of inhibition and potency (19, 20, 22, 25). The compounds we synthesized had comparable to lower IC$_{50}$ from 1.10 to 5.91 µg/ml, and 4.19 to 29.82 µM (Figure 5.3, Table 5.1), and similar cytotoxicity levels from 107.88 to 448.20 µM (Table 5.1). Interestingly, all eight of the synthetic atracylodinol and analog compounds had higher potency and lower IC$_{50}$ in vitro than cryptoporus volvatus extract, the only anti-PRRSV compound reported to date that has been tested in pigs. In MARC-145 cells, the cryptoporus volvatus extract had a reported EC$_{50}$ of 340 to 360 µg/ml and inhibited PRRSV $10^4$-fold at a concentration of 3 mg/ml (22), whereas our most potent PRRSV inhibitors, atracylodinol and compound 19, inhibited >99.9% of $10^5$ infectious units at an incubation concentration of 62.5 µg/ml. In treated pigs, cryptoporus volvatus extract was capable of slowing PRRSV replication and lessening clinical disease compared to untreated control pigs (22). It is important to note that the cryptoporus volvatus extract likely required higher concentrations because it was not a homogenous synthetic compound, but rather a naturally derived mushroom extract. Nevertheless, these results provide promising evidence that anti-PRRSV activity in vitro can translate to PRRSV inhibition in vivo.

Compound 19 inhibited PRRSV primarily by targeting a post-entry step in the virus replication cycle. Compound 19 inhibited some attachment of PRRSV to the cell, however the observation that the amount of PRRSV RNA remained relatively constant prior to 12
hpi suggests that the main target of compound 19 was a post-entry step that inhibited genome replication. If a post-attachment step in entry was targeted, we would expect to see a more drastic reduction in RNA from 0 to 4 hpi, representing inhibition of the virus entering the cell during the eclipse phase and subsequent degradation of the viral RNA. In addition, the lack of RNA replication in the compound-treated cells at 8 hpi, when rampant RNA replication occurred in the virus-only cells, further suggests that the compound is directly inhibiting a step in RNA replication (Figure 5.5B). The lack/delay of PRRSV RNA synthesis could potentially be due to compound 19 acting to inhibit translation of the nonstructural proteins, which are required for PRRSV genome replication. In addition, compound 19 could directly target the PRRSV RNA-dependent RNA polymerase, thus preventing synthesis of new genomic RNA, as well as minus-strand intermediates and subgenomic RNAs. The increase in genome replication by 12 hpi in the compound-treated wells was likely due to the imperfect nature of the compound, leading to a small amount of PRRSV genome replication by the later stages of infection. However, the genomic RNA replication at the 12 and 24 hpi time points still represented a reduction of >2.5 log₁₀, or >99.9% inhibition.

The proposed mechanism of action of compound 19 targeting a post-entry step of PRRSV replication is consistent with findings from the time of addition studies. When cells were treated with compound 19 up to 6 hours after infection, when genome replication is likely starting, the compound was still effective at inhibiting 80% of FFU compared to the virus-only control (Figure 5.5A), supporting the proposed mechanism of the compound targeting a post-entry step in PRRSV replication. When cells were co-treated with the compound and virus, >99.9% PRRSV inhibition was observed. The reduction in inhibition
when cells were treated at 6 hpi was likely due to the inability of the compound to inhibit attachment, as well as a low-level of PRRSV genomic RNA replication likely occurring by 6 hpi.

Directly acting antivirals that target steps in virus replication post-entry have been successfully used to treat HIV-1 and HCV in humans (11, 13), demonstrating their potential for treating PRRSV infected herds and prevention of the spread of the virus to other barns and farms. However, because many of the pigs that would be treated for PRRSV infection with antivirals would likely enter the food supply, extensive testing in pigs is required to determine the efficacy and safety in animals and humans. In addition, definitive “withdrawal” periods must be established for any compound used in animal production, establishing the period of time required to rid the animal of the compound (28). Therefore, the use of antivirals may not be possible in late-production animals, but temporary treatment of pregnant sows and growing pigs with antivirals during PRRSV outbreaks, including the atractylodinol analogs described here, could help to contain the outbreak and prevent spread to additional farms in the region, thus lessening the economic burden of PRRSV infection.

**Literature Cited**


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Table 5.1 Summary of the potency and cytotoxicity of anti-PRRSV compounds.  
ND ("not done") represents compounds that could not be done due to cytotoxicity from higher dilutions killing control cells (compound 1), or too little compound left to test (compound 18). DMSO CC<sub>50</sub> represents the equivalent compound concentration from the DMSO percentage that killed 50% of cells. 95% confidence interval (CI) reported for each mean.

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<td>391.80</td>
<td>229.50, 604.06</td>
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<td>13</td>
<td>99.8</td>
<td>1.74</td>
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<td>7.24</td>
<td>5.54, 9.20</td>
<td>75.98</td>
<td>58.07, 101.60</td>
<td>316.24</td>
<td>241.70, 422.88</td>
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<td>17</td>
<td>99.2</td>
<td>1.56</td>
<td>1.42, 1.72</td>
<td>5.95</td>
<td>5.41, 6.56</td>
<td>79.02</td>
<td>68.21, 92.08</td>
<td>301.25</td>
<td>260.04, 351.04</td>
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<td>19</td>
<td>100.0</td>
<td>1.68</td>
<td>1.27, 2.14</td>
<td>4.82</td>
<td>3.64, 6.14</td>
<td>37.59</td>
<td>28.80, 55.34</td>
<td>107.88</td>
<td>82.65, 158.82</td>
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<tr>
<td>18</td>
<td>100.0</td>
<td>1.18</td>
<td>1.09, 1.26</td>
<td>4.47</td>
<td>4.12, 4.77</td>
<td>ND</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>23</td>
<td>99.1</td>
<td>1.10</td>
<td>0.62, 1.58</td>
<td>4.19</td>
<td>2.36, 6.02</td>
<td>89.37</td>
<td>74.96, 107.40</td>
<td>340.70</td>
<td>285.77, 409.44</td>
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<tr>
<td>DMSO</td>
<td>5.0</td>
<td>NA</td>
<td>NA</td>
<td>95.36</td>
<td>64.71, 148.00</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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</table>
Table 5.2 Summary of anti-PRRSV potency of compounds up to $10^5$ FFU virus. Mean PRRSV inhibition and standard deviations from two independent experiments reported. Differences between virus treatments within compound were analyzed via one-way ANOVA, and p-values reported. TNTC ("too many to count") represents wells that had too many foci to count.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$10^2$</th>
<th>$10^3$</th>
<th>$10^4$</th>
<th>$10^5$</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100.00 ±0.00</td>
<td>100.00 ±0.00</td>
<td>99.99 ±0.01</td>
<td>100.00 ±0.00</td>
<td>0.07</td>
</tr>
<tr>
<td>19</td>
<td>99.96 ±0.14</td>
<td>99.94 ±0.00</td>
<td>99.93 ±0.01</td>
<td>99.93 ±0.01</td>
<td>0.85</td>
</tr>
<tr>
<td>13</td>
<td>99.28 ±0.66</td>
<td>99.35 ±0.10</td>
<td>99.04 ±0.31</td>
<td>98.75 ±0.15</td>
<td>0.21</td>
</tr>
<tr>
<td>23</td>
<td>98.00 ±2.83</td>
<td>97.98 ±0.71</td>
<td>97.56 ±0.99</td>
<td>97.86 ±0.69</td>
<td>0.66</td>
</tr>
<tr>
<td>18</td>
<td>98.46 ±2.18</td>
<td>98.06 ±0.31</td>
<td>97.43 ±0.28</td>
<td>98.86 ±0.00</td>
<td>0.65</td>
</tr>
<tr>
<td>17</td>
<td>97.69 ±1.09</td>
<td>97.95 ±2.38</td>
<td>98.76 ±1.28</td>
<td>99.62 ±0.00</td>
<td>0.63</td>
</tr>
<tr>
<td>16</td>
<td>97.00 ±1.15</td>
<td>95.69 ±2.39</td>
<td>95.66 ±1.60</td>
<td>96.04 ±0.53</td>
<td>0.67</td>
</tr>
<tr>
<td>2</td>
<td>93.64 ±2.85</td>
<td>89.83 ±3.45</td>
<td>92.48 ±2.63</td>
<td>TNTC</td>
<td>0.21</td>
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We started the synthesis from 3-(2-furyl)acrolein 4, as shown in Scheme 2. We chose to utilize Corey-Fuchs reaction to obtain the terminal alkyne from the aldehyde in two steps. Treatment of 4 with carbon tetrabromide and triphenylphosphine gave us dibromo compound 6, which was converted to furyl enyne 2.

The two-step conversion

Scheme 2. Total synthesis of 1-(E)-atractylophilin (compound 1)

Due to the failure of the original plan at the final stage, a new route was required to continue our project. We did not want to start from the beginning, so enyne 2 was kept in the alternate plan. Instead of a coupling reaction between furyl iodoalkyne 11 and ester 12, iodoalkyne 15 and enyne 2 were carried on to the coupling reaction. The compound was made from terminal alkyne 12 with morpholine hydroiodide salt 10, followed the same procedure described before. The conversion yield was 99%, surprisingly high, compared to 66% yield for compound 11. The same copper catalyzed coupling condition between 2 and 15 gave us the target molecule ester 13 in 35% yield.

Scheme 4. Alternate synthetic route of compound 13

Figure 5.1
Figure 5.1 continued

Scheme 5. Synthesis of dimers, compounds 16, 17, 18, and 19

Scheme 6. Synthesis of compounds 21 and 23
Figure 5.1 continued

**Scheme 8. Synthesis of compounds 26 and 27**

**Scheme 9. Other small molecules**

**Figure 5.1** Synthesis routes for atracylodinol and analogs. Synthesis of **A)** atracylodinol (compound 1) and compound 2; **B)** compounds 12 and 13; **C)** compounds 16, 17, 18, and 19; **D)** compounds 21 and 23; and **E)** compounds 26 and 27. **F)** Structures of other small molecules tested for anti-PRRSV activity. Scheme numbers match the detailed synthesis routes from (26). Red boxes surround compounds tested for anti-PRRSV activity.
Figure 5.2 Anti-PRRSV activity screens of the fifteen synthesized compounds and a DMSO control. 10 μg compounds were incubated with 2x10^2 FFU PRRSV and infected on MARC-145 cells at a final concentration of 5 μg/ml. Percent virus inhibition represents the reduction in PRRSV FFU compared to virus-only control wells. Means from two independent experiments are reported. Error bars represent ± standard deviation of the means.
Figure 5.3 Inhibition assays to determine IC₅₀s of the eight compounds with inhibitory activity of PRRSV. Compounds were serially diluted and incubated with 2×10² FFU PRRSV and infected on MARC-145 cells. Percent virus inhibition represents the reduction in PRRSV FFU compared to virus-only control wells. Means from two independent experiments are reported. Error bars represent ±standard deviation of the means.
Figure 5.4 Potency assays of the eight anti-PRRSV compounds up to $10^5$. Serial dilution of PRRSV from $10^5$ to $10^2$ FFU were pre-treated with 10 μg compounds at a final concentration of 5 μg/ml. Percent virus inhibition represents the reduction in PRRSV FFU compared to virus-only control wells. Means from two independent experiments are reported. Error bars represent ± standard deviation of the means. NC=”Not Counted” because of too many FFU. No statistically significant differences between virus treatments within compound were detected via one-way ANOVA; results are reported in Table 5.2.
Figure 5.5: Mechanism of Action of compound 19

Panel A: Percent Virus Inhibition over hours post infection.
Panel B: Log(10) PRRSV gRNA copies over hours post 4°C incubation.
Panel C: Log(10) reduction of gRNA over hours post 4°C incubation.
**Figure 5.5** Mechanism of action of compound 19. **A)** Time of addition assay with compound 19. Hours post infection represents the time after infection cells were treated with 10 μg per well of the compound, except -1 hr represents a one hour pre-treatment of compound and virus prior to inoculating cells. Means from two independent experiments are reported. Error bars represent ±standard deviation of the means. **B and C)** Attachment and PRRSV RNA replication assays in the presence and absence of compounds 19. **A)** Average RNA copy number per well as determined by RT-qPCR. Elution represents the RNA copy number present in elutions of attached virions after incubation at 4°C. Cells were also harvested at 0, 4, 8, 12, and 24 hpi, RNA isolated, and RNA copy per well determined. Asterisks represent statistically significant differences between virus-only and compound-treated cells as determined by a t-test. **B)** The associated log_{10} reduction in RNA in compound-treated samples compared to the virus
CHAPTER 6: GENERAL DISCUSSION

General Summary

This work provided the first direct evidence of porcine reproductive and respiratory syndrome immune escape variants arising during the course of infection \textit{in vivo}. The four identified neutralizing antibody escape variant viruses from two pigs utilized multiple genetic pathways of NAb escape that targeted GP5 and/or the GP2/3/4 trimer. The mutation or combination of mutations that mediated escape was unique to each escape virus. Only one of the escape viruses had a decrease in infectivity, and none had dramatically decreased growth rates. We also identified potent small molecule inhibitors of PRRSV \textit{in vitro}. Mechanism studies revealed that both neutralizing antibody and the anti-PRRSV compounds targeted post-attachment steps of virus entry.

Importance

Virus variation poses a major challenge to the prevention and treatment of many viral diseases. The production of effective vaccines is hampered by the high amount of variation in many human and animal viruses, including HIV-1 and PRRSV. Many RNA viruses employ myriad strategies and genetic pathways for immune escape from the host's antibody response, including HIV-1 and Influenza (1-4). Longitudinal analyses of long-term HIV-1 infected patients has shown that patient sera can neutralize autologous HIV-1 isolates from previous times in infection, but cannot neutralize concurrent virus (1, 2, 5).
Similarly, the equine lentivirus equine infectious anemia virus (EIAV) can undergo a continual pattern of immune escape from host antibody, leading to periods of viral recrudescence, or “viral blips” from the replication of the immune escape variants, which are then subsequently neutralized by newly produced antibodies (6).

Despite well-documented mechanisms of immune escape in many RNA viruses, direct evidence of immune selection and the existence of immune escape variants had previously been elusive in PRRSV. For the first time, the work in this dissertation provides direct evidence for selective pressure by the immune system, specifically by neutralizing antibody, contributing to PRRSV variation during infection in vivo. This selective pressure can result in variants capable of escaping from concurrent autologous neutralizing sera from pigs. Notably, both the major envelope protein GP5 and the minor envelope protein trimer consisting of GP2, GP3, and GP4 were targets of neutralization, consistent with the findings of others (7-10). There was not a single mechanism or genetic pathway of NAb escape in the PRRSV variants. Even different virus variants that occurred simultaneously within the same animal utilized different mutations and/or envelope proteins to mediate escape, similar to HCV NAb escape (11-12). Additionally, in three of the four identified NAb escape variants, escape was not mediated through a single amino acid change, but rather required a unique and specific combination of mutations occurring together across one or more envelope proteins to mediate escape, suggesting that neutralizing antibody targeted conformational epitopes. One escape virus mediated escape through a single amino acid change, GP5 A27V, and this mutation was also seen in a different escape virus, however it did not mediate escape on its own in that virus. This result indicates that the epitopes that get recognized in each pig are dependent upon that specific pig’s immune response, which
likely contributes to the lack of effective vaccines against PRRSV. Given the complex
process of antibody maturation, this is not an uncommon phenomenon during viral
infections. While broadly neutralizing antibodies have been discovered against HIV-1 and
Influenza and their specific binding sites have been identified, thus far they have not been
reliably elicited via immunization and the development of strategies to guide their specific
production are in their infancy (4, 13, 14).

Neutralizing antibody escape is often associated with decreased infectivity and/or
replication fitness of the immune escape variants. This is usually due to changes in the
neutralizing epitope also being in functionally important regions for the virus, so variation
that disrupts antibody binding to the epitope also alters the protein’s function. This has
been well characterized in HIV-1 bNAb escape variants (15, 16). Assessment of the
replication phenotypes of the identified PRRSV NAb escape variants revealed that escape
was not associated with a loss in infectivity or replication kinetics. Only one of the four
escape variants had a significant reduction in infectivity, but none of the escape viruses had
significant reductions in growth rates. These results, combined with the results that the
escape variants were susceptible to heterologous broadly neutralizing PRRSV anti-serum
and pooled sera, indicate that the mutations that mediated escape in these viruses did not
occur in functionally important regions of the PRRSV envelope proteins. Therefore, the
neutralizing antibody produced by these pigs likely functions through steric hindrance
rather than directly binding to crucial envelope protein domains. It is possible that escape
of these viruses resulted because the neutralizing antibody from these pigs did not target
crucial domains of the virus. NAb that targeted functionally important regions of the virus,
such as the receptor-binding site, may elicit sterilizing immunity that the virus cannot easily escape from due to deleterious mutations and functional losses.

Previous studies have established that the current PRRSV vaccine strategies using modified live virus (MLV) vaccines are not effective against heterologous PRRSV strains, and their ability to reduce transmission and/or lessen clinical signs in animals outside of experimental conditions is not well characterized (17-19). We found that the replication fit immune escape variants arose in PRRSV populations with >99% nucleotide sequence identity, indicating that vaccines may not be fully protective against even homologous challenge. This finding supported a previous study that showed that three different MLV vaccines lessened clinical signs from homologous challenge, but did not protect against infection (20). Virus from vaccinated animals or replication competent immune escape variants could potentially lead to transmission of virus to other pigs, and the development of clinical disease in non-vaccinated and vaccinated animals. Additionally, the transient nature of the immune escape variants identified in this study may be a mechanism of PRRSV persistence, similar to the mechanism of persistence in EIAV (6). Currently, the molecular mechanisms that facilitate PRRSV persistence are not known, in part due to limited sampling during persistence studies (21-23). Therefore, a better understanding of the molecular mechanisms of PRRSV immune escape and the contribution of immune selection on PRRSV diversity and persistence are needed to facilitate the design of new vaccine strategies.

Because of the difficulty in developing effective vaccines due to viral variation and inconsistent and unpredictable immune responses, many human viruses have been successfully treated with antiviral drugs. Antiretroviral therapy has dramatically improved
the prognosis for HIV-positive individuals (24, 25), and direct acting antivirals against HCV have actually cured many patients with chronic infections (26, 27). The use of antiviral drugs in livestock animals is not currently widespread due to concerns of unwanted compounds entering the food supply. However, with the proper safety protocols, antivirals could potentially be good treatment options during outbreaks of viral diseases. Antivirals could be added to feed in the case of an outbreak, thus treating a large number of animals very quickly. Reducing viral load could help prevent further spread of the infection to other animals and/or barns in the area, lessening the impact of the outbreak. We synthesized and characterized small molecule compounds with inhibitory activity against PRRSV based on the previously described anti-PRRSV natural compound atractylochin (28). Atractylochin and seven other analogs had potent anti-PRRSV activity in vitro at low μM concentrations. Interestingly, compound 19 appeared to inhibit a small amount of attachment, but primarily acted to block a post-entry step in PRRSV replication. All of the analogs were less cytotoxic and had lower inhibitory concentrations than atractylochin. Currently, only one anti-PRRSV compound, cryptoporus volvatus extract, has been tested both in vitro and in pigs (29). The atractylochin analogs all were more potent inhibitors of PRRSV in vitro compared to in vitro inhibition assays of cryptoporus volvatus extract (29). While any of the analogs have the potential to be use to treat PRRSV in vivo, the effectiveness and easily scalable synthesis strategy of one of the compounds, compound 19, makes it an ideal candidate for future trials in pigs.
Limitations and Alternative Strategies

This dissertation work provided evidence and characterization of immune escape variants in PRRSV and the characterization of anti-PRRSV compounds in vitro. However, there are limitations to the work presented here, which are discussed below.

Evaluation of immune selection

Chapters 3 and 4 presented work on the identification and characterization of immune selection, neutralization, and immune escape variants. While this work provided the first evidence of immune escape variants arising during in vivo infection of PRRSV, the analyses were limited by a small number of animals and a limited amount of serum from each pig. We selected five pigs with differing virological outcomes; two with prolonged viremia over 35 days of infection and three that initially cleared the virus then experienced a subsequent rebound in viremia by 42 dpi. Our initial sequencing results from a small number of virus clones from each of the pigs revealed very little variation in both the envelope protein-encoding region of ORF2-6 and nonstructural protein 2, and that the variation that did occur appeared to be largely pig-specific. Therefore, due to time and cost constraints, we focused our strategy on sequencing more virus clones from the five pigs rather than sequencing virus from additional pigs. Including virus from additional pigs would have increased our statistical power to detect selection in the virus variants, especially when comparing virus from the prolonged pigs and rebound pigs. However, previous studies of immune selection in PRRSV using a larger number of animals and bigger sequencing data sets have had conflicting results, underscoring the difficulty in
evaluating immune selection in PRRSV by sequencing and statistical approaches alone (30-33).

**Characterization of NAb escape variants**

Additionally, while each of the immune escape variants we identified utilized different mutation(s) for escape from NAb, the characterization of virus from additional rebound pigs may have revealed patterns or virus preferences in the mutations that were used to mediate escape. We also identified one rebound pig that had detectable neutralizing antibody to the inoculum virus, but the concurrent rebound day virus did not escape the autologous serum, suggesting an alternative strategy of virus rebound. Evaluation of virus from additional rebound pigs may have identified additional rebound viruses that did not result from NAb escape. Characterization of these viruses may have revealed alternative strategies of PRRSV rebound. While we gained valuable information from the characterization of NAb escape in two pigs, additional insights into the mechanism of escape likely could be gained by the assessment of rebound virus from additional pigs.

Precise mapping of the neutralizing epitopes involved in neutralization and escape of the four NAb escape viruses was hindered by the very limited amount of autologous serum from the rebound pigs. Previous studies mapping epitopes in PRRSV have relied on phage-display or peptide inhibition studies, limiting detection to linear epitopes (10, 34). Our finding that rebound virus was predominated by distinct ORF2-6 viral haplotypes, and that three of the four escape variants required multiple mutations occurring together suggests that neutralizing antibody likely targeted conformational epitopes. While we were
able to narrow down the envelope protein(s) involved in escape and the sets of amino acid mutations involved in NAb escape for each virus, we did not have enough serum to perform neutralization assays on all possible combinatorial mutations to specifically identify the amino acids that mediated escape. We found that the escape variants only escaped from autologous serum, however given enough time to test additional pig sera from the PHGC trials, we may have been able to find sera from different pigs that targeted the same epitopes and could be used for further epitope mapping.

The work presented in this dissertation utilized the African Green Monkey kidney MARC-145 cells, the only cell line susceptible to PRRSV that is regularly used for in vitro studies of PRRSV infection (35). However, using primary cultures of the natural PRRSV host cell, the porcine alveolar macrophages (PAMs) would have more closely resembled the in vivo biology of PRRSV pathogenesis (36). Although there has been much debate about the true PRRSV receptor, recently it was shown that CD163 is a required PRRSV receptor in both MARC-145 cells and PAMs, so entry into the cell is likely facilitated through similar processes in the two cell types (37-40). However, there are reported differences in PRRSV pathogenesis in PAMs versus MARC-145 cells, most notably in the induction of the innate immune response (41). Therefore it is possible that there may be differences in PRRSV neutralization and replication kinetics between MARC-145 cells and PAMs.

**Mechanism of PRRSV neutralization**

Because of the limited amount of rebound pig serum, we also did not have enough of the autologous escape day serum to use in the characterization of the mechanism of neutralization studies. Ideally, we would have used the rebound serum that targeted GP5
and/or GP2/3/4 of the inoculum virus in neutralization mechanism studies to identify the step(s) in PRRSV replication targeted by antibody to the different envelope proteins. The specific immune escape variants would have been tested for a rescue phenotype of viral replication to verify the NAb targeted replication step. However, because we did not have enough rebound serum to do this, we used the pooled PHGC serum, which did not allow for the differentiation between GP5-targeted NAb and GP2/3/4-targeted NAb steps in replication. An alternative strategy would have been to generate monospecific antibody in rabbits to each glycoprotein using immunogenic peptides from the NVSL97-7895 inoculum sequence that were involved in neutralization and escape of the immune escape variants, as described in (42). The monospecific antibodies could be screened for neutralizing activity against the inoculum and immune escape variants, and the neutralizing monospecific antibodies used for neutralization mechanism studies to identify the specific step in PRRSV replication targeted by the NAb.

Characterization of anti-PRRSV compounds

Chapter 5 reported the results of identifying and characterizing potent small molecule compounds with anti-PRRSV activity in vitro. The major limitation of this work is that it was only performed in vitro, and follow-up characterization of the compounds’ efficacy and safety in animals is needed. Several potent anti-PRRSV compounds have been described recently in the literature (28, 29, 43-45). However, to date only one has been tested in vitro and in pigs. The compound, *cryptoporus volvatus* extract, was successful in decreasing both clinical disease and viral load in animals, however the animals were pre-treated twice daily for eight days prior to inoculation (29). These results provide proof of
concept that compounds with anti-PRRSV activity in vitro have the potential for in vivo protection. However, success of a compound in vitro by no means guarantees success in vivo. A recent evaluation of approval for compounds in clinical trials for humans estimated that only 10-15% of all compounds make it to market (46). While this was an estimate of all compounds, not just antivirals, it highlights the difficulty of translating in vitro results to safe and effective products. Currently, there are no antivirals approved for the treatment of livestock diseases. While there is great potential in treating PRRSV with antivirals, actually getting a compound approved for commercial use will be challenging. In addition to proving the compound is safe and effective for use in the animal, verification that the compound does not enter the food supply and is safe to humans presents a major challenge for the approval of livestock antivirals.

**Future Work**

Several unanswered questions and hypotheses from this work remain that warrant further study. The first question is if the NAb escape variants are capable of causing productive infection in pigs. Because the escape variants did not have a marked decrease in infectivity or replication fitness, it is likely that they could be transmitted to other pigs. To determine if the escape variants result in productive infection in pigs, studies involving the inoculation of PRRSV naïve pigs with the escape variants could be performed. Virus from the FL12 infectious molecular clone, from which the chimeric escape viruses were made, replicates efficiently in pigs and therefore infectious studies comparing FL12 and the escape viruses could be performed in vivo (47). While we did not observe drastic infectivity
or replication fitness losses *in vitro*, it is possible that the escape variants have altered replication phenotypes and/or pathogenicity *in vivo*. In order to compare infection between the different variants, serum samples to evaluate viremia would be taken from pigs daily throughout infection to track infection dynamics.

We hypothesized that transient and continual NAb escape may be a contributing mechanism of PRRSV persistence. In order to determine if this may be the case, additional experimental infections with the NVSL97-7895 inoculum virus could be carried out, but would extend the trial period beyond the 42 days of the PHGC trials in order to monitor for multiple rebound events. Due to the brief period of escape observed, frequent samples would need to be taken from the pigs so that the periods of recrudescence would not be missed. Previous studies of persistence have taken serum samples infrequently or not at all, and therefore may have missed these periods of viremia due to replication of escape variants (21-23).

In addition to the potential *in vivo* studies, there are additional *in vitro* assays that could be done to further characterize the mechanism of PRRSV neutralization and escape. As mentioned previously, neutralization mechanism studies utilizing neutralizing monospecific antibodies directed against the different PRRSV envelope proteins could be generated in order to determine the mechanisms of NAb directed against each envelope protein. This approach would also help to elucidate each envelope protein’s role during PRRSV entry into the cell. This strategy, while it would be the most informative, is also high-risk because neutralizing monospecific antibody may not be generated. Regardless, the neutralizing mechanism of the pooled serum, or the broadly neutralizing serum from Harrisvaccines, could be further characterized by a series of assays designed to target
specific steps in replication, not just follow PRRSV genomic RNA replication during infection. These would include assays that specifically target the steps of internalization and uncoating in the presence and absence of the neutralizing PRRSV anti-serum (48).

The small molecule inhibitors of PRRSV characterized in Chapter 5 have the potential to be used to treat PRRSV in the field. As previously mentioned, *in vivo* trials are still needed to evaluate their efficacy and safety. Despite their high potency *in vitro*, most of the compounds did not inhibit 100% of input PRRSV. However, their inhibitory activity remained constant up to $10^5$ FFU PRRSV, raising the question of whether these were drug-resistant variants, or simply the byproduct of the kinetics of the compounds. In order to address this question, the virus that was not inhibited by the compounds could be isolated via plaque assay, passaged, and again used in the compound inhibition assays to determine if the isolated virus was less susceptible to the compound. Additionally, the PRRSV diversity that contributes to vaccine failure could potentially hinder antiviral activity as well. It would be informative to further screen the anti-PRRSV compounds we synthesized for inhibitory activity against a variety of genetically diverse strains of PRRSV in order to determine if their PRRSV inhibition activity is specific to a narrow range of strains, or if they are more broadly acting anti-PRRSV compounds.

**General Conclusions**

The results from this dissertation work indicate that selective pressure by the immune system contributes to the vast genetic diversity of PRRSV through the selection of immune escape variants. These escape variants have the potential to be transmitted pig-to-
pig and contribute to the continual circulation of PRRSV within and between herds.

However, there is potential for the treatment of outbreaks in herds with antivirals. Further characterization and understanding of the molecular mechanisms of variation and immune escape are needed to make successful vaccines to PRRSV.

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