Porcine reproductive and respiratory syndrome virus (PRRSV): Immunization strategies, virulence of various isolates, and efficacy of DNA vaccination

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Porcine reproductive and respiratory syndrome virus (PRRSV): Immunization strategies, virulence of various isolates, and efficacy of DNA vaccination

by

Wesley Scott Johnson

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Program of Study Committee:
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2009

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Dedication

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CHAPTER 1. GENERAL INTRODUCTION

Thesis organization

The following thesis consists of a general introduction in chapter 1, a literature review manuscript prepared for submission to Animal Health Research Reviews in chapter 2, a manuscript published in Veterinary Immunology and Immunopathology 102 (2004) 233–247 in chapter 3, a manuscript prepared for submission to Vaccine in chapter 4, and general conclusions in chapter 5. All of the chapters contained within this thesis were written by Wesley Scott Johnson as primary author.

Introduction

Porcine reproductive and respiratory syndrome virus is an enveloped single-stranded positive-sense RNA virus classified in the family Arteriviridae (Cavanagh 1997). Much of the research that has been done at both the genetic and protein levels for PRRSV has already been reviewed previously (Snijder and Meulenberg 1998; Dea, Gagnon et al. 2000). Snijder et al. provided an early detailed description of the base knowledge known about the viral genome as well as the composition and function of the viral proteome. Porcine reproductive and respiratory syndrome virus encompasses a very diverse collection of isolates; even within its own genus, PRRS viruses possess great differences in their genomes and can be very different from one another at the individual isolate level with base changes, additions, or even deletions. When comparing nucleotide sequences of the VR-2332 and the LV isolates, very different sequences were seen with only about 61% identity in the 5’ leader sequence and only about 55% identity in the ORF1a region was observed (Nelsen, Murtaugh et al. 1999). Alternately, the ORF1b region was
found to be more similar between these same isolates with about 63% sequence identity, and 75% amino acid identity. An earlier genomic comparison of the EU PRRSV LV isolate and the NA PRRSV VR-2332 isolate focusing on the viral envelope proteins ORF 5 and 6 found that sequence homology ranged from 55% in ORF5 to 79% in ORF6 (Murtaugh, Elam et al. 1995). The fluidity of the PRRSV RNA genome and a lack of fidelity of the RNA dependant polymerase contributes to quasispecies where PRRSV exists not as a single isolate or even as a group of isolates, but more as a collection of very similar genotypes on a farm or even within a single animal (Goldberg, Lowe et al. 2003) (Rowland, Steffen et al. 1999). This characteristic of PRRSV produces genomic changes coding for changes within the PRRSV proteome, which in turn provides for the seemingly infinite diversity of the virus. There is no doubt that this diversity contributes to the challenge of vaccine development and PRRS control.

As implied by its name, the diseases caused by PRRSV infection can take two forms, each of which were thought to be separate multifactorial diseases before the etiologic agent was identified (Zimmerman, Yoon et al. 1997). The respiratory disease can be seen in all age groups of pigs and in some cases can be very severe, leading to death. The diversity in viral pathogenicity among PRRSV isolates has been reported by evaluating several different isolates each applied at a standardized dose (Johnson, Roof et al. 2004) and revealed that in vivo replication rate of the different PRRSV isolates was correlated to the level of pathogenesis within the host. The highly virulent isolates contributed to a 50% mortality rate in young pigs, while others that represented the more attenuated isolates did not induce clinical illness and were quickly cleared by the piglets. The reproductive disease can range from a lack of conception, early farrowing of congenitally-infected piglets with increased
mummies and dead born piglets, to abortion storms with significant piglet death loss and even sow death. The timing of sow infection has a great deal of influence on the severity and presentation of PRRSV-associated disease. When examining the reproductive disease around mid-gestation, time frame of 50 days or less, it was demonstrated that sows were susceptible to infection by intranasal exposure but their fetuses were for the most part not susceptible to viral infection through the placental barrier (Christianson, Choi et al. 1993). The study went on to show that direct infection in utero of the fetuses was possible within this same mid-gestation time frame. Infection of a sow with PRRSV during late-gestation, the middle of the third trimester of pregnancy, was found to result in the congenital infection of the fetuses leading to increased fetal death and weak born piglets, followed by PRRSV transmission from the infected piglets to littermates before weaning (Mengeling, Lager et al. 1998).

References


CHAPTER 2. IMMUNIZATION STRATEGIES FOR PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS

A paper to be submitted to Animal Health Research Reviews

Wesley Johnson

Abstract

This review is intended to discuss published work focused on the immunization of swine against porcine reproductive and respiratory syndrome virus (PRRSV). Many different strategies have been pursued by researchers in the quest for control and ultimately the eradication of porcine reproductive and respiratory syndrome (PRRS). Some of the strategies have lead to the licensure of commercial products while others have provided insight into the biology of PRRSV. Many of the strategies examined here have contributed to the overall understanding of the host’s immune response to PRRSV. Some strategies have provided unconventional or in some cases controversial methods of protecting swine from PRRS. The main area of interest in this review will be the clinical protection of swine from virulent PRRSV challenge. Gross lung pathology and viremia along with virus neutralizing antibody titers will serve as the primary parameters for protection in the respiratory challenge model, whereas the number of live born pigs and the number of weaned pigs along with piglet viremia will serve as the primary parameters for protection in the reproductive challenge model. Despite the incomplete reporting of these primary parameters, some publications will be discussed that have focused on protection against PRRSV by either evaluating the immune responses in non-target animals or studies conducted in swine, some without a virulent challenge. It is important to note that for the purposes of this review a homologous PRRSV challenge will be defined as a challenge with the same viral isolate from which the
vaccine was derived, and a heterologous PRRSV challenge will be defined as a challenge with a PRRSV isolate that is not the isolate from which the vaccine was derived. After evaluating the strategies based on these parameters the only immunization strategy that has provided efficacy against both a homologous and a heterologous challenge is the modified live vaccine.

1. Introduction

Nearly 20 years after its characterization, Porcine Reproductive and Respiratory Syndrome (PRRS) is one of the most economically important diseases in the swine production industry. In the US, it was estimated in 2005, that the disease was responsible for over 66 million dollars of lost income from breeding animals and a loss of over 490 million dollars in growing-pig populations (Neumann, Kliebenstein et al. 2005). Retrospectively, porcine reproductive and respiratory syndrome virus (PRRSV) was first described in the swine populations of North America, but initially the disease was referred to as “mystery swine disease” (Hill 1990). The first PRRSV isolate to be well characterized was designated as the Lelystad (LV) isolate, which would later become the prototypical European (EU) PRRSV isolate, or type I PRRSV (Wensvoort, Terpstra et al. 1991). The reproductive disease observed in the case from which the virus originated was reproduced under controlled conditions, fulfilling Koch’s postulates that the LV EU PRRSV isolate was the cause of the clinical disease (Terpstra, Wensvoort et al. 1991). Soon after the discovery of PRRSV in Europe, a detailed description of both the growth properties and the viral structure was published for the isolate ATCC VR-2332, which was later considered the prototypical North American (NA) PRRSV isolate, or type II PRRSV (Benfield, Nelson et al. 1992).
Porcine reproductive and respiratory syndrome virus is an enveloped single-stranded positive-sense RNA virus classified in the family *Arteriviridae* (Cavanagh 1997). Much of the research that has been done at both the genetic and protein levels for PRRSV has already been reviewed previously (Snijder and Meulenberg 1998; Dea, Gagnon et al. 2000). Snijder et al. provided an early detailed description of the base knowledge known about the viral genome as well as the composition and function of the viral proteome. Porcine reproductive and respiratory syndrome virus encompasses a very diverse collection of isolates; even within its own genus, PRRS viruses possess great differences in their genomes and can be very different from one another at the individual isolate level with base changes, additions, or even deletions. When comparing nucleotide sequences of the VR-2332 and the LV isolates, very different sequences were seen with only about 61% identity in the 5’ leader sequence and only about 55% identity in the ORF1a region was observed (Nelsen, Murtaugh et al. 1999). Alternately, the ORF1b region was found to be more similar between these same isolates with about 63% sequence identity, and 75% amino acid identity. An earlier genomic comparison of the EU PRRSV LV isolate and the NA PRRSV VR-2332 isolate focusing on the viral envelope proteins ORF 5 and 6 found that sequence homology ranged from 55% in ORF5 to 79% in ORF6 (Murtaugh, Elam et al. 1995). The fluidity of the PRRSV RNA genome and a lack of fidelity of the RNA dependant polymerase contributes to quasispecies where PRRSV exists not as a single isolate or even as a group of isolates, but more as a collection of very similar genotypes on a farm or even within a single animal (Goldberg, Lowe et al. 2003) (Rowland, Steffen et al. 1999). This characteristic of PRRSV produces genomic changes coding for changes within the PRRSV proteome, which in turn provides for the seemingly infinite diversity of the virus. There is no
doubt that this diversity contributes to the challenge of vaccine development and PRRS control.

As implied by its name, the diseases caused by PRRSV infection can take two forms, each of which were thought to be separate multifactorial diseases before the etiologic agent was identified (Zimmerman, Yoon et al. 1997). The respiratory disease can be seen in all age groups of pigs and in some cases can be very severe, leading to death. The diversity in viral pathogenicity among PRRSV isolates has been reported by evaluating several different isolates each applied at a standardized dose (Johnson, Roof et al. 2004) and revealed that in vivo replication rate of the different PRRSV isolates was correlated to the level of pathogenesis within the host. The highly virulent isolates contributed to a 50% mortality rate in young pigs, while others that represented the more attenuated isolates did not induce clinical illness and were quickly cleared by the piglets. The reproductive disease can range from a lack of conception, early farrowing of congenitally-infected piglets with increased mummies and dead born piglets, to abortion storms with significant piglet death loss and even sow death. The timing of sow infection has a great deal of influence on the severity and presentation of PRRSV-associated disease. When examining the reproductive disease around mid-gestation, time frame of 50 days or less, it was demonstrated that sows were susceptible to infection by intranasal exposure but their fetuses were for the most part not susceptible to viral infection through the placental barrier (Christianson, Choi et al. 1993). The study went on to show that direct infection in utero of the fetuses was possible within this same mid-gestation time frame. Infection of a sow with PRRSV during late-gestation, the middle of the third trimester of pregnancy, was found to result in the congenital infection of the fetuses
leading to increased fetal death and weak born piglets, followed by PRRSV transmission from the infected piglets to littermates before weaning (Mengeling, Lager et al. 1998).

Many of the aspects associated with PRRSV transmission are well established and have been reviewed previously (Zimmerman, Yoon et al. 1997). More recent studies of PRRSV transmission have provided new insight into the complexity of virus transmission. The infectious-dose-fifty for the VR-2332 PRRSV isolate in three-week-old piglets was found to be about $2.0 \times 10^5$ virus particles by the oral route, but only about one twentieth that level, or $1.0 \times 10^4$ virus particles by the intranasal route (Hermann, Munoz-Zanzi et al. 2005). Although the study was only examined qualitatively, the shedding of PRRSV in mammary gland secretions has been observed (Wagstrom, Chang et al. 2001). In another study the experimental spread of PRRSV via the airborne route from one pig unit to another was accomplished with controlled air exchange of as little as 1% of the total air intake (Kristensen, Botner et al. 2004). A later study found PRRSV to be present in just over 25% of oral and nasal swab samples collected from pigs experimentally inoculated with virulent PRRSV at three weeks of age, but the virus was not detected in aerosol samples from the same animals (Hermann, Brockmeier et al. 2008). The detection of PRRSV in oral fluids was examined, looking for either the virus or antibodies to the virus by using real time qRT-PCR or ELISA respectively, and found that PRRSV could be detected in the oral fluid by real time qRT-PCR for about four weeks after experimental inoculation (Prickett, Simer et al. 2008). The transmission of PRRSV was studied using persistently infected (PI) sows that were defined as such by a progression beyond acute disease after a wild-type viral exposure, but were found to be positive for PRRSV at necropsy (Bierk, Dee et al. 2001). Among other parameters, the absence of viremia by virus isolation helped define the end of acute disease at
42 days post exposure. PRRSV-naïve sows were then put into pens adjacent to the PI sows, allowing nose-to-nose contact and three out of four of the contact sows developed viremia and antibodies to PRRSV. It was confirmed that the virus obtained from the contact sows was the same as the wild-type isolate used during the exposure of the PI sows. Indicating that non-viremic PI sows, as defined in this study, were capable of transmitting PRRSV to naïve adjacentely penned sows.

Porcine reproductive and respiratory syndrome virus immunology is very complex and an in-depth discussion will not be attempted here as several publications are available that can provide a more detailed perspective on PRRSV immunology. Briefly, PRRSV infects and replicates very efficiently in macrophages, which is thought to lead to both immunosuppression and predisposition to secondary infections (Molitor, Bautista et al. 1997). The virus induces a weak interferon alpha response by the host, but there is some evidence of early innate immune responses with increases in NK cell numbers within five to seven days after infection (Murtaugh, Xiao et al. 2002). The virus is eventually cleared after a specific antibody and cell mediated response by the host to the virus. The initial antibody response by the host provides little aid in viral clearance but alternatively a virus neutralizing (VN) antibody response occurs and the virus is generally cleared (Lopez and Osorio 2004). Re-exposure of a host to PRRSV will generate a response that is generally protective for closely related viruses but less protective for viruses that are of the opposite type and thus considered heterologous to the vaccine isolate (Mateu and Diaz 2008).

Many different methods and combinations of methods have been used in an attempt to control PRRSV spread both on an individual farm and between different farms including depopulation, test and removal, and mass vaccination all of which are popular control
methods. For example, nursery depopulation was evaluated in an attempt to reduce the economic losses associated with PRRSV on 34 farms and it was determined that this method was a cost effective way of improving the profitability of PRRS-positive herds, especially if a producer had only limited capital, or could not use several sites to control the disease (Dee, Joo et al. 1997). Variations of the test and removal method have been employed by different producers with some success, using a systematic phased testing and culling of breeding stock, followed by testing and culling of nursery pigs (Yang, Moon et al. 2008), or testing of all animals on a single day then removing only the ones testing positive (Dee, Bierk et al. 2001). The control of PRRSV within an entire region of France for several years was accomplished using a variation of the test and removal method, which completely depopulated whole farms that tested positive for PRRSV (Le Potier, Blanquefort et al. 1997). Combining methods such as pairing of the test and removal method for the elimination of persistently-infected breeding animals along with the depopulation method in the nursery and finisher facilities, allowed elimination of PRRSV from a test farm (Dee and Molitor 1998). By combining a breeding stock vaccination program with nursery depopulation, PRRSV was eliminated from a seedstock farm for 16 months after which the virus reappeared in only the finisher population (Dee, Joo et al. 1998).

The challenges associated with the development of a broadly effective and economical method to control PRRSV that can also be differentiated from a wild-type viral infection has fueled scientific interest in the characterization of PRRSV. The financial impact of the disease caused by PRRSV has also provoked the swine industry to push for more and better options in the control of PRRSV. With these motivators in place today, it is no surprise that the amount of research that has been done on PRRSV immunization is immense. An
important call for some much needed standardization of the protocols used by all researchers 
evaluating new PRRSV vaccine prototypes (Murtaugh, Dee et al. 2007) was again recently 
reiterated and explained further (Rowland 2008). Some of the strategies examined here have 
been discussed at a recent conference, but only in a very limited context and in reference to 
only a few specific studies (Roof 2008). The goal of this review is to provide a broad 
presentation of published work and the associated strategies used to immunize swine against 
PRRS.

In the quest to address the question of how PRRSV can be controlled or eliminated 
there have been many strategies and ideas tested. The clinical protection of swine from 
virulent PRRSV challenge using a vaccination or immunization strategy will serve to focus 
the topics included in this review. Each section of the review will discuss a strategy in an 
attempt to categorize the published work, and provide an organizational framework of the 
ongoing efforts toward protection from PRRSV. The hope of the author is that the ultimate 
ew PRRSV vaccine would be one that is very safe, broadly efficacious, easily differentiated 
from wild type exposure, and still economical for swine producers. A common theme 
repeated in this review is that each of the discussed strategies are missing at least one of the 
above attributes, that if were present would in turn identify the preferred strategy for vaccine 
development.

2. PRRSV Passive Protection

2.1 Background

Passive protection from disease is among the oldest strategies employed to protect a 
host from disease, but it can mean different things to different researchers. For the purposes
of this review, the term “passive protection” will be used in reference to the administration of a substance that is intended to provide protection from PRRSV by a means outside of invoking the host’s own immune system to provide the protection. The most common method for providing passive immunity for PRRSV is the parenteral administration of hyper-immune serum to the susceptible host with the intent to confer protection from later or concurrent viral infection. Other forms of passive protection would include the transfer of maternal immunity through colostrum or the administration of antiviral compounds.

2.2 Passive Protection from Respiratory Disease

Protection of pigs from PRRSV viremia using intraperitoneally instilled passive immunity was shown to be dose dependant with serum virus neutralizing (VN) antibody titers of 1:8 being required for protection of piglets from viremia after a respiratory challenge (Lopez, Oliveira et al. 2007). INF-α was tested for its protective effects during PRRSV infection using a human adenovirus expressing porcine INF-α (Brockmeier, Lager et al. 2009). The administration of the INF-α expressing adenovirus one day prior to a virulent PRRSV challenge provided for a decrease in the percent lung involvement and a delay in viremia when compared to the control group. A different approach to passive protection utilized an adenovirus that expressed small interfering RNAs targeted to the PRRSV ORF1b, ORF5 and ORF7 genes to suppress the viruses replication (Li, Jiang et al. 2009). The study was conducted in vitro so no conclusions on the protective efficacy of this approach can be made until a subsequent challenge study is conducted in swine.

2.3 Passive Protection from Reproductive Disease

By utilizing specific VN avian antibodies to a farm isolate of PRRSV, a somewhat unorthodox method of controlling PRRSV infection in a production setting was discovered in
central Mexico (Francos, Lucio et al. 2008). This new protection method was initiated by injecting all of the animals on the farm twice with the PRRSV specific VN avian antibodies about two weeks apart with the subsequent injection of sows between the 11th and 13th weeks of each gestation. In addition to sow treatments the piglets on the farm were also given the avian antibodies orally within the first 12 hours after birth. This use of passive protection was found to provide the farm with economic benefits by reducing both mortalities and the use of medication. The passive protection of pregnant animals from a virulent PRRSV reproductive challenge has been proven successful previously (Osorio, Galeota et al. 2002). In this study the administration of PRRSV-convalescent hyperimmune serum to sows on day 87 of gestation provided protection from an experimental virulent PRRSV challenge on day 90 of gestation. In a later study the challenge of either vaccinated or un-vaccinated gilts with 20 different PRRSV field isolates showed that passive protection could be provided to piglets if the mother had been vaccinated prior to conception (Lager, Mengeling et al. 2003). However this protection was found to be incomplete, with two of the PRRSV isolates being found in the piglets from the vaccinated gilts.

3. PRRSV Killed Virus Vaccination

3.1 Background

Growing PRRSV in vitro or in vivo and inactivating it for use as a vaccine is one of the oldest strategies to be used when vaccinating pigs for the virus. Killed PRRSV vaccination is one of the two strategies that have lead to the development of commercially available vaccines. One of the fundamental benefits of a killed vaccine is its safety, with the virus being inactivated there is no chance for viral replication or vaccine shed, and thus no
chance for reversion or recombination to virulence. Another benefit to the use of killed vaccines is the fact that this type of vaccine can be quickly generated using the newest field isolates more quickly and safely than other strategies since these isolates will be inactivated in the final product. The use of killed PRRSV vaccines is not without challenges, for example, eliciting an immune response sufficient for protection by this method has proven difficult with the host’s antibody response not being detectable after the use of a killed vaccine. A vaccinated animal therefore is not readily differentiated from a naïve animal. It seems that for PRRSV there may be a fundamental tradeoff between safety and efficacy, because the same killed PRRSV vaccine that is completely safe seems to have little or no efficacy.

3.2 North American PRRSV killed vaccines

There is only one killed NA PRRSV vaccine that has ever been licensed in the US (Vaccine A; table 1) and it is intended for use as a two-dose presentation in breeding age females, with two-dose boosters at each gestation. In the vaccine licensing trials (Vaccine A) provided for both a significant increase in the number of normal piglets and a significantly greater weight gain for those piglets born to vaccinated sows (Thacker, Thacker et al. 2003). In a later study it was determined through the vaccination (Vaccine A) of ten-week-old pigs that no decrease in the magnitude or duration of viremia was provided when comparing vaccinated and unvaccinated groups (Nilubol, Platt et al. 2004) (table 2). However, a significantly increased level of VN antibodies and a slightly increased number of INF-γ producing cells was observed in the vaccinated animals. The host’s reaction to vaccination was also studied by performing repeated vaccinations with either a killed vaccine (Vaccine A) or a licensed North American PRRSV modified live virus (MLV) vaccine (Vaccine B;
table 1) (Bassaganya-Riera, Thacker et al. 2004). This study found that regardless of the vaccine type used, repeated vaccination over an extended length of time resulted in a diminished immune responsiveness to specific PRRSV antigens.

Research has also been done looking at killed vaccines using North American isolates with a focus on protection from virulent PRRSV challenge with a very closely related isolate. For example, the vaccination of sows in China with a killed vaccine made from a virulent isolate provided for about 85% protection from a challenge with vaccine isolate while the immunity afforded to the unvaccinated piglets of vaccinated sows was found to provide about 92% protection from the same challenge (Cai, Guo et al. 2002) (table 2). Both of these levels of protection were based on reductions in the number of animals positive for live virus by isolation after the virulent challenge. An earlier study examining a killed autogenous vaccine showed that some protection was conferred to the vaccinated animals after a subsequent challenge with the same isolate used in the formulation of the vaccine (Osorio, Zuckermann et al. 1998) (table 3). However, the level of protection afforded by the killed vaccine was not as good as the protection provided by the modified live vaccines that were also examined in the study.

3.3 EU PRRSV killed vaccines

Killed PRRSV vaccines are marketed in the EU, with varying degrees of protection from various challenge isolates. The vaccination of either naïve or previously PRRSV-exposed piglets with a commercially available killed EU PRRSV vaccine (Vaccine C; table 1), lead to a significant reduction in the percentage of animals with viremia after a challenge with a homologous isolate (Reynaud, Charreyre et al. 2004) (table 2). A study evaluating INF-γ secreting T cell levels in pigs showed that vaccination (Vaccine C) could induce an
increase in the production of these cells soon after vaccination (Piras, Bollard et al. 2005) (table 2). Unfortunately, no post challenge clinical data were presented for this study, so a correlation between the increase in the INF-\(\gamma\) secreting T cells and clinical protection of the animals from disease could not be made. In a recently conducted study it was concluded that vaccination (Vaccine C) provided no protective immunity from a virulent LV challenge (Zuckermann, Garcia et al. 2007) (table 2). The effectiveness of a different EU PRRSV killed vaccine, Cyblue® (formally marketed by Cyanamid) had also been brought into question when it was tested in boars to determine if the level and duration of viremia could be reduced after a virulent challenge (Nielsen, Nielsen et al. 1997) (table 3). The vaccine was found to provide no reduction in the level or duration of viremia when compared to the challenge control boars. In a more recent vaccination (Vaccine C) study conducted in boars it was shown that there were no significant changes to the production of sperm or its quality, but without a virulent challenge in the study no conclusions could be made about the efficacy of the vaccine (Papatsiros, Alexopoulos et al. 2006). The vaccination (Vaccine C) of sows with in a field setting resulted in a significantly increased number of both live born and weaned pigs (Papatsiros, Koptopoulos et al. 2004) (table 3). After an extensive study evaluating the reproductive benefits provided by the extended use of vaccine (Vaccine C) in field exposed sows, it was determined that for the sow characteristics, only the premature farrowings were reduced in the four parities tested (Papatsiros, Alexopoulos et al. 2006). Also observed in this study, for reasons described as unclear by the authors, was a statically significant increase in the farrowing rate of the vaccinated group. As for the litter characteristics observed in this study, the number of weaned pigs was increased in vaccinated animals as compared to the unvaccinated animals. In order to evaluate its efficacy a study was conducted with a different
commercial EU PRRSV killed vaccine, Suvaxyn® PRRS (formerly marketed by Fort Dodge Animal Health) (Scortti, Prieto et al. 2007) (table 3) and it was determined that the level of protection provided by the vaccine to a virulent reproductive challenge was nearly nonexistent, with the only statically significant difference between the challenge control animals and the vaccinated animals being the pre-weaning mortality of piglets, which was reduced in the vaccinated animals.

Other studies have been conducted using unlicensed killed PRRSV vaccine prototypes that were again based on EU isolates which have also shown varying degrees of protection from virulent challenge as well. A commercialized EU type PRRSV killed research prototype provided no protection to pigs after a subsequent respiratory PRRSV challenge with the virulent I2 isolate while an EU PRRSV MLV vaccine (Vaccine D; table 1) provided good protection from the same challenge (Gozio and Woensel 2006) (table 2). The vaccination of sows and gilts with an inactivated EU PRRSV vaccine containing an oil adjuvant resulted in a significant increase in both live born and weaned piglets after a field exposure of the animals to a wild type PRRSV isolate prevalent on the farm where the study was conducted (Reynaud, Brun et al. 2000). In an earlier study, the use of an inactivated EU type PRRSV vaccine containing an oil adjuvant that forms a double oil emulsion, also showed up to 80% protection of sows after a virulent reproductive challenge with the same PRRSV isolate used to generate the killed vaccine (Plana-Duran, Bastons et al. 1997).

The protective efficacy provided by killed PRRSV vaccination is questionable at best with nearly no protection seen after most respiratory evaluations and conflicting results in reproductive evaluations. Perhaps the future discovery of a unique immune response in the host after an exposure to replicating virus will provide some insight into the deficiencies in
the response after exposure to killed virus. This type of discovery could lead to some much needed improvements in killed PRRSV vaccines, but at this time killed vaccines do not provide adequate protection from PRRS.

4. PRRSV Subunit Vaccination

4.1 Background

The formulation of a prototype vaccine containing a single PRRSV protein, combinations of two or more proteins, fusions of whole viral proteins, or parts of viral proteins to either non-viral proteins or other carriers all describe PRRSV subunit vaccination. One of the biggest advantages of this strategy is its inherent ability to be used as a differentiating infected from vaccinated animals (DIVA) vaccine. This strategy has proven very effective for many other viral pathogens, usually ones that contain a single characterized antigen or group of antigens that have been shown to be protective when the immune response of the host is directed to them. With PRRSV being such a diverse group of viruses that have genomes predisposed to change and the fact that no definitive protective antigen is established for the virus, it is no surprise that this method of vaccination has shown only limited efficacy to date.

4.2 Importance of and focus on the PRRSV Gp5 antigen

The consensus among many PRRSV researchers is that the viral Gp5 protein, encoded by the viruses ORF5 gene, plays a key roll in the interaction of the virus with the host cell, but a difference in the level of glycosylation of a glycoprotein could lead to differences in the interaction of antibodies with that protein. This idea has led to work focused on finding the neutralizing epitopes of Gp5 and also identifying VN monoclonal
antibodies (Mab’s) to the Gp5 protein. An in depth examination of the Gp5 ectodomain was performed using overlapping synthetic peptides to measure peptide-specific antibodies in experimentally infected pigs as well as field serum that showed VN antibodies (Plagemann 2004). It was shown that antibodies to nine specific amino acids localized in the ectodomain of Gp5 were responsible for neutralization of the virus. Further testing was done looking at alteration of this peptide at specific amino acid positions and it was found that two regions consisting of three amino acids were required for neutralization of the virus. A more recent examination of the Gp5 protein was performed also using peptides, but focusing on immunodominant T-cell epitopes (Vashisht, Goldberg et al. 2008). Two of the 96 peptides tested were shown to have immunodominant T-cell epitopes and when the sequence of these two peptides from the isolate tested were compared to the sequences of other isolates it was found that there was a good level of homology with at most only two amino acid substitutions being observed. An evaluation of Mab’s to the Gp5 protein of a Canadian PRRSV isolate indicated that the Mab’s were in fact type specific because none of the Mab’s neutralized the EU LV PRRSV isolate and only two of the five Mab’s showed neutralization of the NA VR-2332 PRRSV isolate (Pirzadeh and Dea 1997). Further evaluation of several Mab’s directed at four PRRSV antigens, N, M, Gp5, and Gp3, showed that Mab’s directed to the N protein and portions of the Gp5 protein tended to cause antibody dependant enhancement while antibodies directed to the M, portions of the Gp5, and the Gp3 proteins showed VN properties (Cancel-Tirado, Evans et al. 2004). Interestingly, it was found that the Mab directed to the Gp3 (ISU45B) was statistically a much stronger inhibitor of viral replication in PAM cells than the Mab that recognized an epitope on the Gp5 protein (ISU25C).
4.3 Subunit vaccine prototypes for PRRSV

The hypothesis that Gp5 plays an important role in PRRSV infection of the host cell has lead to its use in several subunit vaccine prototypes, but Gp5 is not used exclusively. Other well-known PRRSV antigens as well as innovative new vaccine platform technologies are all currently being evaluated as vaccine prototypes. Purified Baculovirus expressed protein products of the PRRSV ORF5 and ORF3 genes were able to confer qualitative protection to sows as measured by an increased number of live born and weaned piglets as compared to the challenge control group (Plana Duran, Climent et al. 1997). It is important to note that the genes expressed by Baculovirus were originally cloned from same isolate that was used for the challenge in this study. The use of a PRRSV N protein fusion with Pseudomonas exotoxin provided partial protection from virulent challenge in piglets with a significant reduction in viremia, but once again no prevention of pneumonia (Liao, Lin et al. 2004) (table 4). When fusion proteins consisting of PRRSV Gp5 and M fused to the Pseudomonas exotoxin were used to vaccinate piglets, the constructs again only provided partial protection from virulent challenge with microscopic lung lesions that were significantly decreased in the vaccinated animals but the reduction in viremia was only marginal and not significant (Liao, Yang et al. 2006) (table 4). The recent evaluation of fusion proteins consisting of PRRSV N and ORF1b to the Pseudomonas exotoxin provided only partial protection from virulent challenge in piglets with significant reductions in viremia, but not complete prevention of viremia (Liao, Weng et al. 2008) (table 4).
5. PRRSV DNA Vaccination

5.1 Background

The reverse transcription of PRRSV RNA into cDNA followed with further genetic manipulations for use as a vaccine is one of the more modern strategies used in the vaccination of pigs for PRRSV. The strategy by design can be both safe and differentiated from a wild type exposure, but perhaps the biggest advantage to this strategy is that the fact that the host itself produces the viral proteins and the proteins are presented to the immune system in a manner similar to that during a natural infection. The drawbacks to this strategy are again a lack of sufficient protection, complexity, cost, and the labor involved in the vaccination process. DNA vaccines hold much potential for the future, but the current limitations of technology along with the complex immunology of PRRSV make this strategy difficult to commercialize at this time.

5.2 Importance of and focus on the PRRSV ORF5 gene

Again, the focus for most PRRSV DNA vaccines has been put on the viral ORF5 gene, either alone or in combination with other viral genes. An early pig challenge study evaluating a DNA vaccine that consisted of a plasmid with the PRRSV ORF5 under control of the cytomegalovirus (CMV) promoter was shown to induce a VN antibody titer sufficient for protection of the animals from a generalized viremia and macroscopic lung lesions following a challenge (Pirzadeh and Dea 1998) (table 4). A plasmid-based DNA vaccine coding for a fusion protein of PRRSV Gp5 to bovine herpes virus 1 VP22 was used to immunize mice which resulted in an increase in VN antibodies that was significantly greater than the increase seen in the group receiving the vaccine containing Gp5 alone (Zhao, Xiao et al. 2005). PRRSV DNA vaccination in piglets was tested using plasmids encoding the Gp5 or
M proteins either alone or in combination (Jiang, Xiao et al. 2006) (table 4). It was shown that VN antibodies could be detected within the animals by 10 weeks after the primary vaccination, but only in the combination group not in the groups given Gp5 or M alone. One of the conclusions of this study was that the heterodimer formed by the Gp5 and M proteins could in fact be an important antigen for developing a VN antibody response. Another DNA vaccination of mice with the PRRSV Gp5 gene, this time modified with a Pan DR T-helper cell epitope between the putative neutralizing epitope and a decoy epitope showed an enhanced level of VN antibodies when compared to the native Gp5 construct (Fang, Jiang et al. 2006). Very recently the PRRSV ORF5 gene was chosen for codon optimization and was used as a cDNA vaccine in a plasmid either with or without the addition of co-expressed swine ubiquitin (Hou, Chen et al. 2008) (table 4). The study found that the protection from virulent challenge was again only partial, even for the vaccine containing swine ubiquitin, which showed improved efficacy as compared to the other vaccines with a greater reduction of the viremia levels. The PRRSV Gp5 protein was once more the focus of a vaccine prototype, as a suicidal DNA vaccine expressing both a modified PRRSV Gp5 and the native PRRSV M proteins (Jiang, Fang et al. 2009) (table 4). In piglets the vaccine prototype elicited some protection from virulent PRRSV challenge, with a reduced incidence and duration of viremia as well as reductions in lung lesions. The vaccination of pigs with DNA vaccines encoding either ORF4, ORF5, ORF6, or ORF7 were able to elicit both a humoral and a cell mediated immune response (Kwang, Zuckermann et al. 1999) (table 4). The immune responses were measured by antibody production for the humoral and either interferon-gamma production or specific lymphocyte proliferation for the cell mediated response but since no virulent challenge was conducted the responses measured cannot be
correlated to protection. Vaccination of piglets with a DNA vaccine encoding a modified and mammalian codon optimized ORF5 gene from a highly virulent Chinese PRRSV isolate induced a significantly increased VN antibody response but again there was no virulent challenge of the pigs so no conclusions about protective efficacy can be made about this vaccine (Li, Xiao et al. 2009) (table 4).

5.3 EU PRRSV DNA vaccine prototypes

Prototype DNA vaccines for PRRSV based on EU isolates have also been evaluated with much the same outcomes as those observed for the prototypes based on the NA isolates. A Danish PRRSV isolate was used to generate DNA vaccine constructs for each of the viral ORF’s including the ~11Kb ORF1a/b replicase, the constructs were then tested either individually or in combination (Barfoed, Blixenkrone-Moller et al. 2004) (table 4). After three vaccinations antibodies were readily detected in the ORF7 group, but only sporadically in the groups receiving the other ORF’s. After six vaccinations the pigs were challenged with the same PRRSV isolate used for the generation of the DNA vaccines, but the resulting disease remained sub-clinical even in the control animals. Measurement of antibodies specific to the Nsp2, Gp4, and Gp5 proteins indicated that a response was induced by the DNA vaccines containing the individual ORF’s and the vaccine consisting of the combination of all the ORF’s. A later study that focused on a EU type PRRSV ORF 7 DNA vaccine either alone or in combination with a porcine secondary lymphoid chemokine DNA vaccine was shown to elicit both a humoral and a cell mediated immune response, but these responses unfortunately did not confer protection (Diaz, Domínguez et al. 2006) (table 4). After challenge with the virulent isolate used to generate the DNA vaccines the typical PRRSV-induced growth retardation was observed along with viremia levels in the vaccinated
pigs that were similar to the levels in the challenge control pigs. The vaccination of pigs with EU PRRSV DNA vaccines encoding either the Gp4 or Gp5 proteins were again shown to be only partially protective (Ferrari, Petrini et al. 2006) (table 4). No measurable humoral response was seen in the vaccinated animals, but interestingly there was a significant increase in the T-cell response observed in these same animals. Again the vaccines were unfortunately not able to prevent or even significantly reduce the levels or duration of viremia after a virulent PRRSV challenge with the same isolate used to generate the DNA vaccine.

Although much has been attempted using the DNA vaccine strategy for PRRSV immunization there are still many promising possibilities for future vaccine development. The advancements being made in the DNA vaccine industry bring this strategy closer to a reality each year. With many of the PRRSV DNA studies utilizing the CMV promoter, the evaluation of a more diverse array of promoters may be advised and will perhaps lead to a breakthrough in this vaccination strategy.

6. PRRSV Vector Vaccination

6.1 Background

The use of a genetically modified organism, which contains a PRRSV gene or group of genes, to vaccinate pigs to the virus is yet another strategy examined by researchers. The idea is that a replicating or non-replicating vector infects the host to facilitate the expression of the PRRSV genes. In some cases the vector itself is able to express the PRRSV genes outside of any assistance from the host while other vectors require assistance from the host to express the proteins of interest. The two major advantages of this strategy are again the safety of the vector and its inherent ability to be differentiated from a wild type exposure. The
drawbacks of vector vaccination include the hosts’ immune response specific to the vector, which makes subsequent vaccinations difficult, and much like the other strategies discussed earlier, the lack of sufficient efficacy remains a problem. The availability of a universally protective PRRSV antigen that could be expressed in a vector system would illustrate the potential usefulness of this strategy.

6.2 Bacterial vectors

Live vector vaccines have been developed for PRRSV utilizing different bacterial species. A recombinant *Mycobacterium bovis* BCG (BCG) was generated that expressed a truncated PRRSV Gp5 and the entire PRRSV M protein (Bastos, Dellagostin et al. 2002). The proteins were expressed so as to allow for localization to either the cytoplasm or surface of the BCG. These BCG constructs were tested in mice resulting in the induction of antibodies to the viral proteins detected by both ELISA and Western blot. A later study of the same BCG vector vaccine in pigs found only partial protection from virulent challenge with a reduction in viremia, pyrexia, and viral load in bronchial lymph nodes but no prevention of these parameters (Bastos, Dellagostin et al. 2004) (table 4). Mice were immunized with attenuated live *Salmonella typhimurium* aroA isolate which was transformed with a PRRSV Gp5 DNA vaccine vector (Jiang, Jiang et al. 2004). The same level of VN antibody titer was seen with this vector vaccine as were seen with the naked plasmid DNA vaccine, but if the neutralizing epitope was removed from the Gp5 within either of the vaccines it was found that the levels of VN antibody titer were significantly reduced. It is important to note that although good expression of the desired viral protein is often achieved when using prokaryotic expression vectors the antigen generated will be free of any glycosylation, which
could present problems in antigenic recognition by the host’s immune system when it is exposed to PRRSV.

6.3 Pseudorabies virus vectors

Pseudorabies virus (PRV) is one of the more widely utilized live viral vectors developed for PRRSV vaccines and has shown varying degrees of protection from challenge. A recombinant PRV vaccine expressing the PRRSV Gp5 was evaluated in the piglet respiratory model and was shown to provide no protection from a virulent EU PRRSV challenge as indicated by the levels of viremia post challenge (Álvarez, Prieto et al. 2004) (table 4). Another evaluation of a recombinant PRV that expressed the PRRSV Gp5 protein that again was evaluated in the piglet respiratory model, only this time along side the parent PRV vaccine and a killed PRRSV vaccine (Qiu, Tian et al. 2005) (table 4). The results of the study indicated that the recombinant PRV vaccine expressing PRRSV Gp5 provided the best level of protection from virulent challenge with no clinical signs of disease, viremia for only two weeks, and only mild lung lesions in the animals receiving the vaccine. A later study of recombinant pseudorabies viruses was designed to evaluate, in mice, prototypes that were generated to express the PRRSV Gp5 or M proteins individually or in combination, as either native or modified proteins (Jiang, Fang et al. 2007) (table 4). The vector that elicited the greatest immune response in mice was found to be the one expressing the combination of both the modified Gp5 protein and the native M protein. This vector was then further tested in pigs for its ability to provide protection from a virulent PRRSV challenge and was shown to provide some protection from challenge, as indicated by a reduced level of lung lesions and a shorter duration of viremia when compared to a commercially available killed vaccine that was included in the study as a control.
6.4 Adenovirus vectors

Adenoviruses have been used for the development of promising viral vectors for PRRSV vaccination. Recombinant adenoviruses expressing the PRRSV Gp5 and M proteins individually or in combination were tested in mice to determine the immunological responses elicited to each (Jiang, Jiang et al. 2006). The virus co-expressing the Gp5 and M proteins provided a superior response, as indicated by significantly higher VN antibody titers and stronger lymphocyte proliferation responses. Again, adenoviruses were evaluated in mice for immunogenicity, one vector expressed the whole Gp3 protein and the other vector expressed a truncated version of Gp3 referred to as modified Gp3 (Jiang, Jiang et al. 2007). The vector expressing the modified Gp3 was found to provide a significantly enhanced immune response when compared to vector with the unmodified Gp3. In a very recent study the expression by adenovirus of Gp3, Gp4, and Gp5 gene fusions of varying combinations in mice were evaluated for both VN antibody production and cell-mediated immune responses (Jiang, Jiang et al. 2008). These responses were shown to be significantly enhanced when the fusions were compared to the viral vectors containing only the individual genes.

Adenoviruses that expressed both the PRRSV Gp3 and Gp5 proteins each fused to the *Haemophilus parasuis* heat shock protein 70 (HSP70) with different linkers were shown to provide partial protection to pigs after being challenged with the same PRRSV isolate used to generate the recombinant vector vaccine (Li, Jiang et al. 2009). The protection of the vaccinated animals was measured by a reduction in, but not the prevention of clinical signs, lung lesions, and viremia when compared to the unvaccinated challenged animals. In a recent study adenoviruses expressing either PRRSV Gp3 and Gp5 alone or with co-expressed swine granulocyte-macrophage colony stimulating factor (GM-CSF) were evaluated for protection
from challenge with the isolate which the Gp3 and Gp5 genes originated from (Wang, Li et al. 2009) (table 4). The level of protection was superior in the group co-expressing PRRSV Gp3, Gp5, and the GM-CSF, with significantly decreased lung lesions and clinical signs.

6.5 Other viral vectors

Several other viral vectors have been used as prototype vector vaccines for PRRSV. For example, a recombinant vaccinia virus expressing the PRRSV ORF2 gene was used to vaccinate piglets, resulting in the production of a significant VN antibody titer (Rogan, Levere et al. 2000) (table 4). The utility of different recombinant modified vaccinia virus constructs expressing the Gp5 and M PRRSV proteins in different arrangements were evaluated through immunizing mice (Zheng, Chen et al. 2007). The vector that expressed the two proteins separately each controled by a different promoter resulted in the greatest VN antibody response and cellular immune response. A transmissible gastroenteritis coronavirus (TGEV) minigenome that expressed PRRSV ORF5 was used to vaccinate pigs resulting in the detection of a humoral immune response in the vaccinated animals (Alonso, Sola et al. 2002) (table 4). Some of the early investigations of the PRRSV LV isolates’ proteins were performed using the semliki forest virus (SFV) expression system (Meulenberg and Petersen-den Besten 1996), (Meulenberg, van Nieuwstadt et al. 1997). More recently the SFV expression system was evaluated for PRRSV ORF5 antigen production with aspirations of using the recombinant virus particles in a vaccine prototype (Jung, Hwang et al. 2002).

Recombinant fowlpox viruses co-expressing the PRRSV Gp3 and Gp5 proteins either with or without porcine IL-18 provided some protection after a virulent PRRSV challenge with the same isolate used to generate the recombinant vector vaccine (Shen, Jin et al. 2007) (table 4). This protection was illustrated by a reduction in viremia and fewer virus isolations from the
bronchial lymph nodes in the vaccinated animals when compared to the control animals. A modified recombinant Baculovirus gene delivery vector expressing both the PRRSV Gp5 and M proteins under the control of independent CMV immediate early promoters was used to vaccinate mice (Wang, Fang et al. 2007). With a significantly enhanced production of VN antibodies and a significantly increased level of INF-\(\gamma\) this vaccination study showed that the vector, even at its lowest dose, was able to outperform a DNA vaccine encoding the same antigens. A recent study of killed TGEV vector prototype vaccine expressing the PRRSV ORF5 and ORF6 gene products was able to induce only partial protection from challenge as illustrated by reduced viremia and faster antibody production in the vaccinated animals, but interstitial pneumonia was still detected in the vaccinated animals (Urniza, Ceriani et al. 2008) (table 4). New approaches to the creation of DIVA PRRSV vector vaccines are currently being developed by Sirrah Bios in collaboration with AlphaVax (Harris, Erdman et al. 2008). A whole new PRRSV vaccine platform is being generated, consisting of virus like replicon particles that can be used to immunize swine to PRRSV. This new approach to the vector vaccination strategy has been shown to provide partial protection to pigs from a virulent respiratory PRRSV challenge (Mogler, Erdman et al. 2008).

Although promising data exists for this strategy, much of it has been generated in the mouse model focusing primarily on immunological parameters. More studies need to be conducted in swine that incorporate virulent challenges before a true understanding of the full potential afforded by vector vaccination against PRRSV can be realized.
7. PRRSV Infectious Clone Vaccination

7.1 Background

Using infectious clones of PRRSV for MLV vaccine prototypes is again a potential strategy for vaccine development. The strategy consists of producing a cDNA construct of the entire viral genome, which can then be genetically manipulated more easily than the RNA genome of the virus. In order to incorporate attenuating changes into the new cDNA clone, bases can be changed, deleted, or exchanged with other clones either systematically or randomly. Infectious PRRSV cDNA clone vaccines again have the potential advantage of being differentiated from wild-type-exposed animals and also the potential to be safe. This strategy is not without risk as it does come with some disadvantages, such as the potential for reversion or recombination to virulence. Porcine reproductive and respiratory syndrome virus infectious cDNA clone work has been done using both single isolate infectious clones and multi-virus chimera clones. The work done with PRRSV infectious clones contributes to the body of knowledge that already exists for Equine Arteritis Virus (EAV) and Poliovirus infectious clones and adds to the understanding of the host’s immune response to the viral infection as well as providing some insight into the functions of the different viral proteins that are encoded by the compact but very complex PRRSV genome (Yoo, Welch et al. 2004).

The first ever cDNA infectious clone made for any PRRSV isolate was based on the LV isolate and incorporated mutations that added a unique PacI restriction site directly following the ORF7 gene (Meulenberg, Bos-de Ruijter et al. 1998). These mutations were found to be present in the virus that was rescued after an RNA transfection of BHK-21 cells and subsequent passage in PAM cells or CL2621 cells. The presence of the unique PacI site in the progeny virus supports the fact that this virus was in fact generated de novo from the
cloned cDNA. The first cDNA infectious clone that was generated for a NA PRRSV isolate was based on the prototypical VR-2332 isolate (Nielsen, Liu et al. 2003). This clone incorporated several genetic changes when compared back to the parent isolate two of which affected amino acid sequence. It was shown that besides a slightly lower titer when grown in Marc-145 cells the cDNA clone behaved the same as the parent isolate. In fact, when this clone was tested in 5.5-week-old pigs it displayed the same clinical signs as the parent virus right down to the observation of the characteristic blue colored ears on the challenged animals.

7.2 Infectious clones for evaluating PRRSV biology

The utilization of PRRSV cDNA clones makes genetic manipulation of the virus much less cumbersome and thus has enabled a new era of research into the biology of PRRSV. For example, reverse genetics were used to manipulate a cDNA clone of PRRSV to produce separate PRRSV mutants each with one of the cysteines within the N protein substituted with a serine (Lee, Calvert et al. 2005). These manipulations were done in order to examine the ability of the virus to replicate when key dimmer sites were removed from the protein. The removal of either of two out of the three cysteine amino acids resulted in a lethal phenotype for the virus, but since cysteine 23 is known to form a covalently bonded homodimimer it was expected that changing it would result in the loss of replication of the virus. Interestingly though, cysteine 90 was not known to be involved with any dimmer formation so when changing it resulted in the loss of viral replication it was hypothesized that this residue could possibly be involved in the formation of a dimmer as well. Recently PRRSV cDNA clones were generated based on a Chinese isolate that is thought to be responsible for the production of a specific set of clinical signs characterized as porcine high
fever syndrome (PHFS) (Lv, Zhang et al. 2008). The overall goal of this work was to unequivocally identify PRRSV as the causative agent behind PHFS and in fact this was accomplished through the fulfillment of Koch’s postulates using the characterized cDNA clone of the PRRSV isolate. In an early infectious clone study, PRRSV was explored as a possible vector to carry small fragments of genetic information encoding peptides from other pathogens like Influenza as a proof of concept (Groot Bramel-Verheije, Rottier et al. 2000). The PRRSV clone which has an A2 autoprotease site added to remove the peptide from the N protein was shown in fact to be able to not only continue its replication and function in cultured alveolar macrophages but it was also shown to efficiently express the added genetic material encoding the nine Influenza amino acids. A study to determine if the EU PRRSV Gp5/M protein heterodimer was responsible for the virus’s cell tropism was designed where by the ectodomain of the M protein was substituted with that of several other viruses including the NA PRRSV VR-2332 isolate and EAV (Verheije, Welting et al. 2002). The changes in the ectodomain of the M protein tested in this study had no influence on the virus’s cell tropism, indicating that the M protein within the Gp5/M protein heterodimer may not be involved in cell tropism. Very recently a cDNA clone of the APRRS isolate was mutated in order to separate the individual coding regions of the virus and to provide for more straight forward manipulations in generating chimeric viruses (Yuan and Wei 2008). This manipulation of the cDNA clone could possibly provide the building blocks needed for generating a better and more cross protective PRRSV vaccine.

7.3 Comparisons of infectious clones to their parental isolates

Much of the research into infectious clone vaccination involves the comparison of the parent PRRSV isolate to its cDNA clone with respect to in vitro and in some cases in vivo
characteristics. The generation of a cDNA clone that was able to replicate both \textit{in vitro} and \textit{in vivo} to levels equivalent to its parent isolate, provided a starting point for the evaluation of the attenuation level of vaccine candidates generated by incorporating mutations into the clone (Truong, Lu et al. 2004). Two other infectious PRRSV cDNA clones, one virulent and one vaccine-derived were evaluated to examine the phenotypes of the clones both \textit{in vitro} and \textit{in vivo} (Kwon, Ansari et al. 2006). The results indicated that the vaccine-derived infectious clone retained the attenuated phenotype of its parental isolate, and the wild type clone retained the virulent phenotype of its parental isolate. The Nsp2 region of the PRRSV genome was evaluated recently using a cDNA clone to determine if it was possible for the virus to accommodate the deletion of specific parts of the Nsp2 gene (Ran, Chen et al. 2008). The virus was in fact able to replicate faster \textit{in vitro} after the deletion, indicating that the deleted portions of the gene were not essential for viral growth in cell culture.

\textbf{7.4 Infectious clone vaccinations}

Full-length cDNA constructs of PRRSV isolates are not only useful for basic research of the biology of PRRSV, they have in fact also been designed and used as vaccine prototypes that have been evaluated directly (without modification from the parent virus), as genetically modified versions of the parent isolate, and as chimera viruses made of more then one PRRSV isolate. Three different PRRSV cDNA clones each based on the LV isolate were evaluated for safety as well as efficacy from challenge with either homologous (EU PRRSV) or heterologous (NA PRRSV) isolates (Verheije, Kroese et al. 2003) (table 4). Because the parental isolate of the cDNA clones was not considered to be virulent the stability of the mutations that were incorporated into the clone served as a standard to evaluate safety. The mutations incorporated into the cDNA clones included both nucleotide deletions and
nucleotide substitutions. In eight-week-old pigs, the changes to the cDNA clones remained stable throughout the *in vivo* test period even while the animals developed viremia, indicating that the cDNA clones were stable and viable. The efficacy of the cDNA clones to a homologous challenge was good with only one pig from each of two of the cDNA vaccinated groups showing viremia post challenge. Unfortunately, the efficacy of the cDNA clones to heterologous challenge was poor with viremia in all of the vaccinated animals that were challenged, but on a more positive note, no viremia was seen in the vaccinated sentinels that were added to two of the challenged groups. In another study, two different cDNA clones that were constructed based on the PRRSV isolate P129 genome and with deleted ORF2 or ORF4 genes, respectively, were evaluated for safety and efficacy (Welch, Jolie et al. 2004) (table 4). The PRRSV deletion clones were shown to be stable in cell lines complementing the deleted genes, but offered no protection in vaccinated pigs from a challenge with the same isolate used to construct the cDNA clones and it was evident that the limited-replicating cDNA clones would not make good vaccines. Even though the clones were not efficacious, they could still serve as useful tools for investigating the function of the respective protein encoded by the deleted genes leading to a better understanding of the overall biology of PRRSV. The manipulation of two different cDNA clones, one generated from a vaccine isolate (Vaccine B) and one from a virulent field isolate (MN184), resulted in the development of several PRRSV chimera clones (Wang, Liang et al. 2008) (table 4). These chimera virus clones represented combinations of the genes encoding the structural proteins (ORF2-7) of one virus and the genes encoding the replication proteins (ORF1a/b) of the other virus. When evaluated *in vivo* the chimera viruses were attenuated as compared to the MN184 isolate cDNA clone but not to the attenuation level of the vaccine (Vaccine B)
cDNA clone. In evaluating their pathogenicity, lung scores in the chimera clone groups were observed to be intermediate to the almost absent scores seen in the animals inoculated with the vaccine (Vaccine B) cDNA clone and the high scores of over 40% seen in the animals inoculated with the MN184 cDNA clone. Another study of PRRSV cDNA chimera clones involved the generation of individual chimera clones using the genetic backbone of a virulent virus and the individual structural proteins of a vaccine isolate, in addition a cDNA clone utilizing the ORF5 of a second virulent virus was also incorporated into this same genetic backbone (Kwon, Ansari et al. 2008). The goal of this research was to determine the possible role of each of the structural genes in the attenuation process of the virus. Safety testing in the reproductive model, found that the ORF5 structural gene provided the most significant level of attenuation, followed by the ORF2 gene and then the remaining genes were found not to contribute to the virulence of the virus, but as a safety study with no virulent challenge of the animals no conclusions as to the efficacy of these vaccines could be made.

A PRRSV DIVA vaccine clone was created by incorporating deletions in the Nsp2 region of the viral genome (de Lima, Kwon et al. 2008). Specific B-cell epitopes within the Nsp2 region were targeted for deletion so as to obtain a unique serological response in the vaccinated animals, which would be easily detected via ELISA. Differentiation of animals infected with the wild type virus from those vaccinated with the deletion mutant proved to be successful, by using the commercially available IDEXX PRRSV ELISA kit for the detection antibodies to the PRRSV N protein, and an ELISA specifically developed to detect antibodies directed to the deleted portion of the Nsp2 gene, but because there was no challenge there is no efficacy data available for this study. Other efforts to investigate a DIVA incorporated differentiating attributes by utilization of deletions in the Nsp2 gene, but
in this study the Green Florescent Protein gene was added to the PRRSV cDNA clone in place of the deletion (Fang, Rowland et al. 2006). The cDNA clone was subsequently used as a vaccine allowing vaccinated animals to be differentiated from those exposed to wild type virus using an ELISA, unfortunately once again no data on protection was presented for this study, as there was no challenge (Fang, Christopher-Hennings et al. 2008). The insertion of the new genetic material into the PRRSV genome proved to again demonstrate the lack of genomic stability in the virus through the mutation of the GFP and its loss of florescence. Other groups have also focused on the Nsp2 gene, using cDNA clones with even larger deletions to produce marker vaccine clones, which also resulted in the easy differentiation of vaccine and wild-type-exposed animals by serology using an ELISA or molecularly using RT-PCR, but again no data was presented for efficacy, because there was no challenge in the study (Kim, Kaiser et al. 2009).

8. PRRSV Modified Live Virus Vaccination

8.1 Background

The use of a PRRSV modified live virus (MLV) vaccine is a common commercial strategy used in vaccination of swine to protect against PRRSV. This strategy has been employed to generate licensed and commercially available PRRSV vaccines, several of which are still being sold today (Vaccine B, Vaccine D, Vaccine E; table 1, and Vaccine F; table 1). The details of the processes employed for generating an MLV vaccine vary, but they generally involve the sequential passage of the virus isolate of interest in a cell line permissive to its replication until it is sufficiently attenuated to the point of not causing clinical disease in the host. The major advantage to this strategy is that it provides an
infectious virion that stimulates an appropriate immune response similar to the response seen after a wild type infection. This advantage of PRRSV MLV vaccines is likely due to the fact that the vaccine isolates are genetically very similar to the virulent parent isolates. A complete genome comparison between the PRRSV isolate VR-2332 and its attenuated vaccine isolate (Vaccine B) showed that only 41 nucleotides were changed in the attenuation process of the virus (Yuan, Mickelson et al. 2001). Of those mutations, ten were silent when looking at the resulting amino acids and an additional five mutations were considered to be conservative changes. This led to an untested theory that the remaining twenty-six mutations provided the attenuated phenotype of the vaccine isolate. Since MLV vaccines became available many different combinations of both herd management practices and vaccination protocols have been successfully established for the proper use of the MLV vaccines against PRRSV in the field (Dee, Joo et al. 1998), (Philips and Dee 2002), (Gillespie 2003), (Gillespie and Carroll 2003), (Wetzell 2004), (Eger 2000), (Philips, Jordan et al. 2000), (Beilage and Beilage 2004), (Angulo, Diaz et al. 2006), and (Lebret and Ridremont 2008). Some drawbacks do still exist for the use of MLV vaccines and include the lack of differentiation from a wild type exposure, and there is a potential for reversion of the MLV to a more virulent form.

8.2 Laboratory respiratory efficacy

In the swine industry the PRRSV MLV vaccine is a common option for vaccination. A summary of 16 controlled lab scale PRRSV MLV vaccination studies each including a virulent challenge provided the collective conclusion that the use of NA PRRSV MLV vaccines (Vaccine B or Vaccine F) significantly reduced the appearance of lung lesions after each challenge, regardless of what challenge isolate was used (Roof 2008). Furthermore, in a
side by side comparison a killed NA PRRSV vaccine (Vaccine A) was unable to induce VN antibodies to PRRSV while the use of either wild type PRRSV exposure or MLV PRRSV vaccine (Vaccine B) to immunize animals resulted in the induction of a VN response (Meier, Galeota et al. 2003). It was noted that this VN response was however delayed when compared to the overall antibody response of the host. Pigs were protected from a challenge with either of two different virulent Japanese PRRSV isolates after being vaccinated (Vaccine B) (Okuda, Kuroda et al. 2008) (table 5). It was determined that this vaccine provided a greater weight gain and reduced both lung lesions and clinical signs after the challenge with either of the isolates. As might be expected pigs challenged with the PRRSV isolate that shared more similarity with the vaccine isolate showed a reduction in virus isolation from serum, bronchoalveolar lavage fluid, and tissues while no reductions were seen for these same parameters in the animals challenged with the more genetically unrelated isolate. Other researchers have shown that high passage of PRRSV field isolates in cell culture can make good attenuated live PRRSV vaccines that tend to confer the best protection to pigs subsequently challenged with the low passage parent isolate of the attenuated MLV vaccine (Mengeling, Lager et al. 2003). Nearly all of the animals in this study cleared the low passage parent challenge isolate while clearance of the other challenge isolates varied, with some variation even among the pigs within the same treatment group. Also supporting this evidence is a study conducted with a prototype Chinese PRRSV MLV vaccine that was shown to provide protection to vaccinated animals from a challenge with the virulent parent isolate of the vaccine (Tian, Zhou et al. 2008), (Tian, An et al. 2009).

The cross-protective immunity provided by PRRSV MLV vaccination has been evaluated in the respiratory model with mixed outcomes. Generally the results of the studies
indicate that there is some degree of cross-protection provided by one type of PRRSV to the other. In a single study both a NA PRRSV MLV vaccine and a EU PRRSV MLV vaccine were evaluated for efficacy against three different virulent EU PRRSV challenge isolates (van Woensel, Liefkens et al. 1998). It was determined that the EU PRRSV MLV vaccine provided the greatest level of protection with no viremia in the group challenged with the German PRRSV isolate and low levels of viremia in the Dutch and Spanish isolate challenged groups. The NA PRRSV MLV vaccine on the other hand only provided a significant reduction in viremia for the pigs challenged with the Spanish isolate. Two PRRSV MLV vaccines, one NA and the other EU, were again evaluated side by side for virological protection of the lung after a challenge with the LV isolate (Labarque, Van Gucht et al. 2003). The number of pigs infected and the levels of viremia were reduced more for the homologous vaccine (EU), but the lung protection was not complete even for this group. The vaccination (Vaccine D) of piglets provided protection from viremia in a homologous EU PRRSV challenge, but not complete protection from viremia in a heterologous NA PRRSV challenge (Thanawongnuwech, Panyathong et al. 2006) (table 5). The vaccination induced the production of VN antibodies to the vaccine isolate, but not to either of the challenge isolates. The vaccination (Vaccine B) of animals has been shown to provide some protection from subsequent challenge with an EU type PRRSV isolate in the respiratory model (Nodelijk, de Jong et al. 2001) (table 5), (Roof, Gorcyca et al. 2000) (table 5).

The use of EU type PRRSV MLV vaccines for evaluating the protection of pigs from respiratory disease has provided insight into the cell mediated response of the host in addition to revealing the variability in the protection provided from various challenge isolates. An in depth examination of the host immune response, focusing on cytokine expression and cell
stimulation as measured by RT-PCR and flow cytometry, after vaccination (Vaccine D) showed that no significant changes in cytokines or in CD4 and CD8 markers could be found (Sipos, Duvigneau et al. 2003) (table 5). Also, a similar CD8 single positive T cell curve could be seen for all vaccinated animals, showing an initial decrease followed by an increase between 22 and 40 days post vaccination with no increase in INF-\(\gamma\). Although this data is interesting at an academic level it would be much more valuable if it were correlated with protection from a virulent challenge. In a respiratory study of vaccination (Vaccine D) the efficacy against challenge with either a closely related isolate or a less related isolate was tested (Labarque, Reeth et al. 2004) (table 5). No virus was detected in the vaccinated group after challenge with the closely related isolate, but after challenge with the less related isolate, virus was detected at levels that were significantly lower than the unvaccinated challenged animals. A vaccination (Vaccine D) study evaluating the protective efficacy provided to a closely clustered virus found that after challenge, all vaccinated animals still showed viremia during at least one sampling day, but the viremia seemed to be more sporadic in the vaccinated than in the un-vaccinated group (Prieto, Alvarez et al. 2008) (table 5). Challenge virus was detected in the tissues of four of five animals in the vaccinated group, and in all of the un-vaccinated animals. Since the vaccine was not completely protective despite the high degree of genetic homology in the ORF5 gene between the MLV and challenge virus it was concluded that ORF5 homology might not be a good indicator of protection. In a later study two EU type PRRSV MLV vaccines were evaluated for their immunological properties and for protection from a subsequent virulent challenge (Diaz, Darwich et al. 2006). After vaccination, the animals had vaccine viremia, but did not develop VN antibodies, and once again the frequency of INF-\(\gamma\) secreting cells remained low. After
challenge with a virulent EU type PRRSV isolate one of the vaccines was shown to provide protection from viremia while the other vaccine did not. The vaccination (Vaccine D or Vaccine G; table 1) of pigs has also been shown in other studies to provide some protection from a subsequent EU PRRSV isolate challenge (Gozio and Woensel 2006) (table 5) and (Zuckermann, Garcia et al. 2007) (table 5) respectively.

Therapeutic PRRSV MLV vaccination of animals, after exposure to virulent NA or EU PRRSV, is a topic of research that looks to mimic the conditions found in a PRRSV positive production setting. Pigs infected with a virulent PRRSV isolate (MN-30100) were vaccinated (Vaccine B) and subsequently challenged with a different highly virulent PRRSV isolate (Cano, Dee et al. 2007) (table 5 & table 7). The vaccination of these infected animals provided for a reduction in the duration of shedding but not in the overall viral load or the proportion of persistently infected animals. The results of the challenge indicated that vaccination provided for a significant reduction in clinical signs and enhanced weight gain, but no prevention of infection or shedding. In a well controlled setting, vaccine (Vaccine B) was administered after a wild-type VR2332 PRRSV infection and was able to reduced the duration of viral shedding and provided some protection from challenge with a highly virulent isolate (Cano, Dee et al. 2007) (table 5 & table 7).

8.3 Respiratory field efficacy

A field study was conducted using 10,000 six-week-old pigs vaccinated (Vaccine D) on a farm known to be PRRSV positive demonstrated that the vaccinated pigs outperformed the unvaccinated pigs by becoming ill less often and suffering less mortality (Mavromatis, Kritas et al. 1999) (table 5). The vaccinated pigs also showed better feed conversion resulting in a higher daily rate of gain. In another vaccination (Vaccine D) field study it was shown
that the vaccine provided a significant reduction in clinical signs after a challenge with a virulent EU PRRSV isolate of about 85% ORF5 sequence homology to the vaccine (Martelli, Gozio et al. 2009) (table 5). Efficacy in the field was evaluated by pig performance from birth through fattening and indicated that the vaccine (Vaccine D) provided limited efficacy with no differences in morbidity during the nursery or finisher phases, but significant reductions in morbidity were seen in the vaccinated animals during the grower phase (Kritas, Alexopoulos et al. 2007) (table 5). An across the board significant improvement in mortality for all ages of vaccinated pigs was observed and when compared to the unvaccinated pigs from unvaccinated sows, a significant improvement in the daily rate of gain was also seen for vaccinated animals.

8.4 Laboratory reproductive efficacy

The reproductive efficacy has been evaluated extensively for both EU and NA isolates of PRRSV MLV vaccines in laboratory studies with varying levels of protection that is generally accepted a being better than the protection afforded by inactivated PRRSV vaccines. For example, when sows were vaccinated with either of two different NA PRRSV MLV vaccines they were better protected then sows vaccinated with an inactivated autogenous vaccine as indicated by increased piglet viability after a virulent PRRSV challenge (Osorio, Zuckermann et al. 1998). Vaccinated (Vaccine E or Vaccine G) pregnant sows produced more live born piglets and more weaned piglets then did the unvaccinated sows after a virulent challenge of all the animals (Scortti, Prieto et al. 2006) (table 6). The efficacy of an attenuated PRRSV isolate was found to be incomplete after it provided a reduction of piglet death loss in the vaccinated/challenged group, compared to the unvaccinated/challenged group, but also an increase in piglet death loss as compared to the
unvaccinated/unchallenged group (Mengeling, Lager et al. 1999). Vaccinated (Vaccine B) and unvaccinated pregnant sows were exposed to a mixture of 20 virulent PRRSV isolates showed that eight of the 20 isolates were found in the unvaccinated sows whereas two of the twenty isolates were recovered from the vaccinated sows. The two isolates found in the vaccinated sows were also among the eight isolates found in the unvaccinated animals. The study concluded that maternal immunization puts some selective pressure on a PRRSV challenge, and that this pressure is insufficient to prevent transplacental infection, and that the 20 challenge isolates used in the study must have differed in their ability to replicate in vivo and differed in their capability to cross the placental barrier in the sows (Lager, Mengeling et al. 2003) (table 6). The vaccination of three individual gilts each with one of three vaccines (Vaccine D, Vaccine E, or Vaccine G) resulted in the production of VN antibodies not only to the vaccine isolate but also to many other isolates with titers varying from those accepted as being protective to levels barely detectable (Mogedas, Martínez-Lobo et al. 2008). With such a small group size and the lack of a proper virulent challenge of the animals in this study it is impossible to make any conclusions on the potential protection provided from the VN antibodies elicited by the vaccines tested. A PRRSV MLV vaccine was evaluated off label to vaccinate boars and it was shown that the vaccine virus was in fact shed in semen, but for a shorter duration than virulent virus (Christopher-Hennings, Nelson et al. 1997). Vaccination of the boars reduced, but did not always eliminate the shedding of PRRSV after a subsequent virulent challenge and also seemed to negatively impact the quality of semen leading to the conclusion that the decision to use the MLV vaccine in boars should be made with caution and is not recommended.
Studies evaluating the reproductive cross protection provided by NA PRRSV MLV vaccination to heterologous challenge have shown that there are varying degrees of cross protective efficacy. The vaccination (Vaccine B) of animals has repeatedly provided protection from subsequent virulent NA type PRRSV challenge, but provides incomplete protection from challenge with EU type PRRSV isolates in the reproductive model (Glávits, Medveczky et al. 2002), (Medveczky, Kulcsár et al. 2002) (table 6), (Canals, Sánchez et al. 2000) (table 6), (Roof, Gorcyca et al. 2000) (table 6).

8.5 Reproductive field efficacy

Field studies have been done which focus on the protection of both sows and their piglets from PRRS using MLV vaccines with mixed results. A placebo controlled field study of a EU PRRSV MLV vaccine in sows and gilts was performed to test the efficacy of the vaccine in pregnant animals (Pejsak and Markowska-Daniel 2006). The vaccinated animals had significant reductions in abortions and stillborn pigs as well as significant increases in live born pigs and weaned pigs per sow when compared to the unvaccinated animals, which contradicts the results of earlier negatively controlled laboratory studies in pregnant animals. Vaccination (Vaccine B) was used to stabilize a herd in China that suffered severe reproductive losses and sow deaths after PRRSV introduction leading to a reduction in stillborn and weak born piglets as well as reductions in death losses after weaning (Guo 2008). Vaccination (Vaccine D) of an endemically PRRSV-infected herd resulted in a significantly increased farrowing rate and fewer returns to oestrus as well as more live born and weaned pigs in the vaccinated animals as compared to the unvaccinated animals (Alexopoulos, Kritas et al. 2005) (table 6).
8.6 Respiratory safety

The safety of PRRSV MLV vaccination has been evaluated in the respiratory model and the indications are that the vaccines pose only limited risk of reversion to virulence (Mengeling, Lager et al. 2003). Studies looking at not only safety but also efficacy in the in the respiratory model evaluating both vaccination (Vaccine B) or a multiple isolate PRRSV MLV vaccine prototype found that the vaccines performed similarly in regard to efficacy. However, the observation of enlarged lymph nodes in the unchallenged multiple isolate vaccinated group brought the safety of that vaccine into question. A study focused on the possibility for reversion to virulence of a vaccine isolate of PRRSV found that in some herds the PRRSV vaccine isolate may in fact persist long enough to mutate over time (Mengeling, Vorwald et al. 1999).

8.7 Reproductive safety

PRRSV MLV vaccine safety has been a topic of research with some evidence of virulent vaccine-like isolates appearing in herds that were not actively vaccinating. Concern was raised in Denmark regarding swine herds that were suffering from the reproductive form of PRRSV caused by isolates that were very similar to vaccine (Vaccine B), and it was claimed that the new isolates were derived from that vaccine isolate (Botner, Strandbygaard et al. 1997). Again, in Denmark the investigation of vaccination (Vaccine B) raised questions about the safety of MLV vaccination. Similarities between some field isolates and vaccine isolates (Vaccine B) from which they were believed to have originated, supported the conclusion that several mutations in the ORF1 gene may have regenerated the genotype of the parent isolate in the vaccine isolate leading to the new more virulent field isolates (Nielsen, Oleksiewicz et al. 2001). An interesting observation was made in a swine herd that
utilized two different MLV vaccines at separate time points in an attempt to control PRRSV on a farm which was positive for PRRSV before beginning the vaccination program (Kiss, Sami et al. 2006). A number of post-vaccination new-outbreak isolates had genetic similarities to one of the vaccine isolates suggesting that the new isolates might have originated from the vaccine.

Forty-seven swine herds in Canada and the midwestern United States set out to determine if the use of a PRRSV MLV vaccine was safe in gestating sows (Dewey, Wilson et al. 1999). The results indicated that sows vaccinated at any point during pregnancy had a reduced number of live born and weaned pigs with an increase in the number of stillborn and mummified pigs when compared to the unvaccinated sows. The largest litter losses were observed in the sows vaccinated in the last four weeks of gestation, but the ultimate conclusion of the study remained that PRRSV MLV vaccines should only be administered to non-gestating animals. The reproductive parameters of sows after an initial vaccination with a NA PRRSV MLV showed that when the vaccine was administered during gestation it seemed to have contributed to a reduction in live born and weaned pigs also indicating that PRRSV MLV vaccines should only be administered to non-gestating animals (Dewey, Wilson et al. 2004).

In contradiction to the previously mentioned studies some researchers have shown the safety of vaccination with PRRSV MLV vaccines. After being administered to naïve gilts on gestation day 60 a PRRSV MLV vaccine was found to be safe, with levels of piglet death loss in the vaccinated/unchallenged group similar to the unvaccinated/unchallenged group (Mengeling, Lager et al. 1999). Vaccination (Vaccine E or Vaccine G) has been shown to have no significant detrimental effects in pregnant gilts even though the vaccine isolates were
able to cross the placental barrier and infect the piglets (Scortti, Prieto et al. 2006). The reasons for the discrepancies between these studies and the ones mentioned previously could be attributed to any number of factors, but likely the problems can be found in the group sizes used.

8.8 Novel applications and approaches to MLV vaccination

New methods of producing MLV vaccines, the co-administration of probiotic bacteria with MLV vaccines, as well as the route of delivery for MLV vaccines have also been evaluated for the potential to provide enhanced efficacy. The growth of MLV vaccines (Vaccine H; table 1) in the ZMAC-1 cell line resulted in a significant reduction of viremia over the same vaccine grown the traditional way on MARC-145 cells after a virulent PRRSV challenge (Calzada-Nova, Husmann et al. 2008). The probiotic bacteria *Lactobacillus casei*, when administered orally in conjunction with PRRSV vaccination (Vaccine B) was found to be able to provide a significant increase in body weight gain when compared to the vaccinated only animals (Kritas and Morrison 2007). The probiotic, although very promising from the producers point of view for increased weight gain during PRRSV infection, was unable to provide any benefits over the MLV alone for reductions in the level or duration of viremia. No differences in the safety or the efficacy were observed for vaccination (Vaccine D), evaluated by the intradermal vaccination route as compared to the traditional intramuscular route of vaccine delivery (Cordioli, Alborali et al. 2004), (Drexler, Laar et al. 2006), (Martelli, Cordioli et al. 2007). Protection from viremia was not complete with the vaccinated pigs being positive for viremia after challenge, but reductions were seen in both the level and the duration of viremia as compared to the unvaccinated challenged animals.
9. PRRSV Wild Type Exposure

9.1 Background

Wild type PRRSV exposure is one of the more controversial strategies used by producers to protect their swine herds from PRRS. In its simplest form, this strategy consists of swine being intentionally exposed to a wild type PRRSV isolate, usually the predominate isolate found on the farm where the animals will be housed. The general theory behind this approach is based on the idea that the potential benefits of true homologous protection outweigh the potential losses associated with deliberately spreading the disease among a herd. This strategy takes on many forms, from simply commingling known positive pigs with naïve pigs, to more advanced methods of harvesting serum from an individual animal that was intentionally exposed to PRRSV, then injecting that animals virus containing serum into other swine. The strategy has evolved over time to become more complex and organized as it has gained following in the industry. It is currently one of the most popular strategies used in the United States (US) today and is likely second only to commercial vaccines for doses applied to swine. This strategy has intrinsic risks associated with its use including but not limited to the complete lack of government regulation, the lack of adequate quality controls, the probability of transmitting microorganisms other than PRRSV to naïve pigs, introduction of PRRSV clinical disease to a herd, the potential for PRRSV mutation or genetic drift, and the accidental exposure of neighboring farms to virulent PRRSV. Because of these risks and the risk of introducing a new PRRSV isolate into a herd, most production farms that use virulent exposure programs close their herds to replacement animals. Another drawback of live virus exposure is that the numbers of controlled laboratory studies specifically exploring the protection provided by this strategy are very limited, with much of the published research
available for this strategy being from field studies on farms that already have PRRSV problems. Additional issues that further confound the review of this strategy are the lack of any established standard dose and the lack of uniform methods for applying that dose. Perhaps the biggest two issues to consider for this strategy are the vast differences among the farm specific isolates used for immunizations along with the flawed hypothesis that each farm has only one isolate to protect from. This idea of one farm one isolate is disproved by the evidence for quasispecies, which would suggest that there are more than one isolate per pig let alone per farm. Because of these issues comparisons of studies to one another are only qualitative at best.

9.2 Various virulence of wild type PRRSV

Controlled laboratory studies have been done to examine the virulence of many different PRRSV isolates as well as the same isolate at different inoculation levels. Other studies have examined the transmission or clearance of PRRSV isolates from the host after a wild type exposure. Inoculation of naïve animals with one of nine different PRRSV isolates showed that differences existed in the abilities of the viral isolates to induce clinical signs, cause fever, and induce both macroscopic and microscopic lung lesions (Halbur, Paul et al. 1996). A later study conducted in piglets using different wild-type (low passage), attenuated (high passage), or vaccine (USDA licensed) PRRS viruses at a low standardized dose replicated these results (Johnson, Roof et al. 2004). The study went further to show that viremia of varying lengths of time, and magnitudes could be statistically correlated to a corresponding difference in the humoral immune response of the piglets. To examine the idea that viremia levels impact disease, pigs were challenged with the same wild-type virus isolate at two different doses (Loving, Brockmeier et al. 2008). The goal was to determine if there
were differences in the hosts immune response to the same isolate at different inoculation doses. The outcome of this research showed that the inoculating dose of challenge virus did not significantly affect the level of viral RNA in the serum or the transcription of INF-γ, but it did affect the cell infiltration of the lung and the body temperature.

Evaluations of wild type isolates from around the world describe vastly different levels of virulence. A highly virulent PRRSV isolate in China, which is characterized by two noncontiguous deletions in the Nsp2 gene was experimentally tested by exposing two-month-old pigs to the virus (Zhou, Hao et al. 2008). The resulting clinical signs were similar to those observed in the field with the death of all pigs by day 21 post-infection. The prevalence of this type of PRRS is extensive in China with reports of the disease in 20 provinces, with 56 field isolates being cataloged. At the opposite end of the spectrum a Chilean isolate of PRRSV was examined by exposing age-matched naïve pigs to one-month-old pigs previously infected with the wild-type PRRSV isolate for a specific and controlled amount of time (Ramirez, Moreno et al. 2008). It was observed that none of the animals in the study showed clinical signs with the exception of transient pyrexia. Viremia was detected in all infected as well as all contact exposed animals. In the sows exposed during late gestation a higher number of congenitally-infected and dead born piglets were seen, while sows exposed during mid gestation did not show the congenitally infected piglets leading to the conclusion that timing of virus exposure is an important consideration. The same results were again observed after a reproductive wild-type PRRSV exposure trial conducted in pregnant sows found that a challenge at day 90 of gestation was more severe than the same challenge at day 45 of gestation (Mengeling, Lager et al. 1998). These differences in the challenge severity were similar to those found by Christianson et al. in 1993 with an increased likelihood of the
virus to cross the placenta and cause adverse affects at day 90 of gestation as compared to
day 45 of gestation resulting in more fetal death after a gestation day 90 challenge.

9.3 Intentional exposure to wild type PRRSV

The deliberate inoculation of naïve or sero-positive pigs with wild type isolates of
PRRSV has resulted in several different outcomes. There is some evidence to support the
idea that exposing swine to a virulent PRRSV isolate can provide up to a lifetime protection
from a subsequent exposure to the same isolate (Lager, Mengeling et al. 1997). An
evaluation of wild type PRRSV exposure as a means to stabilize sow herds concluded that
the strategy “may have a place in some multiple site production systems” (Wagner 2005).
Wagner explained the use of wild-type exposure in two scenarios; lingering clinical signs
after a new isolate introduction to a farm or subpopulations of susceptible animals after a
severe clinical break. In both of these examples abortions were observed after the sows were
exposed to the wild-type PRRSV isolates, but to a lesser extent than those seen after the
initial PRRSV break. A different examination focused on wild-type PRRSV inoculation of
both naïve and previously exposed pigs and showed a significant reduction in lung lesions
after exposure to a new virulent PRRSV challenge (Opriessnig, Baker et al. 2007) (table 7).
The study also showed that exposure of pigs to wild type PRRSV isolates at either a low
dosage or a high dosage resulted in a significantly reduced daily weight gain. The use of a
wild type PRRSV inoculation for acclimation of PRRSV-naïve gilts to a farm specific
PRRSV isolate was achieved by intramuscularly injecting serum from selected wild-type
PRRSV positive animals (Fano, Olea et al. 2005). Infections among pigs on that farm were
subsequently eliminated from within the fully segregated flow, but PRRSV was never
eliminated from the partially segregated flow at the same farm. Again the acclimation of
PRRSV-naïve pigs on an endemic farm was accomplished, this time by commingling intentionally wild-type PRRSV inoculated and uninoculated age matched pigs at 6.5 weeks of age and at 10.5 weeks of age (Vashisht, Erlandson et al. 2008). The farm evaluating this method saw a slight improvement in the average number of pigs weaned only after it was depopulated, cleaned, and disinfected. The inoculation of sows with sera containing wild-type PRRSV for the first time on a farm resulted in an acute reproductive failure with about 600 abortions and even a few sow deaths, but once the herd was stabilized, pigs free of PRRSV were again weaned (Bruner 2007). A subsequent unexpected PRRSV break occurred on this same farm after a second round of sow inoculations took place with sera containing the same wild type PRRSV isolate. A study comparing wild type exposure to MLV vaccination demonstrated an increased level of VN antibodies and an earlier generation of INF-γ secreting cells in sows after wild type exposure as compared to the MLV vaccination, but a mean increase of 2.45 pigs per litter in the MLV group over the wild type exposure group was also observed (Lowe, Zuckermann et al. 2006). This increase in the pigs per litter was attributed mainly to the increased rate of conception failure in the wild type PRRSV exposed group.

The intentional inoculation of swine with wild type PRRSV is to say the least risky, and without the publication of more controlled laboratory studies examining the use of this strategy it becomes more difficult to differentiate between the attempted protection from PRRS and its cause. If this strategy is ever to be better understood, properly controlled laboratory studies will need to be conducted in which many of the complicating factors associated with field studies can be eliminated. Until these studies are done this strategy will remain an enigma as to its true potential for immunizing swine against PRRSV.
10. Conclusion

Killed PRRSV vaccines have the obvious advantage of safety, but the efficacy of these vaccines is poor with only limited protection demonstrated in rare cases. Since killed PRRSV vaccination is clearly safe, efforts in the development of improved killed vaccines would best focus on increasing efficacy. Adjuvants will likely to be the best option for this improvement, but other options that may provide adjuvant-like effects would be supplementation of the killed PRRSV vaccine with the addition of purified PRRSV antigens or combination vaccines including antigens that may help enhance the immune response to the PRRSV component within the vaccine.

Again, as with killed vaccines, subunit vaccines for PRRSV are very safe, but they currently lack adequate efficacy to be viable vaccine candidates. Subunit vaccines have been traditionally focused on the PRRSV structural proteins and more specifically the Gp5 protein. The further investigation of the non-structural proteins for use as antigens is warranted. The focus of subunit PRRSV vaccine research should be the development of a more efficacious subunit vaccine with perhaps a more broadly applied approach than has been used in the past. Incorporation of both structural and non-structural antigens into new vaccine prototypes may provide an added benefit to subunit vaccination.

Both DNA and vector vaccines for PRRSV have the potential to be safe but seem to again provide incomplete protection. Only limited ranges of antigens have been evaluated using these methods so perhaps the use of different PRRSV antigens or combinations of antigens would provide increased efficacy. Another approach for the DNA and vector vaccinations could be the use of a prime-boost vaccination method. This method is often employed in cancer vaccination studies where a purified protein subunit vaccine is used to
prime the immune response of the individual, which is then followed by a boost with either a DNA vaccine or vector vaccine that is designed to express the same protein that was used in the prime. Perhaps broader ranges of either DNA vaccination plasmids or vector platforms need to be evaluated using the same PRRSV antigens that have already been evaluated in previous studies.

The safety and efficacy of PRRSV vaccines generated using infectious cDNA clones are likely comparable to a traditional MLV vaccine, but it is plausible that both could be improved over a traditional PRRSV MLV vaccine with proper design. The potential for the inclusion of specific DIVA characteristics in a PRRSV cDNA clone for use as a vaccine is intriguing, but needs to be tested for efficacy from a virulent challenge and if the DIVA site can be maintained after *in vivo* passage. If such a DIVA PRRSV vaccine were generated, shown to be safe and efficacious, and was subsequently licensed it is likely that it would quickly become the preferred method of PRRSV vaccination for many producers.

The commercially available traditional PRRSV MLV vaccination strategy has been shown to nearly always outperform other strategies when evaluated in a head to head challenge study. PRRSV MLV vaccination strategies may not work flawlessly against every PRRSV isolate every time they are applied, but after this review it is clear that this strategy is the best commercial option available to swine producers relative to a balance of safety and efficacy.

At best, both the safety and efficacy of exposing pigs to wild type PRRSV is questionable and should only be used after careful consideration. This strategy not only produces clinical disease, and reductions in weight gain, but also abortions and death of sows in some more severe cases. Both the variability among the isolates tested and the variability
in the standards used for evaluation of this strategy are troublesome for comparing studies to one another. However, better comparisons could be made if whole genome sequence information were to be provided for the isolates used in a study and if a standard set of evaluation criteria was established for the presentation of data on wild-type-exposure. Furthermore, if more controlled laboratory style challenge studies were applied to the evaluation of the wild-type exposure strategy as opposed to the more traditional field challenge studies currently employed, more meaningful and more broadly applicable comparisons between studies of both this strategy and others could be made. The fact that even a wild type exposure does not provide broad immunity to PRRSV illustrates the scope of the challenge faced in the development of a broadly efficacious vaccine.

While writing this review, it became very evident that there was a great need for standardization among all PRRSV researchers, not only for the study designs used to evaluate prototype PRRSV vaccines, but also for the primary parameters that should be included in all studies. In order to keep the development of PRRSV vaccines moving forward it is very important to remind all PRRSV vaccine researchers about a few very good suggestions, but one in particular could be considered the most important of all; always include a group vaccinated with a licensed commercial PRRSV vaccine in all study designs to serve as a direct comparison of the prototype vaccine being tested to something available in the marketplace today (Murtaugh, Dee et al. 2007). While examining the tremendous body of work published on PRRSV protection strategies it was absolutely astonishing to find that so much has already been tested and learned about PRRSV since its classification, yet so little is truly known about protection from PRRSV. Simply put, PRRSV has been, is now,
and will continue to be for at least the near future, one of the most significant and elusive pathogens researched in swine health.

Acknowledgements

The author would like to thank Dr. Eric Vaughn, Dr. Michael Roof, Dr. James Roth, Dr. Hank Harris, and Dr. Jeffery Zimmerman for their valuable insight and critical reviews of this manuscript.
### Table 1. Commercial PRRSV vaccines.

<table>
<thead>
<tr>
<th>Vaccine Trade Name</th>
<th>Manufacturer</th>
<th>Vaccine Isolate Type</th>
<th>Vaccine Category</th>
<th>Vaccine Designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRRomiSe®</td>
<td>Intervet</td>
<td>Type II</td>
<td>Killed</td>
<td>Vaccine A</td>
</tr>
<tr>
<td>Ingelvac® PRRS MLV</td>
<td>Boehringer Ingelheim</td>
<td>Type II</td>
<td>Modified Live</td>
<td>Vaccine B</td>
</tr>
<tr>
<td>PROGRESSIS®</td>
<td>Merial</td>
<td>Type I</td>
<td>Killed</td>
<td>Vaccine C</td>
</tr>
<tr>
<td>Porcilis® PRRS</td>
<td>Intervet</td>
<td>Type I</td>
<td>Modified Live</td>
<td>Vaccine D</td>
</tr>
<tr>
<td>Amervac® PRRS</td>
<td>Hipra</td>
<td>Type I</td>
<td>Modified Live</td>
<td>Vaccine E</td>
</tr>
<tr>
<td>Ingelvac® PRRS ATP</td>
<td>Boehringer Ingelheim</td>
<td>Type II</td>
<td>Modified Live</td>
<td>Vaccine F</td>
</tr>
<tr>
<td>Pyrsvac-183®</td>
<td>SYVA Laboratories</td>
<td>Type I</td>
<td>Modified Live</td>
<td>Vaccine G</td>
</tr>
<tr>
<td>PrimePac® PRRS</td>
<td>Intervet</td>
<td>Type II</td>
<td>Modified Live</td>
<td>Vaccine H</td>
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</table>
Table 2. PRRSV killed virus vaccination and subsequent respiratory challenge.

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Vaccination</th>
<th>Challenge</th>
<th>Results</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a</td>
<td>b</td>
<td>c</td>
<td>d</td>
</tr>
<tr>
<td>Vaccine A</td>
<td>N</td>
<td>10</td>
<td>IM</td>
<td>2</td>
</tr>
<tr>
<td>Prototype</td>
<td>N</td>
<td>0.5</td>
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<td>1</td>
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<tr>
<td>Vaccine C</td>
<td>N</td>
<td>10</td>
<td>IM</td>
<td>2</td>
</tr>
<tr>
<td>Vaccine C</td>
<td>E</td>
<td>6</td>
<td>IM</td>
<td>2</td>
</tr>
<tr>
<td>Vaccine C</td>
<td>N</td>
<td>6 to 8</td>
<td>IM</td>
<td>2</td>
</tr>
<tr>
<td>Vaccine C</td>
<td>N</td>
<td>20</td>
<td>IM</td>
<td>2</td>
</tr>
<tr>
<td>n/a</td>
<td>N</td>
<td>5</td>
<td>IM</td>
<td>1</td>
</tr>
</tbody>
</table>

*Sero-status (N = naïve, E = exposed), †Animal age (weeks), ‡Route of vaccination (IM = intramuscularly), §Number of vaccinations, ¶Challenge virus, ‖Challenge timing (Study day), ‖Lung lesions, ‖Viremia, ‖VN antibody, n.d. = Not determined, ↔ = Not significant, ↑ = Significant increase, ↓ = Significant decrease, n/a = Not available.
Table 3. PRRSV killed virus vaccination intramuscularly and subsequent reproductive challenge.

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Vaccination</th>
<th>Challenge</th>
<th>Results</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autogenous</td>
<td>N gilts</td>
<td>3 IA-1-4-2</td>
<td>↑ ns</td>
<td>(Osorio, Zuckermann et al. 1998)</td>
</tr>
<tr>
<td>CyBlue®</td>
<td>N boars</td>
<td>2 18794/93</td>
<td>n.d. n.d.</td>
<td>(Nielsen, Nielsen et al. 1997)</td>
</tr>
<tr>
<td>Vaccine C</td>
<td>E sows</td>
<td>Field</td>
<td>↑ ↑ n.d.</td>
<td>(Papatsiros, Koptopoulos et al. 2004)</td>
</tr>
<tr>
<td>Suvaxyn®PRRS</td>
<td>N gilts</td>
<td>2 2156</td>
<td>↔ ↑ ↔ n.d.</td>
<td>(Scortti, Prieto et al. 2007)</td>
</tr>
</tbody>
</table>

*Sero-status (N = naïve, E = exposed), *Animal age, *Number of vaccinations, *Challenge virus, *Field = Exposure to the PRRSV isolate(s) on the test farm, *Challenge timing (Gestation day(s)), *Challenge timing (Study day(s)), *Live born, *Live at wean, *Viremia, n.d. = Not determined, ↔ = Not significant, ↑ = Significant increase, ↓ = Significant decrease, n/a = Not available, ns = in conjunction with an arrow specifies a change but no statistical difference was indicated.
Table 4. PRRSV prototype vaccination and subsequent respiratory challenge.

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>d</th>
<th>e</th>
<th>f</th>
<th>g</th>
<th>h</th>
<th>i</th>
<th>References</th>
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<tr>
<td>Subunit</td>
<td>N</td>
<td>1</td>
<td>IM</td>
<td>2</td>
<td>ND-1</td>
<td>42</td>
<td>↓</td>
<td>ns</td>
<td>↓</td>
<td>n.d. (Liao, Lin et al. 2004)</td>
</tr>
<tr>
<td>Subunit</td>
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<td>2</td>
<td>IM</td>
<td>2</td>
<td>MD-1</td>
<td>42</td>
<td>↓</td>
<td>ns</td>
<td>↓</td>
<td>n.d. (Liao, Yang et al. 2006)</td>
</tr>
<tr>
<td>Subunit</td>
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<td>4</td>
<td>IM</td>
<td>2</td>
<td>TC-01</td>
<td>42</td>
<td>n/a</td>
<td>↓</td>
<td>n.d. (Liao, Weng et al. 2008)</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>N</td>
<td>3</td>
<td>IM</td>
<td>2</td>
<td>IAF-Klop</td>
<td>51 &amp; 65</td>
<td>↓</td>
<td>ns</td>
<td>↓</td>
<td>ns</td>
</tr>
<tr>
<td>DNA</td>
<td>N</td>
<td>3</td>
<td>ID</td>
<td>1</td>
<td>IAF-Klop</td>
<td>51 &amp; 65</td>
<td>↓</td>
<td>ns</td>
<td>↓</td>
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<td>3</td>
<td>IM</td>
<td>2</td>
<td>ND</td>
<td>-</td>
<td>n.d.</td>
<td>n.d.</td>
<td>↔</td>
<td>(Jiang, Xiao et al. 2006)</td>
</tr>
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<td>IM</td>
<td>3</td>
<td>Ch-1a</td>
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<td>↓</td>
<td>ns</td>
<td>↓</td>
<td>ns</td>
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<td>IM</td>
<td>3</td>
<td>YA1</td>
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<td>↓</td>
<td>ns</td>
<td>↓</td>
<td>↑</td>
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<td>n.d.</td>
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<td>3</td>
<td>IM</td>
<td>2</td>
<td>ND</td>
<td>-</td>
<td>n.d.</td>
<td>n.d.</td>
<td>↑</td>
<td>(Li, Xiao et al. 2009)</td>
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<td>6</td>
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<td>↑</td>
<td>ns</td>
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<td>IM</td>
<td>3</td>
<td>5710</td>
<td>56</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>(Diaz, Domínguez et al. 2006)</td>
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<td>IM</td>
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<td>BS/114/L/2000</td>
<td>56</td>
<td>n.d.</td>
<td>↓</td>
<td>ns</td>
<td>↔</td>
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<td>c</td>
<td>d</td>
<td>e</td>
<td>f</td>
<td>g</td>
<td>h</td>
<td>i</td>
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<td>16244B</td>
<td>60</td>
<td>n.d.</td>
<td>▼ ns</td>
<td>▲ ns</td>
<td>(Bastos, Dellagostin et al. 2004)</td>
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<td>IN&amp;IM</td>
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<td>5710</td>
<td>0</td>
<td>n.d.</td>
<td>▼ ns</td>
<td>▲ ns</td>
<td>(Álvarez, Prieto et al. 2004)</td>
</tr>
<tr>
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<td>Ch-1a</td>
<td>30</td>
<td>▼ ns</td>
<td>▼ ns</td>
<td>↔</td>
<td>(Qiu, Tian et al. 2005)</td>
</tr>
<tr>
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<td>N</td>
<td>4</td>
<td>IM</td>
<td>2</td>
<td>YA1</td>
<td>30</td>
<td>▼ ns</td>
<td>▼ ns</td>
<td>▲ ns</td>
<td>(Jiang, Fang et al. 2007)</td>
</tr>
<tr>
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<td>N</td>
<td>3</td>
<td>IM</td>
<td>2</td>
<td>SY0608</td>
<td>42</td>
<td>▼</td>
<td>▼</td>
<td>▲</td>
<td>(Wang, Li et al. 2009)</td>
</tr>
<tr>
<td>Vector</td>
<td>N</td>
<td>3</td>
<td>IM</td>
<td>3</td>
<td>nd</td>
<td>-</td>
<td>n.d.</td>
<td>n.d.</td>
<td>▲</td>
<td>(Rogan, Levere et al. 2000)</td>
</tr>
<tr>
<td>Vector</td>
<td>N</td>
<td>1</td>
<td>IN&amp;IG</td>
<td>3</td>
<td>nd</td>
<td>-</td>
<td>n.d.</td>
<td>n.d.</td>
<td>▲</td>
<td>(Alonso, Sola et al. 2002)</td>
</tr>
<tr>
<td>Vector</td>
<td>N</td>
<td>3</td>
<td>IM</td>
<td>2</td>
<td>Chang Chung</td>
<td>60</td>
<td>n.d.</td>
<td>▼</td>
<td>▲</td>
<td>(Shen, Jin et al. 2007)</td>
</tr>
<tr>
<td>Vector</td>
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<td>3-4</td>
<td>n/a</td>
<td>2</td>
<td>Olot 91</td>
<td>70</td>
<td>▼ ns</td>
<td>▼ ns</td>
<td>↔</td>
<td>(Urniza, Ceriani et al. 2008)</td>
</tr>
<tr>
<td>Clone</td>
<td>N</td>
<td>8</td>
<td>IM</td>
<td>1</td>
<td>SDSU73 or LV</td>
<td>28</td>
<td>n.d.</td>
<td>▼ ns</td>
<td>n.d.</td>
<td>(Verheije, Kroese et al. 2003)</td>
</tr>
<tr>
<td>Clone</td>
<td>N</td>
<td>3</td>
<td>IM or IT</td>
<td>2</td>
<td>P129</td>
<td>42</td>
<td>▼</td>
<td>▼</td>
<td>n.d.</td>
<td>(Welch, Jolie et al. 2004)</td>
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<tr>
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<td>3</td>
<td>IM</td>
<td>1</td>
<td>SDSU73</td>
<td>21</td>
<td>▼</td>
<td>▼ ns</td>
<td>n.d.</td>
<td>(Wang, Liang et al. 2008)</td>
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</table>
Table 4. (continued)

aSero-status (N = naïve, E = exposed), bAnimal age(weeks), cRoute of vaccination (IN = intranasal, IM = intramuscularly, ID = intradermal, GG = gene gun, SC = subcutaneous, IG = intragastric, IT = intratracheal), dNumber of vaccinations, eChallenge virus, fChallenge timing (Study day(s)), gLung lesions, hViremia, iVN antibody, n.d. = Not determined, ↔ = Not significant, ↑ = Significant increase, ↓ = Significant decrease, n/a = Not available, ns = in conjunction with an arrow specifies a change but no statistical difference was indicated.
Table 5. PRRSV modified live virus vaccination and subsequent respiratory challenge.

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>d</th>
<th>e</th>
<th>f</th>
<th>g</th>
<th>h</th>
<th>i</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccine B</td>
<td>N</td>
<td>4</td>
<td>IM</td>
<td>1</td>
<td>wt-7 or wt-11</td>
<td>28</td>
<td>↓</td>
<td>↓&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>n.d.</td>
<td>(Okuda, Kuroda et al. 2008)</td>
</tr>
<tr>
<td>Vaccine E</td>
<td>N</td>
<td>3</td>
<td>IM</td>
<td>2</td>
<td>02SB3</td>
<td>28</td>
<td>n.d.</td>
<td>↓&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>↑</td>
<td>(Thanawongnuwech, Panyathong et al. 2006)</td>
</tr>
<tr>
<td>Vaccine E</td>
<td>N</td>
<td>3</td>
<td>IM</td>
<td>2</td>
<td>01NP1</td>
<td>28</td>
<td>n.d.</td>
<td>↓&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>↑</td>
<td>(Thanawongnuwech, Panyathong et al. 2006)</td>
</tr>
<tr>
<td>Vaccine B</td>
<td>N</td>
<td>4</td>
<td>IM</td>
<td>1</td>
<td>LV variable days</td>
<td>n.d.</td>
<td>↓</td>
<td>n.d.</td>
<td>n.d.</td>
<td>(Nodelijk, de Jong et al. 2001)</td>
</tr>
<tr>
<td>Vaccine B</td>
<td>N</td>
<td>5</td>
<td>IM</td>
<td>1</td>
<td>CDI-NL-2.19</td>
<td>28 or 42</td>
<td>n/a</td>
<td>↓</td>
<td>n.d.</td>
<td>(Roof, Gorcyca et al. 2000)</td>
</tr>
<tr>
<td>Vaccine D</td>
<td>N</td>
<td>6</td>
<td>IM</td>
<td>1</td>
<td>nd</td>
<td>-</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>(Sipos, Duvigneau et al. 2003)</td>
</tr>
<tr>
<td>Vaccine D</td>
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<td>5</td>
<td>IM</td>
<td>1</td>
<td>LV or Italian</td>
<td>49</td>
<td>n.d.</td>
<td>↓</td>
<td>n.d.</td>
<td>(Labarque, Reeth et al. 2004)</td>
</tr>
<tr>
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<td>4</td>
<td>IM</td>
<td>3</td>
<td>5710</td>
<td>0</td>
<td>n.d.</td>
<td>↓</td>
<td>↑&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>(Prieto, Alvarez et al. 2008)</td>
</tr>
<tr>
<td>Vaccine D</td>
<td>N</td>
<td>5</td>
<td>IM</td>
<td>1</td>
<td>I2</td>
<td>35</td>
<td>n.d.</td>
<td>↓</td>
<td>n.d.</td>
<td>(Gozio and Woensel 2006)</td>
</tr>
<tr>
<td>Vaccine G</td>
<td>N</td>
<td>20</td>
<td>IM</td>
<td>2</td>
<td>LV</td>
<td>28</td>
<td>↔</td>
<td>↓</td>
<td>n.d.</td>
<td>(Zuckermann, Garcia et al. 2007)</td>
</tr>
<tr>
<td>Vaccine B</td>
<td>E</td>
<td>6-8</td>
<td>IM</td>
<td>1-3</td>
<td>MN-184</td>
<td>97</td>
<td>n.d.</td>
<td>↓</td>
<td>n.d.</td>
<td>(Cano, Dee et al. 2007)</td>
</tr>
<tr>
<td>Vaccine B</td>
<td>E</td>
<td>6-8</td>
<td>IM</td>
<td>1-3</td>
<td>MN-184</td>
<td>97</td>
<td>n.d.</td>
<td>↓</td>
<td>n.d.</td>
<td>(Cano, Dee et al. 2007)</td>
</tr>
<tr>
<td>Vaccine D</td>
<td>E</td>
<td>6</td>
<td>IM</td>
<td>1</td>
<td>Field</td>
<td>-</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>(Mavromatis, Kritas et al. 1999)</td>
</tr>
<tr>
<td>Vaccine D</td>
<td>N</td>
<td>5</td>
<td>IM/ID</td>
<td>1</td>
<td>Field</td>
<td>45</td>
<td>n.d.</td>
<td>↓</td>
<td>n.d.</td>
<td>(Martelli, Gozio et al. 2009)</td>
</tr>
<tr>
<td>Vaccine D</td>
<td>E</td>
<td>5</td>
<td>IM</td>
<td>1</td>
<td>Field</td>
<td>-</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>(Kritas, Alexopoulos et al. 2007)</td>
</tr>
</tbody>
</table>
Table 5. (continued)

aSero-status (N = naïve, E = exposed), bAnimal age (weeks), cRoute of vaccination (IM = intramuscularly, ID = intradermal),
dNumber of vaccinations, eChallenge virus, Field = Exposure to the PRRSV isolate(s) on the test farm, fChallenge timing (Study
day(s)), gLung lesions, hViremia, iVN antibody, n.d. = Not determined, ↑ = Significant increase, ↓ = Significant decrease, n/a = Not available, ns = in conjunction with an arrow specifies a change but no statistical difference was indicated.
Table 6. PRRSV modified live virus vaccination and subsequent reproductive challenge.

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>d</th>
<th>e</th>
<th>f</th>
<th>g</th>
<th>h</th>
<th>i</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccine E</td>
<td>N</td>
<td>gilts</td>
<td>IM</td>
<td>1</td>
<td>2156</td>
<td>90</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
<td>(Scortti, Prieto et al. 2006)</td>
</tr>
<tr>
<td>Vaccine G</td>
<td>N</td>
<td>gilts</td>
<td>IM</td>
<td>1</td>
<td>2156</td>
<td>90</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
<td>(Scortti, Prieto et al. 2006)</td>
</tr>
<tr>
<td>Vaccine B</td>
<td>N</td>
<td>gilts</td>
<td>IM</td>
<td>2</td>
<td>pool of 20 viruses</td>
<td>90</td>
<td>↑</td>
<td>ns</td>
<td>n.d.</td>
<td>↓ns</td>
</tr>
<tr>
<td>Vaccine B</td>
<td>N</td>
<td>gilts</td>
<td>IM</td>
<td>1</td>
<td>n/a</td>
<td>90</td>
<td>↑</td>
<td>↑</td>
<td>n.d.</td>
<td>(Medveczky, Kulcsár et al. 2002)</td>
</tr>
<tr>
<td>Vaccine B</td>
<td>N</td>
<td>pregnant sows</td>
<td>IM</td>
<td>1</td>
<td>5710</td>
<td>90</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
<td>(Canals, Sánchez et al. 2000)</td>
</tr>
<tr>
<td>Vaccine D</td>
<td>E</td>
<td>gilts &amp; sows</td>
<td>IM</td>
<td>1</td>
<td>Field</td>
<td>-</td>
<td>↑</td>
<td>↑</td>
<td>n.d.</td>
<td>(Alexopoulos, Kritas et al. 2005)</td>
</tr>
</tbody>
</table>

*Sero-status (N = naïve, E = exposed), *b*Animal age, *c*Route of vaccination (IM = intramuscularly), *d*Number of vaccinations, *e*Challenge virus, *f*Challenge timing (Gestation day(s)), Field = Exposure to the PRRSV isolate(s) on the test farm, *g*Live born, *h*Live at wean, *i*Viremia, ↑ = Significant increase, ↓ = Significant decrease, n.d. = Not determined, , n/a = Not available, ns = in conjunction with an arrow specifies a change but no statistical difference was indicated.
Table 7. Initial exposure of naïve swine to wild type PRRSV followed by a subsequent challenge.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Exposure</th>
<th>Challenge</th>
<th>Results</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autogenous</td>
<td>8 IM</td>
<td>VR3285, Day 70</td>
<td>↓ ↓ ↑</td>
<td>(Opriessnig, Baker et al. 2007)</td>
</tr>
<tr>
<td>SDSU-73 &amp; Autogenous</td>
<td>3 &amp; 8 IN</td>
<td>VR3285, Day 70</td>
<td>↓ ↓ ↑</td>
<td>(Opriessnig, Baker et al. 2007)</td>
</tr>
<tr>
<td>MN-30100</td>
<td>6 to 8 IN</td>
<td>MN-184, Day 97</td>
<td>n/a ↓ n.d.</td>
<td>(Cano, Dee et al. 2007)</td>
</tr>
<tr>
<td>VR-2332</td>
<td>6 to 8 IN</td>
<td>MN-184, Day 97</td>
<td>n/a ↓ n.d.</td>
<td>(Cano, Dee et al. 2007)</td>
</tr>
</tbody>
</table>

*aAnimal age (weeks), bRoute of exposure (IN = intranasal, IM = intramuscularly), cLung lesions, dViremia, eVN antibody, ↑ = Significant increase, ↓ = Significant decrease, n/a = Not available, n.d. = Not determined.*
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CHAPTER 3. PATHOGENIC AND HUMORAL IMMUNE RESPONSES TO PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (PRRSV) A RE RELATED TO VIRAL LOAD IN ACUTE INFECTION


Wesley Johnson, Michael Roof, Eric Vaughn, Jane Christopher-Hennings,
Craig R. Johnson, Michael P. Murtaugh

Abstract

Although much research has been performed on porcine reproductive and respiratory syndrome virus (PRRSV), little quantitative information is available on the relationships between virulence and in vivo virus replication, among isolates recovered at different times in the history of PRRS, or the relative levels of virulence associated with individual virus isolates. In this study the in vivo growth properties of virulent field isolates and attenuated PRRSV isolates were compared. The results show that virulent PRRSV isolates exhibit longer and more elevated levels of viremia, induce faster and more intense humoral immune responses, negatively affect body weight gain, induce higher death rates, and cause more severe clinical signs in a respiratory disease model. We found that the more virulent field isolates grew to significantly higher levels in pigs than did cell-culture adapted isolates. We concluded that the pathogenic consequences and immunological responses of pigs to PRRSV are directly related to viral load in acute infection as reflected in viral titers in blood.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped single stranded RNA virus classified in the family Arteriviridae (Cavanaugh, 1997). It causes a widespread disease of swine that was first described as ‘mystery swine disease’ in the USA
in 1987 (Hill, 1990). The disease manifests as respiratory illness in all age groups of swine leading to death in some younger pigs and severe reproductive problems in breeding age females.

The dynamic nature of PRRSV allows for constant change in the disease and provides ample opportunity for the appearance of new strains (Andreyev et al., 1997; Murtaugh et al. 1998; Meng, 2000). The fact that PRRSV changes so readily, coupled with its ability to cause devastating problems for swine producers makes it an important subject for research (Mengeling et al., 1998; Pejsak et al., 1997). Variation in levels of isolate virulence were demonstrated in lung lesions, and death in swine (Halbur et al., 1996), but efforts to link biological and immunological differences to specific genetic differences has been largely unsuccessful (Albina et al., 1998; Key et al., 2001; Yuan et al., 2001; Murtaugh et al., 2002; Grebennikova et al., 2004). Studies examining the safety and efficacy of PRRS vaccines include the work of Labarque et al. (2003), Mengeling et al. (2003a), and Nodelijk et al. (2001). These studies show that under experimental conditions modified live PRRS vaccines reduce the amount and duration of viremia as well as fever and lung lesions after virulent challenge.

Opriessing et al. (2002) showed that isolates with high amino acid sequence homology in open reading frame 5 (ORF5) caused significantly different levels of pneumonia in pigs. Variation in swine responses to PRRSV also are affected by host variation (Mengeling et al. 2003b). Virulence has been examined in relation to replication rates and distribution of PRRSV in pigs (Haynes et al., 1997), to macrophage copper clearing capabilities (Thanawongnuwech et al., 1998), and the anemia levels of the host animal (Halbur et al., 2002). This study expands on the previous work by examining virulent field
isolates, their attenuated vaccine derivatives and highly virulent field isolates collected recently.

The purpose of the present work was to examine the *in vivo* growth characteristics of and antibody responses to eight different PRRSV isolates with various known levels of virulence, so as to establish the relationship between *in vivo* replication of the virus, virulence and antibody response, and to test the hypothesis that *in vivo* replication of PRRSV is a key determinant of virulence and the level of humoral antibody response.

2. Materials and Methods

2.1 PRRS virus isolates

The PRRSV isolates used in this study are shown in Table 1. The isolates were chosen to span the history of PRRS and a range of virulence levels, and to represent relevant clinical disease manifestations. All of the virus isolates used in the study grew readily on CL2621 cells. Three of the primary field isolates also had attenuated forms of low or undetectable virulence that were derived by *in vitro* passage. The PRRSV isolate ATCC VR-2332 was isolated in 1991 in Minnesota and was used at cell culture passage three, the attenuated form of this virus is commercially available under the trade-name Ingelvac® PRRS MLV (Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO). PRRSV SDSU 73 was recovered in Iowa from a severe case of reproductive disease in 1996 and was used at cell culture passage one. The attenuated form of SDSU 73, designated Abst-1, was obtained by 52 passages. The PRRSV isolate 17198-6 was obtained from Oklahoma in 1997 from a herd experiencing severe reproductive disease and was used at passage level four. The PRRSV isolate JA 142, kindly provided by William Mengeling, National Animal Disease Center,
Ames, Iowa, was isolated in 1997 in Iowa from a severe “abortion-storm” case of reproductive failure and was used at cell culture passage five. The attenuated form of JA 142 is commercially sold under the trade-name Ingelvac® PRRS ATP (Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO). The PRRSV MN 184 isolate was obtained in 2001 from a swine farm experiencing severe reproductive disease and sow mortality in southern Minnesota and was provided by Kurt Rossow, University of Minnesota, St. Paul, and was used at a cell culture passage of one. Additionally, a pool of all isolates was produced and each pig in test group 9 was inoculated with two ml containing $10^{3.71}$ total virus/ml. The virulent field isolates are quite distinct and represented a diverse group of PRRSV isolates, whereas the parental and vaccine PRRSV pairs were nearly genetically identical, as shown by the pairwise comparison in Table 2 and the dendrogram in Figure 1. The pairwise comparison and dendrogram were generated using the Lasergene software suite of sequence analysis tools (DNASTAR, Inc, (Madison, WI).

2.2 Study Design

One hundred healthy 2-3 week-old pigs were obtained from a PRRS-free commercial herd and were maintained at Veterinary Resources, Inc., Ames, Iowa, under the supervision of a veterinarian. Animals received food and water ad libitum. All of the animal care and laboratory personnel involved with the study were blinded to the treatments given to the various groups of animals. Pigs were tested negative by HerdCheck® PRRS ELISA 2XR (IDEXX Laboratories Inc. Westbrook, ME) and divided randomly by weight into 10 groups with 10 pigs per group. On day 0 each of the eight PRRSV isolates and the PRRSV pool were diluted to approximately $3.0 \log_{10} \text{TCID}_{50}/\text{ml}$ in Eagle’s Minimum Essential Medium (EMEM) (JRH Bioscience, Lenexa, KS) containing 4% FBS (JRH Bioscience, Lenexa, KS)
and administered intranasally to pigs at a dose of 2 ml (1 ml per nostril). The untreated control group received 2 ml of media. The inocula were retitrated on 96-well plates containing three-day-old CL2621 cells for titer confirmation using the Reed-Muench method (Reed et al., 1938). The observed titers administered to pigs are shown in Table 1.

2.3 Evaluation of Viremia

Blood samples were collected by vacutainer on days 0, 1, 3, 7, 15, 21, 28, 35, 42, and 49. Serum was separated from clotted whole blood by centrifugation at 3200xg for 20 minutes. Serum samples were divided for analysis by virus isolation, Log$_{10}$ TCID$_{50}$/ml, quantitative reverse transcriptase-polymerase chain reaction (RT-PCR), HerdCheck® PRRS ELISA 2XR, and PRRSV protein-specific ELISA. The serum samples in this study were processed immediately after collection and were chilled on ice within 3 hr. The samples were stored for a maximum of 24 hr at 4°C and at -70°C thereafter. Serum tested by RT-PCR was frozen at -70°C the day of collection and stored until the testing could be performed at which time only the number of samples that could be tested within 24 hours were thawed, extracted, and tested.

Virus isolation was performed on three-day-old CL2621 cells (an MA-104 monkey kidney cell line) for samples collected on days 0, 1, 42, and 49. Viral titration was performed on days 3, 7, 14, 21, 28, and 35 using 100 μl of serum from each pig. Serum was diluted serially by ten-fold dilutions to a final dilution of 10$^{-6}$ in tubes containing 900 μl of EMEM, 2% FBS, 50 μg/ml gentamicin (Sigma Chemical Co. St. Louis, MO), and 2.5 μg/ml Fungizone (Invitrogen Corporation, Grand Island NY). Four replicates of each dilution were incubated on 96-well plates containing CL2621 cells, at 37°C and 4.5% CO$_2$ for eight days. Each well then was examined for cytopathic effect (CPE) and the titers were determined.
using the Reed-Muench calculation.

To obtain viral RNA for quantitative RT-PCR the QIAamp Viral RNA Mini-Kit® (Qiagen Inc. Valencia, CA) was used as described in the kit instructions. A commercially available real-time, single-tube, RT-PCR assay for the detection of U.S. PRRSV was provided by Tetracore Inc. (Gaithersburg, MD) and used to detect PRRSV RNA. A minor groove binding (MGB) 5’ nuclease probe and primers were designed by alignment of GenBank isolates and based on conserved areas of the 3’ untranslated region (UTR). PRRSV RNA was reverse transcribed in a 25 μl single tube reaction consisting of Tetracore U.S. PRRSV Master Mix (18.9 μl Master mix, 2 μl Enzyme mix 1, 0.1 μl Enzyme mix 2) and 4 μl of extracted RNA. The reaction tubes were loaded into the Smart Cycler II® block (Cepheid, Sunnyvale, CA) and software settings of fluorescent detection were set for automatic calculation of the baseline with the background subtraction on. The thermal cycler program consisted of 52°C for 1800 s, 95°C for 900 s, and 45 cycles at 94°C for 30 s, 61°C for 60 s and 72°C for 60 s. A PCR reaction was considered positive if the cycle threshold (Ct) level was obtained at ≤ 45 cycles. For quantitation, known amounts of serially diluted \textit{in vitro} transcript RNA product (1 x 10^{-1} through 1 x 10^{8} copies/μl) were used to generate a standard curve. Copy/ml concentrations of the unknown samples were determined by linear extrapolation of the Ct values plotted against the known concentration of the 3’UTR transcript product.

2.4 Antibody Measurement

ELISA S/P ratios were generated by performing the HerdCheck® PRRS ELISA 2XR according to the manufacturer’s instructions. PRRSV protein-specific ELISA was performed with recombinant isolate VR2332 nucleocapsid (N) and nonstructural protein 4 (nsp 4) which
were expressed in BL21 (DE3)-RP cells (Stratagene) from the plasmid pET 24b as fusion proteins containing an amino terminal myc-tag and a carboxyl terminal 6x histidine tag. Denatured proteins were dialyzed in 0.1 M Tris HCl, pH 8.0, 6 M guanidine-HCl, 2 mM EDTA and adjusted to a concentration of 3 mg/ml. DTT was added to 300 mM and the solution was filtered through a 0.45 µm membrane. Reduced protein was added into refolding buffer (100 mM Tris HCl, pH 8.0, 0.5 M L-arginine, 8 mM oxidized glutathione, 2 mM EDTA, 10 µM pepstatin A, 10 µM leupeptin, and 1 mM PMSF), filtered (0.22 µm) and stirred overnight. The purified protein was concentrated by tangential flow filtration (Pellicon XL Ultracel PLC 5 kd, Millipore) and dialyzed against 20 mM Tris HCl, pH 8.0. Proteins were analyzed on an Agilent 2100 Bioanalyzer with the Protein LabChip. Purified protein solutions were stored at –80 °C.

Protein-specific ELISAs were performed by coating microtiter plates with 100 ng recombinant protein in carbonate buffer, pH 9.6, or with buffer alone. Plates were blocked with 2.5% nonfat dry milk in phosphate-buffered saline containing 0.1% Tween 20 (PBST). One hundred µl of a 1:2000 dilution of serum was applied to duplicate wells for 2 h, after which plates were washed with PBST and antibody binding was detected by incubation with horseradish peroxidase-conjugated goat-anti swine IgG heavy + light chains (KPL, Gaithersburg MD) diluted 1:5000 for 1 hr, followed by washing and color development with 100 µl of TMB substrate (KPL). Reactions were stopped with 1 M phosphoric acid and plates were read at 450 nm.

2.5 Body Weights

All pigs were weighed on day 0 (first day of study) and day 49 (end of study). Pigs were weighed on a portable electronic weigh-bar scale system Weigh-Tronix™ model
615XL, (Weigh-Tronix Inc., Fairmont, MN). The scale was calibrated using certified test weights prior to and after each use.

2.6 Clinical Scores

On every day of the study each pig was scored by a veterinarian for respiratory signs, behavior, and coughing on a scale of one to four for each clinical sign. A normal animal was given a score of three, maximum clinical illness was a score nine and a dead animal received a score of 12. Samples from all animals that died in the study were submitted to the Iowa State University Veterinary Diagnostic Laboratory for pathological examination.

2.7 Statistical Analysis

All data were imported into SAS version 8.02 for data management and analysis. Summary statistics including mean, standard deviation standard error, median and frequency distributions were generated for all outcome variables as appropriate. Weight, RT-PCR, and Log$_{10}$ TCID$_{50}$/ml data were analyzed by one way ANOVA for overall differences among the treatment groups with pairwise testing for differences between treatment groups by Least Significant Difference t test. All tests for differences between groups were designed as two-sided tests. Differences were considered statistically significant at $p \leq 0.05$.

Some changes were made to the data to facilitate correlation analyses. The Log$_{10}$ TCID$_{50}$/ml values listed as $<2.00$ were set to 1.0. Negative RT-PCR values were set to 1.0 and all RT-PCR values were normalized by transformation to log base 10 before analysis. Control group results were not included in the correlation analyses. Results for each pig were converted to an approximate area under the curve using trapezoidal rule (Hennen 2003). Area under the curve was computed for the entire study period, from the first observation to day
15, and from day 15 to the last observation, although only the entire study period is shown in the figures.

3. Results

3.1 Virus isolation and Log_{10} TCID_{50}/ml quantification

Before exposure on the day of infection no animals tested positive for PRRSV. At 1 day after intranasal infection, only 13 animals in 5 groups tested positive for virus. However, at 3 days after infection all animals that were infected with field isolates, except for isolate 17198-6, were virus positive with mean log_{10} TCID_{50}/ml values ranging from 2.1 (SDSU-73) to 3.9 (MN 184). By contrast, animals inoculated with attenuated isolates were uniformly negative by cell culture (Figure 2). Peak levels of viremia, from 3.6 to 4.6 log_{10} TCID_{50}/ml were attained on day 7 for four of five virulent isolates and titers remained near or above 2 log_{10} TCID_{50}/ml in all virulent virus groups for 21 days except for JA 142-infected pigs which had titers below that level.

The levels of viremia in the pigs inoculated with attenuated PRRSV isolates were lower than in pigs inoculated with virulent field isolates. The Abst-1 isolate, with the exception of day 3 post inoculation, was never re-isolated. Ingelvac® PRRS MLV fluctuated between 0.5 and 1.0 log_{10} TCID_{50}/ml from days 7 to 28, and Ingelvac® PRRS ATP varied between 0.4 and 1.2 log_{10} TCID_{50}/ml from days 7 to 28. Attenuated isolate viruses were not recovered from serum after day 28, and virus was recovered from only two of the virulent field isolate groups, the pool-infected and MN 184-infected pigs through day 35 (Figure 2). Nearly all pigs were nonviremic by virus isolation at days 42 and 49.

Overall, the more virulent isolates were observed to replicate faster and to higher
titers in pigs than were the attenuated isolates. Pigs infected with the MN 184 isolate, in particular, showed a very rapid increase in virus replication beginning before day 3 and reaching a peak of over $4.5 \log_{10} \text{TCID}_{50}/\text{ml}$ on day 7. After peaking, the MN 184 viremia steadily decreased but still maintained a significantly higher titer ($t$-test, $p \leq 0.05$) than all other isolates on days 28 and 35. A similar trend was observed in all of the remaining virulent groups, namely VR2332, JA 142, SDSU 73, and the pool (Figure 2). Pigs infected with 17198-6 followed the same general trend described for the MN 184 infected group but not as closely. Groups of pigs administered the attenuated isolates (Ingelvac® PRRS MLV, Ingelvac® PRRS ATP, and Abst-1) followed a different trend. They showed a moderate increase in viral titer beginning after day 3 that reached a peak between days 7 and 15 at a viral titer more than a log less than any of the virulent exposure groups and several orders of magnitude less than the MN 184-infected group. The titers observed in these attenuated exposure groups then declined to zero on or before day 35 (Figure 2).

3.2 Virus quantification by real time RT-PCR

Levels of viremia were also determined by real time RT-PCR since it was possible that growth on CL2621 cells was not the same for all strains and because real time RT-PCR might be a more sensitive measure than growth on cells for viremia. The virulent exposure groups showed a dramatic increase in average concentration on day 1 and all groups peaked above 8 logs/ml between days 7 and 15 (Figure 3). The virulent exposure group concentrations then gradually tapered off through the next several weeks, reaching concentrations below 4 logs/ml by day 49. The attenuated strain exposure groups showed a much less dramatic increase in concentration that also began around day 1 and the average group titer never reached or exceeded 7 logs/ml (Figure 3). The concentrations observed for
the attenuated exposure groups were maintained at fluctuating levels showing a wide range in values in the weeks following the exposure. The fluctuations were due to sporadically high values in a single pig. The three attenuated strain exposure groups all peaked on different days of the study. The Ingelvac® PRRS MLV group peaked at a concentration of 4.31 logs/ml on day 28, the Ingelvac® PRRS ATP group peaked at 6.58 logs/ml on day 3, and the Abst-1 group peaked at 6.85 logs/ml on day 35 (Figure 3). The average concentration of the virulent isolate groups was observed to be significantly higher (P<0.05) than the average concentration of the attenuated strain groups on days 3 and 15, but on day 49 the average concentration of the virulent isolate groups was significantly lower (P<0.05) than that of the attenuated isolate groups.

3.3 HerdCheck® PRRS ELISA 2XR

The humoral immune response to PRRSV, as measured by HerdCheck® PRRS ELISA 2XR S/P ratios, showed that the virulent isolate exposure group averages rose above the 0.4 cutoff for a positive result on day 15. By contrast, the attenuated strain exposure group averages were negative and all three groups remained below 0.4 until after day 21. The Ingelvac® PRRS MLV and Ingelvac® PRRS ATP groups showed positive results on day 28, but the Abst-1 group did not show an average S/P ratio over 0.4 until day 42 (Figure 4). In comparing the humoral response of groups infected with virulent isolates or the pool to groups inoculated with attenuated strains, it was clear that the kinetics and magnitude of the antibody response was associated with the level of viremia, particularly between 14 and 35 days after infection. This observation is further supported by the correlation between viremia levels and humoral antibody responses determined by paired comparisons of HerdCheck® PRRS ELISA 2XR S/P ratios to either virus titration or RT-PCR. Figure 5 and Figure 6 show
that the humoral antibody response is closely associated with viral load over the entire study period with a correlation coefficient $r=0.858$ for virus titration and $r=0.794$ for RT-PCR. These associations were highly significant ($p<0.0001$ in each case). Moreover, attenuated strains showed low antibody responses and viral loads, whereas virulent strains show high responses (Figures 5 and 6).

3.4 PRRSV protein-specific ELISA

To gain additional insight into the relationship between differences in PRRSV inocula and humoral immune responses, the antibody titers against N, the major structural protein, and nsp 4, an essential but minor nonstructural protease, were determined. The kinetics of the anti-N IgG response were nearly identical in all groups of pigs, with a peak titer on day 28 followed by a sharp decline in the next 7-14 days, after which the levels were maintained or rose slightly between days 42 and 49 (Figure 7A). The magnitude of the response was similar to the HerdCheck® PRRS ELISA 2XR results, and consistent with the levels of viremia. The lowest peak titers at day 28 were observed in the groups inoculated with attenuated strains, and the highest titer was attained in pigs infected with the highly virulent MN 184 isolate. By day 49 the anti-N titer was equivalent in all groups except for MN 184 and the pool, suggesting that the humoral response to MN 184 may be qualitatively different. Also, only 5 pigs survived to day 49 in each of these two groups, which is reflected in the increased standard error at day 49 in the MN 184 group.

The IgG response to nsp 4 was substantially different than to N. No anti-nsp 4 antibody was detected before day 21, the overall response was much weaker, and no significant response was detected in the groups receiving Ingelvac® PRRS MLV and Abst-1 (Figure 7B). Moreover, the magnitude of the anti-nsp 4 response was not associated with
level of viremia. The responses to VR 2332, JA 142, MN 184, and the pool were all equivalent, with a peak at day 28, followed by a decline at day 35, then rising again at day 42, whereas the magnitude, time course and duration of viremia varied among these four groups. When examining the summary measures analysis, presented as area under the curve, data for the nsp 4 ELISA compared to the Log_{10} TCID_{50/ml} data, no significant correlation was observed between level of viremia and nsp 4 humoral antibody response (Figure 8).

3.5 Body weight

There was no significant difference in the mean weight of any of the groups on day 0 of the experiment (P = 0.099). On day 49 pigs inoculated with the attenuated strain Abst-1 had the highest mean weight, which was significantly higher then all other groups except for the control (Table 3). Also on day 49 the mean weights of all the virulent isolate exposure groups except for the 17198-6 group were significantly lower than the control group (Table 3). The mean weights of the attenuated strain exposure groups Ingelvac® PRRS MLV and Ingelvac® PRRS ATP and the control group were statistically equivalent (Table 3).

3.6 Clinical Scores

Increases in average clinical scores were observed in only four of the virulent exposure groups: JA 142, SDSU 73, MN 184, and Pool. These higher scores were maintained throughout the study while the remaining groups, both virulent and attenuated exposures, had essentially normal clinical scores for the duration of the study. The only major cause of changes in the average clinical scores observed in this study occurred when one or more animals died in the associated treatment group (Table 4). The severity of clinical disease was highly associated with viral load (p<0.001 for virus titration). When examining the area under the curve as shown in Figure 9, the clinical scores were highest for the groups infected
with MN 184 and the Pool. Fifty percent of the pigs in each group died, and virus titration indicated that the level of infection was substantially higher than for all other groups (Figure 9). The differences in viral load as determined by RT-PCR were less marked (data not shown) and the correlation of clinical signs with viral load by RT-PCR was less than with virus titration ($r=0.556$ versus $r=0.803$, respectively). The clinical scores in group 10 (Control) increased after the death of two pigs from bacterial pneumonia. Both pigs were shown to be PRRSV negative by immunohistochemical staining of lung tissue, negative virus isolation and real-time PCR analyses, and the complete lack of seroconversion by HerdCheck® PRRS ELISA 2XR or protein-specific ELISA. The summary of findings from the Iowa State University Veterinary Diagnostic Laboratory indicated that various bacterial pathogens were present in animals that died unexpectedly during the study and these deaths were likely attributable to secondary bacterial infection (Table 5).

4. Discussion

The objective of this study was to examine various PRRSV isolates with known levels of virulence to determine if there was a relationship with in vivo replication that could be used to predict the virulence of PRRSV isolates without the necessity of performing controlled challenge experiments. In addition, it was of interest to determine the relationship between isolate virulence, levels of viremia, and the humoral antibody response.

In order to test PRRSV isolates under the same conditions it was necessary to use dosages of licensed vaccines that were below the minimum immunizing dose established with the USDA and that were not representative of a commercial dose. Also, the intranasal route of administration of the MLV vaccines used in the study was not in accordance with the
USDA label and was only used to mimic a more natural exposure. The experimental doses used in the present study were not expected to and did not induce an immune response equivalent to the much higher commercial doses. Although not specifically addressed in this study, the effect of dose is likely much more significant for an attenuated or less virulent virus than it is for a virulent field virus that can quickly grow in and be recovered at over 4 logs/ml in pig serum within 3-7 days of exposure. The higher recommended intramuscular commercial dose induces HerdCheck® PRRS ELISA 2XR S/P ratios above the 0.4 cutoff by 14 days post vaccination which is one-half the amount of time observed for the doses used in this study (Roof et al., 2003). The nominal dose used in this study, $2 \times 10^3$ TCID$_{50}$ per animal, caused 50% mortality in groups that received isolate MN 184, and anti-nucleocapsid responses in all groups. Higher doses were not tested since excessive mortality in groups challenged with highly virulent strains would have compromised the study objectives. In addition, previous studies had shown no difference in clinical signs and viremia in young pigs inoculated with PRRSV isolate VR2332 at doses of $10^{2.2}$, $10^{3.2}$ and $10^{4.2}$ TCID$_{50}$ per animal (unpublished data).

Both the Log$_{10}$ TCID$_{50}$/ml and real time RT-PCR results showed that the viremia levels vary significantly among groups following PRRSV exposure, indicating that the growth rate of PRRSV in pigs is a phenotypic characteristic of the virus independent of possible variation in pig susceptibility to infection. In addition, attenuation of PRRSV by adaptation to growth on CL2621 cells reduced not only its ability to grow in pigs, but altered the kinetics of viral replication so that peak viremia occurred at later times. A similar observation was also made by Chang et al. (2002), who showed that even a limited period of cell culture passage of the moderately virulent PRRSV isolate VR 2332 reduced viral growth
in pigs and delayed significantly the time to peak viremia. However, a delayed time to peak viremia is not diagnostic for in vitro cell culture passage or for attenuation, since the highly virulent isolate 17198-6 also showed a delayed time to peak viremia.

Overall, virulent isolates showed substantially higher viremia levels in serum than did attenuated strains at equivalent doses of inoculation. For example, the highest observed virus titer in any of the attenuated isolate exposure groups was 1.22 logs on day 15 in pigs given Ingelvac® PRRS ATP, whereas the lowest titer of any virulent group on day 15 was 2.40 logs in the SDSU 73 group. The peak of viremia at days 3-7 and the levels of virus detected (all >3.5 logs/ml) was highly consistent among virulent PRRSV isolates, though MN 184 was significantly greater in its magnitude and duration, with virus titers still present on days 28 and 35. Our results support the concept that highly virulent PRRSV isolates replicate to a substantially higher titer in vivo than do attenuated or lowly virulent isolates, but they do not establish a direct quantitative relationship between level of virulence and level or rate of in vivo growth among wild-type PRRSV (Haynes et al. 1997).

The real time RT-PCR results were statistically very similar to the Log_{10} TCID_{50}/ml results, indicating that both methods measure relative levels of infectious virus among groups (data not shown). The Pearson correlation coefficient (r) between the RT-PCR and Log_{10} TCID_{50}/ml day 7 data was 0.89 and for the average real time RT-PCR and Log_{10} TCID_{50}/ml results was 0.88. The concentration values determined by real time RT-PCR may have been several orders of magnitude higher than log_{10} TCID_{50}/ml values for several reasons, including differences between the frequency of viral particles containing the target amplicon and particles that are fully infectious on CL2621 cells, and the presence of neutralizing antibodies that could lower infectivity (Dianzani et al., 2002). However, neutralizing
antibody is unlikely to account for the difference, since it was observed at all time points, including times before which an anti-PRRSV antibody response had been produced.

In general, the ELISA observations support the concept that the magnitude of the humoral immune response is related to the level of viral replication during acute infection. The trend indicated in Figures 5 and 6 illustrates this relationship, a slower and less intense humoral immune response was triggered by the cell-culture attenuated virus isolates, whereas a faster and more intense humoral immune response was triggered by the virulent isolates. In addition these observations also demonstrate that at least two factors, isolate type and infectious dose, impact relative S/P ratio values in the HerdCheck® PRRS ELISA 2XR. Although the ELISA results shown in Figure 4 indicate a clear positive or negative average group response, it is important to note the variability among individual animals, some pigs within attenuated virus groups were positive before day 21, and some pigs in the virulent groups remained negative up to day 21. This information also shows that caution must be exercised to quantitatively evaluate and compare S/P ratios. Such comparisons are impacted by a variety of host, viral, and technical factors which limit their interpretation and could cause false conclusions.

Analysis of specific antibody responses to N and nsp 4 show that immune responses to PRRSV vary in intensity independently of the inoculating isolate. Antibody responses to the N protein in animals that were inoculated with the highly virulent isolates MN 184 and JA 142 showed a trend similar to that of all the isolates but to a higher magnitude. Pigs inoculated with MN 184 and JA142 also had the highest viral titers, as shown in Figures 1 and 2. Thus, it suggests that the level of humoral immune response may be related to the viral load in acute infection as measured by viral titer. Interestingly, the time course of response
was the same in all groups, even though the time to peak titer was delayed for highly virulent strain 17198-6 and the attenuated strains. The nsp 4 antibody response, by contrast, was low at all of the time points and for all of the isolates, both attenuated and virulent. However, the time course of anti-nsp 4 response was equivalent in all of the groups despite differences in the time to peak viral load among groups, as observed for the anti-N antibody response. All pigs regardless of treatment had low anti-nsp 4 responses relative to the anti-N response (Figure 7B).

These observations indicate that some of the PRRSV proteins elicit a more robust response from the host immune system regardless of exposure isolate virulence. However, the observations also indicate that the magnitude of the immune response to the more immunogenic proteins is likely related to the virulence of the exposure isolate, or the ability of the isolate to replicate in vivo. It also is possible that differences in antibody response might be due simply to genetic differences among isolates that result in differences in antigenic reactivity such that antibodies directed against N and nsp 4 of other isolates do not react or react poorly to the recombinant proteins expressed from isolate VR2332 that were used to coat the ELISA plates. However, several lines of evidence suggest that the observed differences in antibody levels reflect immunologically relevant responses. Isolate MN 184 shows the greatest genetic difference from VR2332, as determined by ORF 5 comparisons, yet has the highest anti-N antibody response. Kapur et al. (1996) showed previously that relative differences among PRRSV isolates in one open reading frame are also present in other open reading frames. Also, individual proteins contain conserved and nonconserved regions (e.g. Kapur et al., 1996) and extensive immunogenic reactivity may be directed toward the conserved epitopes (Ostrowski et al., 2002). Nevertheless, ELISA results based on
antibody reactions with purified PRRSV proteins may be affected by genetic and antigenic variation, and these effects must be considered. Refolding of recombinant proteins was performed since antigenic reactivity of N was higher in plates coated with refolded N, but no differences were observed between nonrefolded or refolded nsp 4 (unpublished data).

It was noted that at approximately 4 to 5 weeks after inoculation a relatively large decrease in the antibody response to both the N and nsp 4 proteins occurred. A similar peak of 1 to 2 weeks followed by a decline of antibody reactivity was previously noted by Foss et al. (2002) for GP5, the major envelope glycoprotein. Taken together, these observations suggest that the response to individual viral proteins likely does not represent the full picture of the pig’s immune response to PRRSV since the humoral immune response as measured by the HerdCheck® PRRS ELISA 2XR does not show a similar transient peak of antibody reactivity.

Reduced growth and mortality were the key correlates of virulence and viral in vivo growth rate. The lower mean weight observed in the virulent isolate exposure groups most likely reflected a difference in the ability of a PRRSV isolate to replicate in vivo and induce a more severe illness in the pig. These observations are consistent with previously reported data that PRRSV infection may cause anorexia with a 25 to 40 percent reduction in daily weight gain (Thacker, 2003). The clinical scores of most of the virulent isolate exposed animals showed rapid increases shortly after the inoculation, whereas there was virtually no change in the scores of the attenuated virus exposure animals. This increase in clinical signs was reflected in the observed death rates of 50%, 20%, and 10% in the virulent exposure groups receiving PRRS isolates MN 184, SDSU 73, and JA 142, respectively. In contrast, the attenuated exposure groups incurred no deaths. The relationship between rapid viral growth
and viral pathogenesis under the same conditions of viral exposure were most evident in comparing the groups exposed to MN 184 and Abst-1. The inoculation titers were virtually the same, 4.10 logs/ml and 4.18 logs/ml, respectively, and yet, as indicated in Figure 9, there were remarkable differences in the way the two isolates affected pigs. The Abst-1 isolate was nearly inert, it hardly replicated in vivo and caused no clinical signs. By contrast, the MN 184 isolate replicated to extremely high titers in vivo and caused severe clinical signs, resulting in the death of 50% of exposed animals. Also notable, the group of pigs exposed to the pool of all virus isolates showed about the same virological, clinical, and immunological responses as pigs exposed to MN 184. This finding indicates that the most rapidly replicating virus in a mixed infection is likely to outcompete other isolates so that the net result is essentially the same as an infection with the single isolate having the highest growth potential.

The notable in vivo differences between virulent and attenuated PRRSV isolates shed light on the relationship between the virulence of an isolate and its in vivo growth and replication. When administered at equivalent doses in pigs the more virulent isolates show Log10 TCID50/ml titers and RT-PCR concentrations that are exponentially higher than the attenuated isolates. The virulent isolates induce a more rapid and intense humoral immune response. The virulent isolates negatively affect weight gain and induce higher death rates and more severe clinical signs as compared to the attenuated isolates.

Of note, this study emphasizes the variability in phenotypic characteristics of PRRSV in vivo. The interpretation of studies on PRRSV persistence, shedding, dissemination, transmission, immunity, and other interactions of PRRSV with pigs may be influenced by the isolates used in the study.

In conclusion, the results of this study indicate that attenuated and virulent PRRSV
isolates induce remarkably different clinical signs, as well as immune responses that differ in intensity. We attribute these differences to the ability of the virus to replicate in vivo, a phenotypic characteristic that can be measured quantitatively in serum samples and may be developed for predicting the virulence of PRRSV isolates.

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Table 1. Virulence and Inoculation Titer of Isolates.

<table>
<thead>
<tr>
<th>Group</th>
<th>Isolate</th>
<th>Year Isolated</th>
<th>Virulence***</th>
<th>Titer Log_{10} TCID_{50}/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>VR 2332</td>
<td>1991</td>
<td>Moderate</td>
<td>3.43</td>
</tr>
<tr>
<td>2</td>
<td>Ingelvac® PRRS MLV*</td>
<td>USDA license 1996</td>
<td>Attenuated VR2332</td>
<td>3.02</td>
</tr>
<tr>
<td>3</td>
<td>JA 142</td>
<td>1997</td>
<td>High</td>
<td>3.13</td>
</tr>
<tr>
<td>4</td>
<td>Ingelvac® PRRS ATP*</td>
<td>USDA license 1999</td>
<td>Attenuated JA 142</td>
<td>4.14</td>
</tr>
<tr>
<td>5</td>
<td>SDSU 73</td>
<td>1996</td>
<td>High</td>
<td>2.75</td>
</tr>
<tr>
<td>6</td>
<td>Abst-1*</td>
<td>Attenuated 1999</td>
<td>Attenuated SDSU 73</td>
<td>4.18</td>
</tr>
<tr>
<td>7</td>
<td>MN 184</td>
<td>2001</td>
<td>High</td>
<td>4.10</td>
</tr>
<tr>
<td>8</td>
<td>17198-6</td>
<td>1997</td>
<td>High</td>
<td>2.81</td>
</tr>
<tr>
<td>9</td>
<td>Pool**</td>
<td>N/A</td>
<td>High</td>
<td>3.71</td>
</tr>
<tr>
<td>10</td>
<td>Control</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

* Attenuated PRRSV isolates.

** Mixture containing all of the eight isolates.

*** Summary of lung lesions reported in Symposium on Emerging Diseases, Rome 2003.
Table 2. Pairwise comparisons of ORF5 nucleotide sequence of virulent and attenuated PRRSV isolates used in the study.

<table>
<thead>
<tr>
<th>Percent Divergence</th>
<th>Percent Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>VR 2332</td>
<td>Ingelvac® PRRS MLV</td>
</tr>
<tr>
<td>VR 2332</td>
<td>99.7</td>
</tr>
<tr>
<td>Ingelvac® PRRS MLV</td>
<td>0.3</td>
</tr>
<tr>
<td>JA-142</td>
<td>9.7</td>
</tr>
<tr>
<td>Ingelvac® PRRS ATP</td>
<td>10.3</td>
</tr>
<tr>
<td>SDSU 73</td>
<td>10.9</td>
</tr>
<tr>
<td>Abst-1</td>
<td>11.5</td>
</tr>
<tr>
<td>MN 184</td>
<td>15.5</td>
</tr>
<tr>
<td>17198-6</td>
<td>10.5</td>
</tr>
</tbody>
</table>

Note: Percent similarity is shown in the upper right and percent divergence is shown in the lower left of the table.
### Table 3. Average Body Weights.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Day 0</th>
<th>Day 49</th>
</tr>
</thead>
<tbody>
<tr>
<td>VR 2332</td>
<td>6.38(^1)</td>
<td>33.5(^b)</td>
</tr>
<tr>
<td>Ingelvac® PRRS MLV</td>
<td>6.56</td>
<td>34.6*</td>
</tr>
<tr>
<td>JA 142</td>
<td>6.42</td>
<td>32.7(^b)</td>
</tr>
<tr>
<td>Ingelvac® PRRS ATP</td>
<td>6.24</td>
<td>35.0*</td>
</tr>
<tr>
<td>SDSU-73</td>
<td>6.59</td>
<td>32.9(^b)</td>
</tr>
<tr>
<td>Abst-1</td>
<td>6.69</td>
<td>39.4(^a)</td>
</tr>
<tr>
<td>MN 184</td>
<td>6.73</td>
<td>23.7(^c)</td>
</tr>
<tr>
<td>17198-6</td>
<td>6.36</td>
<td>34.5*</td>
</tr>
<tr>
<td>Pool**</td>
<td>6.51</td>
<td>23.0(^c)</td>
</tr>
<tr>
<td>Control</td>
<td>6.48</td>
<td>38.4*</td>
</tr>
</tbody>
</table>

\(^1\) Weights are in kg. There were no significant differences in mean wt at day 0.

* Indicates statistically equivalent weights among these groups on day 49.

** Pool was a mixture containing all eight isolates.

\(^a\) Significantly greater than all groups except the Control group (p\(\leq\)0.05).

\(^b\) Significantly less than the Control group.

\(^c\) Significantly less than all other groups.
Table 4. Mortality of Pigs after Exposure

<table>
<thead>
<tr>
<th>Group</th>
<th>Strain</th>
<th>Mortality</th>
<th>Day(s) of Death(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>VR 2332</td>
<td>0/10</td>
<td>N/A</td>
</tr>
<tr>
<td>2</td>
<td>Ingelvac® PRRS MLV</td>
<td>0/10</td>
<td>N/A</td>
</tr>
<tr>
<td>3</td>
<td>JA 142</td>
<td>1/10 = 10%</td>
<td>17</td>
</tr>
<tr>
<td>4</td>
<td>Ingelvac® PRRS ATP</td>
<td>0/10</td>
<td>N/A</td>
</tr>
<tr>
<td>5</td>
<td>SDSU 73</td>
<td>2/10 = 20%</td>
<td>9, 23</td>
</tr>
<tr>
<td>6</td>
<td>Abst-1</td>
<td>0/10</td>
<td>N/A</td>
</tr>
<tr>
<td>7</td>
<td>MN 184</td>
<td>5/10 = 50%</td>
<td>14, 14, 17, 23, 41</td>
</tr>
<tr>
<td>8</td>
<td>17198-6</td>
<td>0/10</td>
<td>N/A</td>
</tr>
<tr>
<td>9</td>
<td>Pool**</td>
<td>5/10 = 50%</td>
<td>12, 16, 17, 21, 21</td>
</tr>
<tr>
<td>10</td>
<td>Controls</td>
<td>2/10*</td>
<td>41, 48</td>
</tr>
</tbody>
</table>

Attenuated PRRSV 0/30 = 0%
Virulent PRRSV 13/60 = 22%

Note: All deaths in treatment groups were attributed to moderate or severe non-suppurative interstitial pneumonia due to PRRSV with secondary bacterial infection.

* Deaths attributed to bacterial pneumonia with no PRRS involvement.

** Pool was a mixture containing all eight isolates.
### Table 5. Cause of Mortality after Exposure

<table>
<thead>
<tr>
<th>Pig #</th>
<th>Group</th>
<th>Cause of Death</th>
<th>Day of Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>993</td>
<td>JA 142</td>
<td>PRRS &amp; <em>Streptococcus suis</em></td>
<td>17</td>
</tr>
<tr>
<td>948</td>
<td>Neg Control</td>
<td><em>Arcanobacterium pyogenes</em> &amp; <em>Pasteurella multocida</em></td>
<td>41</td>
</tr>
<tr>
<td>983</td>
<td>Neg Control</td>
<td><em>A. pyogenes</em> &amp; <em>P. multocida</em></td>
<td>48</td>
</tr>
<tr>
<td>922</td>
<td>SDSU 73</td>
<td>PRRS &amp; bacterial pneumonia*</td>
<td>9</td>
</tr>
<tr>
<td>918</td>
<td>SDSU 73</td>
<td>PRRS &amp; <em>Escherichia coli</em></td>
<td>23</td>
</tr>
<tr>
<td>973</td>
<td>MN 184</td>
<td>PRRS &amp; <em>Actinobacillus suis</em></td>
<td>14</td>
</tr>
<tr>
<td>992</td>
<td>MN 184</td>
<td>PRRS &amp; <em>A. suis</em></td>
<td>14</td>
</tr>
<tr>
<td>980</td>
<td>MN 184</td>
<td>PRRS &amp; <em>E.coli</em></td>
<td>17</td>
</tr>
<tr>
<td>971</td>
<td>MN 184</td>
<td>PRRS &amp; <em>E.coli</em></td>
<td>23</td>
</tr>
<tr>
<td>958</td>
<td>MN 184</td>
<td>PRRS &amp; <em>E.coli</em></td>
<td>41</td>
</tr>
<tr>
<td>976</td>
<td>Pool</td>
<td>PRRS &amp; <em>A. suis</em></td>
<td>12</td>
</tr>
<tr>
<td>970</td>
<td>Pool</td>
<td>PRRS &amp; <em>S. suis</em></td>
<td>16</td>
</tr>
<tr>
<td>972</td>
<td>Pool</td>
<td>PRRS &amp; <em>S. suis</em></td>
<td>17</td>
</tr>
<tr>
<td>995</td>
<td>Pool</td>
<td>PRRS &amp; <em>S. suis</em></td>
<td>21</td>
</tr>
<tr>
<td>969</td>
<td>Pool</td>
<td>PRRS &amp; <em>A. pyogenes</em></td>
<td>21</td>
</tr>
</tbody>
</table>

* The diagnostic report indicated “bacterial pneumonia” with no specific agent listed.
Figure 1. Genetic relatedness of the PRRS isolates.

The bar indicates 1 nucleotide change per 100 residues. VR2332 is the parent isolate of Ingelvac PRRS MLV, JA 142 is the parent strain of Ingelvac PRRS ATP and SDSU 73 is the parent strain of Abst-1.
Figure 2. Mean virus titers in serum expressed as log_{10} TCID_{50}/ml.

The average titer of the MN 184 infected group at its peak on day 7 and on days 28 and 35 was significantly higher (p \leq 0.05) than all of the other isolates (asterisks). Viral titration was not performed on days 0, 1, 42, and 49 since less than 15% of the animals were virus-positive.
Figure 3. Mean PRRSV concentrations in serum by real time RT-PCR.

The asterisk indicates the peak concentration of the virulent infected groups on day 7. The double asterisk indicates the highest titer achieved by an attenuated isolate (Abst-1 on day 35). The treatment groups legend is the same as in Figure 2.
Figure 4. Mean HerdCheck® PRRS ELISA 2XR S/P ratios.

All the virulent isolate groups showed HerdCheck® PRRS ELISA 2XR S/P ratios greater than 0.4 (positive result) by day 15 whereas all the attenuated isolate groups still show S/P ratios less than 0.4 (negative result) on day 15. The treatment groups legend is the same as in Figure 2.
Figure 5. Correlation analysis of the HerdCheck® PRRS ELISA 2XR and Log$_{10}$ TCID$_{50}$/ml.

Individual group values for the HerdCheck® PRRS ELISA 2XR S/P ratios and the Log$_{10}$ TCID$_{50}$/ml were determined from the area under the curve across all time points for each treatment group in the experiment as described in Materials and Methods. The X- and Y-axis values are relative units. Points are labeled according to treatment group.
Figure 6. Correlation analysis of the HerdCheck® PRRS ELISA 2XR and RT-PCR concentration.

Individual group values for the HerdCheck® PRRS ELISA 2XR S/P ratios and the RT-PCR concentration were determined from the area under the curve across all time points for each treatment group in the experiment as described in Materials and Methods. The X- and Y-axis values are relative units. Points are labeled according to treatment group.
Figure 7. Effect of PRRSV isolate or strain on antigen-specific IgG response.

Sera were tested at a 1:2000 dilution on ELISA plates coated with 100 ng of recombinant VR 2332 nucleocapsid or nsp 4. Data are the mean values of 10 animals, except where animals died. (A) Response to nucleocapsid. Error bars in the MN 184 data points are standard errors of the mean. (B) Response to nsp 4-specific IgG response.
Figure 8. Correlation analysis of the nsp 4 curve and Log_{10} TCID_{50}/ml.

Individual group values for the nsp 4 and the Log_{10} TCID_{50}/ml were determined from the area under the curve across all time points for each treatment group in the experiment as described in Materials and Methods. The X- and Y-axis values are relative units and it should be noted that the Y-axis scale is different than the scale shown in Figure 5 and Figure 6. Points are labeled according to treatment group.
Figure 9. Correlation analysis of the clinical scores and Log$_{10}$ TCID$_{50}$/ml.

Individual group values for the clinical scores and Log$_{10}$ TCID$_{50}$/ml were determined from the area under the curve across all time points for each treatment group in the experiment as described in Materials and Methods. The X- and Y-axis values are relative units. Points are labeled according to treatment group.
References


CHAPTER 4. DNA VACCINATION OF PIGLETS AGAINST PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (PRRSV) USING SEQUENTIAL EXPRESSION LIBRARY IMMUNIZATION (SELI)

A paper to be submitted to Vaccine

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Abstract

PRRSV causes an economically important swine disease and is an elusive target for vaccine development. Two prototype PRRSV DNA vaccines were investigated; with expression either driven by the muscle-specific synthetic SPc5-12 promoter, or by the ubiquitous CMV promoter constitutively. Ingelvac® PRRS MLV, a commercially available modified-live virus (MLV) vaccine, served as a positive vaccination control. Both DNA vaccines significantly reduced the level of lung lesions after virulent PRRSV challenge when each group was compared to the challenge control group. The MLV provided the best level of efficacy and significantly reduced the lung lesions as compared to all other challenged groups. The level of efficacy provided by the SELI method of DNA vaccination validates this vaccination concept and allows for more focused efforts on determining the most relevant immunogenic regions of the PRRSV.

1. Introduction

PRRSV is classified in the family Arteriviridae (Cavanagh 1997). This enveloped, positive sense single-stranded RNA virus causes widespread disease in swine first known as ‘mystery swine disease’, which was described in the US in 1987 (Hill 1990); (Swenson, Hill et al. 1995). The respiratory form of the disease is found in all age groups of swine, and may
be severe enough to lead to death in some younger pigs (Johnson, Roof et al. 2004) or cause reproductive problems that in some cases can be very severe in breeding age females (Lowe, Zuckermann et al. 2006).

Modified-live virus (MLV) vaccines are able to induce both cellular and humoral immune response in the vaccinated animal (Charerntantanakul, Platt et al. 2006). The use of PRRSV vaccines comprised of inactivated antigens has not been as successful at eliciting high levels of efficacy in vaccinated animals (Zuckermann, Garcia et al. 2007). When a killed vaccine is administered, the resulting host immune response is typically humoral, with little to no induction of a host cellular immune response (Meier, Galeota et al. 2003). A major benefit of DNA vaccination is that the plasmid constructs have the ability to stimulate both humoral and cellular immunity in the vaccinated animal (Kutzler and Weiner 2008) and (Dhama, Mahendran et al. 2008). Furthermore, the use of novel delivery methods, such as \textit{in vivo} electroporation (EP) has allowed for high expression levels, as well as humoral and cellular immune responses to antigens at relatively low plasmid quantities (Luxembourg, Evans et al. 2007). This increase in efficacy favored the testing of DNA formulations containing a large number of plasmids, rather than single antigen-encoding sequences (Laddy, Yan et al. 2008).

Finding a next generation vaccine for PRRSV is a difficult task. Work has been done with cDNA infectious clones of the virus as a new method of generating a more characterized MLV-vaccine (Wang, Liang et al. 2008); (de Lima, Kwon et al. 2008). DNA vaccination is also a subject of investigation; several studies have been done utilizing DNA vaccination with a focus on the structural genes of the virus (Pirzadeh and Dea 1998); (Jiang, Xiao et al. 2006); (Rompato, Ling et al. 2006); (Xue, Zhao et al. 2004). Some researchers
have even looked at DNA vaccination with the ORF1 region as a whole individually and in combination with viral structural genes (Barfoed, Blixenkrone-Moller et al. 2004). The study described here looks to comprehensively examine the PRRSV ORF1 gene in combination with the structural genes as a plausible antigen able to provide protection from challenge.

Sequential Expression Library Immunization (SELI) is a new application of the established Expression Library Immunization (ELI) DNA vaccination concept (Johnston and Barry 1997). Rather than utilizing random fragments of DNA as is done with ELI, the basis of SELI is that the DNA fragments of interest are designed in a systematic and sequential manner. This strategy allows a single extensive open reading frame to be expressed as many small protein fragments within the host. The host, because of their smaller size, can more readily express the SELI constructs. In this study, the very large PRRSV replication complex ORF1a/b (approximately 12,000bp) was divided into cDNA clones that sequentially represented the entire open reading frame. Earlier investigations found that intramuscular administration of SELI DNA vaccines with expression via the CMV promoter combined with an aluminum phosphate-based adjuvant conferred partial efficacy from virulent PRRSV challenge (Vaughn and Stammer 2006). In the current study electroporation was investigated as a more efficient method for ensuring DNA vaccine plasmids entry into cells.

As shown in Table 1 the cDNA clones comprising the ORF1a/1b regions were designated (A) through (M). Clone (A) utilized the authentic ORF1a ATG start codon. The remaining ORF1a/1b clones (B) through (M) have had an ATG start codon added to their respective 5’ coding regions. Additional cDNA clones representing the PRRSV structural proteins ORFs 2a, 2b, 3, 4, 5, 6, and 7 were also generated. By utilizing a combination of the SELI clones and structural clones, it was possible to vaccinate pigs with PRRSV DNA
vaccines that encompassed the PRRSV genome in its entirety (i.e. ORF 1a/1b (A) through (M) and ORFs 2 through 7).

The results of this study indicate that the SELI DNA vaccination strategy via electroporation was beneficial in determining that this approach could confer partial protection from virulent heterologous PRRSV challenge, and that the expression pattern driven by the muscle-specific or ubiquitous promoters evaluated did not affect the degree of efficacy provided by the DNA vaccine prototypes.

2. Materials and Methods

2.1 PRRSV Plasmid DNA Vaccines

RNA was purified from the PRRSV isolate 17198-6 or VR-2332 (see Table 1) by performing, as per kit instructions, the QIAmp Viral RNA Mini Kit, (QIAGEN Inc. Valencia, CA).

Reverse transcription (RT) was performed on the extracted viral RNA using random hexamers (PerkinElmer, Waltham, MA) as per manufacturer’s instructions. As shown in Table 2 specific primers were designed and utilized in the PCR step using reagents from PerkinElmer (Waltham, MA) for amplification of the cDNA. The DNA resulting from this amplicication was then gel purified using Easy Clean DNA Spin Filters (Primm Labs, Inc. Boston, MA). Each of the purified DNA fragments was ligated into the pGem®-T Easy Vector System (Promega Corporation, Madison, WI) and then transformed into MAX Efficiency® DH5-α™ Competent Cells (Invitrogen Inc. Carlsbad, CA).

All of the PRRSV SELI coding regions and structural genes were respectively sub-cloned from the pGem®-T Easy Vector into the DNA expression vector pVC1650
(Abruzzese, Godin et al. 1999). The pVC1650 vector contains the CMV promoter to direct the expression of the PRRSV SELI constructs and structural genes in a broad range of eukaryotic cell types in a constitutive manner.

Additionally, the PRRSV SELI coding regions and structural genes were respectively sub-cloned from the pVC1650 vector into the DNA expression vector pAV0245BNH (pAVBNH). The pAVBNH vector contains the VGX SPc5-12 promoter to direct the expression of the PRRSV SELI coding regions and structural genes in muscle cells (Li, Eastman et al. 1999).

After sub-cloning each of the fragments into the respective vectors, endotoxin-free plasmid preparations were generated by performing, as per kit instructions, the EndoFree Plasmid Giga Kit (QIAGEN Inc. Valencia, CA).

### 2.2 PRRSV Modified Live Vaccine (Ingelvac® PRRS MLV)

Ingelvac® PRRS MLV, (Boehringer Ingelheim Vetmedica Inc. St. Joseph, MO) was the modified live vaccine used in this study as per product insert.

### 2.3 Study Design

The study consisted of pigs that were three-weeks-old at day 0 which were divided into five groups of ten animals each. One group was treated with the twenty-three SELI pAVBNH plasmids via electroporation on both day 0 and day 21 of the study. A second group was treated with the twenty-three SELI pVC1650 plasmids via electroporation on day 0 and day 21 of the study. A third group received Ingelvac® PRRS MLV as per label indications on day 0 of the study. Finally two groups were not treated with a vaccine prototype, and served as challenge and strict controls.
Whole blood samples were collected for serum generation from each animal on study days 0, 21, 44, 45, 47, 51, 54, and 58. The serum was then stored at -70°C until analyzed.

Pigs receiving the DNA plasmid vaccination regime were anesthetized just prior to electroporation of their respective vaccines with a combination product containing ketamine, xylazene and Telazol® (Fort Dodge Animal Health Inc. Fort Dodge, IA) at a dosage of 0.4 mL to 0.6 mL per pig dependant upon animal weight. Plasmids were formulated in sterile water at a concentration of 0.33 mg/mL each for the pAVBNH vector and 0.66mg/mL each for the pVC1650 vector. Electroporation (EP) was performed using a CELLECTRA® constant current electroporation device and its intramuscular (IM) applicator and arrays (Person, Bodles-Brakhop et al. 2008). Briefly, the arrays consist of five electrodes (21 gauge, 2 cm in length) arranged in a 1 cm circular display. The electrode array was inserted into the target tissue, and after verifying impedance; the plasmids were injected through a port situated in the middle of the circular array delineated by the electrodes. Four seconds after the plasmid administration, the EP pulses were applied: 3 pulses at 0.5 Amps, 52 milliseconds/pulse, and 1 second between pulses. On the day of each DNA vaccination, the entire plasmid formulation was split into four and the EP injections were performed in four different sites on each of the anesthetized pigs to accommodate the injection volume. The area chosen for EP was the inside of the hind legs, targeting the semimembranosous muscle. Approximately 0.75 mL of pooled plasmid constructs was administered during each of the four EP injections.

On study day 44 animals, all groups, excluding the strict control group, were challenged intranasally (IN) with 2.0 mL of the virulent PRRSV isolate JA142 at 4.44 logs/mL, 1.0 mL per nostril. The titer of the virus was determined by tissue culture infectious
dose fifty on MA104 cells using the Reed-Muench calculation (Reed and Muench 1938). The study was terminated 14 days after this virulent PRRSV challenge (study day 58).

2.4 Study Evaluation Criteria

At the study termination the lungs of each animal were scored for total percent consolidation due to PRRSV infection. A general description of lung pathology observed and the percentage of pathology for each lung lobe was recorded at the time of necropsy. The total percent of lung pathology for each pig was determined by summation of percent lung pathology for each lung lobe (Straw, Backstrom et al. 1986).

Virus isolations were performed on pre-challenge sera collected on day 44 and on all bleed days after the challenge. Briefly, 100µl of serum was used to inoculate three-day-old MA104 cells grown on 48-well tissue culture plates with EMEM, 2% FBS, 50 µg/ml gentamicin (Sigma Chemical Co. St. Louis, MO), and 2.5 µg/ml Fungizone (Invitrogen Corporation, Grand Island NY). The cells were incubated in a humidified chamber at 37°C with 4.5% CO₂ for 8 days after inoculation with the study serum they were then observed for cytopathic effect (CPE).

Real-time RT-PCR for detection of PRRSV was performed on pre-challenge sera collected on day 44 and on all bleed days after the challenge. Extractions were accomplished by utilizing the QIAGEN BioRobot Universal (QIAGEN Inc. Valencia, CA) for purification of viral RNA from serum using, as per kit instructions, the QIAmp Virus BioRobot MDx Kit, (QIAGEN Inc. Valencia, CA). The one step qRT-PCR was performed using a commercially available assay for the detection of U.S. PRRSV as per kit instructions (Tetracore Inc. Gaithersburg, MD).
HerdCheck® PRRS ELISA 2XR (IDEXX Laboratories Inc. Westbrook, ME) was performed to prescreen all animals prior to beginning the study and on all study bleed days, according to the manufacturer’s instructions.

All animals were weighed on the day of first vaccination, the day of second vaccination, the day of challenge, and at study termination. Pigs were weighed on a portable electronic weigh-bar scale system Weigh-Tronix™ model 615XL, (Weigh-Tronix Inc. Fairmont, MN). The scale was calibrated using certified test weights prior to and after each use.

2.5 Statistical Analysis

All data were imported into SAS version 9.1 for management and analysis. Summary statistics by treatment group including mean, median, standard deviation, standard error, confidence intervals, coefficients of variation, and frequency distributions were generated for all variables, where appropriate.

Pairwise comparisons were made among and between treatment groups and the challenge control group. The strict control group was not included in the analysis. Two-sided results were reported and all comparisons were evaluated at \( \alpha = 0.05 \).

The primary parameter, percent total lung lesion score, were not normally distributed data and were analyzed using the nonparametric Kruskal-Wallis / Wilcoxon Two Sample Test.

Continuous variables serum RT-PCR, ELISA, and weights / average daily weight gains were analyzed by Analysis of Variance. The equality of variances among groups was evaluated by the likelihood ratio test. If group variances were significantly different, an adjustment for unequal variances was made.
Binomial data (present/absent) for sera virus isolations were analyzed by Fisher’s Exact Test.

3. Results

3.1 Lung Lesion Scores

As illustrated in Figure 1 both the pAVBNH SELI vaccinated group and the pVC1650 SELI group had significantly reduced group average lung scores when compared to the challenge control group. The Ingelvac® PRRS MLV group had the largest reduction in group average lung scores when compared to the challenge controls and also had significantly reduced group average lung scores when compared to all other PRRSV-challenged groups in the study. Because of the variability in lung scores within groups the individual animal lung scores are provided and can be found in Table 3. Although statistics were not performed on individual animal data, it is interesting to note that the pAVBNH SELI group had only one animal with a total lung score contributing to much of the group average score.

3.2 Virus isolations

As expected, only the MLV-vaccinated pigs were found to be viremic for PRRSV prior to challenge. All other pigs remained virus isolation negative until after PRRSV challenge. As shown in Figure 2 there was little difference in the postchallenge virus isolation between the PRRSV-challenged groups, with the Ingelvac® PRRS MLV group being the only group to show a significant reduction in viremic animals after challenge. Although not statistically significant, it is interesting to note that the Ingelvac® PRRS MLV group had the lowest percent virus isolation positive on every day post challenge, with no
MLV-vaccinated animals found to be viremic at necropsy.

3.3 Virus quantification by real time RT-PCR

Figure 3 illustrates that all of the challenged groups in this study showed the same general trend for the level of PRRSV RNA in the sera after challenge. All PRRSV-challenged groups showed an increase in the level of PRRSV RNA in the sera until peaking at three or seven days post challenge, after which there is a decrease in the level of PRRSV RNA until necropsy at 14 days post challenge.

One exception to this trend would be the Ingelvac® PRRS MLV group which not only had detectable levels of PRRSV RNA on the day of challenge, but then actually had a decrease in PRRSV RNA level in the sera one day post challenge. The levels of PRRSV RNA on Day 3 through Day 10 post challenge were comparable to the other PRRSV-challenged groups. The Ingelvac® PRRS MLV group also had the lowest level of PRRSV RNA, less then $10^6$ genomic equivalences (g.e.), of any challenged group at the end of the study. Even though the DNA vaccinated groups did show a reduction in the level of PRRSV RNA at the end of the study, both still had a group average of more than $10^{7.5}$ g.e. of PRRSV RNA detected in sera.

3.4 HerdCheck® PRRS ELISA 2XR

As shown in Figure 4 the Ingelvac® PRRS MLV group showed a typical humoral immune response after vaccination, while the SELI groups showed no seroconversion until after virulent challenge. The lack of detectable seroconversion using the IDEXX PRRS ELISA could possibly be explained, at least in part, by low levels of expression of the DNA vaccines by the pigs or an insufficient dose to elicit a response. The two SELI DNA vaccine groups had group average (S/P) ratios above the 0.4 positive cut off level on day 7 post
challenge, which is sooner than the challenge control group. A possible explanation for this earlier seroconversion would be the expression of PRRSV ORF 7 at a low level in the DNA vaccinated animals resulting in an anamnestic response.

3.5 Body weight

No group had a significant increase in average daily weight gain as compared to the challenge control group.

4. Discussion

The objective of this study was to evaluate the efficacy of prototype PRRSV DNA vaccines utilizing either the SPc5-12 or CMV promoter administered via electroporation against that of a commercially available PRRSV MLV vaccine using the host animal in a controlled trial.

It is apparent from the lung lesion data that either of the promoters (SPc5-12 or CMV) in PRRS SELI DNA plasmids were able to provide a significant level of lung protection, as both of these groups showed a significant reduction in lung lesions when compared to the challenge control group. It is interesting to note that, with the exception of one animal, the individual lung consolidation scores of the pAVBNH SELI group were similar to those seen in the Ingelvac® PRRS MLV group following challenge. It has been observed before that expression of DNA vaccines in muscle cells can provide for antigen presentation and subsequent cell mediated protection from influenza challenge in mice (Ulmer, Deck et al. 1997). When the Ingelvac® PRRS MLV-vaccinated group was evaluated, it was clear that the MLV provided the highest level of efficacy and was able to significantly reduce the group average lung lesions when compared to all the other test
groups in the study. As this study was only designed to test the effect of the mode of delivery and promoter usage on the SELI concept, future studies will be needed to focus on the elucidation of the specific clones that are important for providing protection as well as the doses of these clones required to provide the protection.

Although a reduction of lung lesions is the main measure of efficacy for a respiratory PRRS vaccine and has been correlated with cellular immunity (Charerntantanakul, Platt et al. 2006), it was not the only parameter measured in this study. The presence of replicating virus in the serum post challenge is also a very good indicator of vaccine efficacy. According to the virus isolation results in this study, the two SELI vaccine groups did not afford a significant reduction in the presence of viable virus in serum following PRRSV challenge when compared to the challenge controls. Both of the SELI groups showed that at least 30% of the animals were virus isolation positive on the five sampling days post challenge. Also, with the exception of the Ingelvac® PRRS MLV-vaccinated group, it was observed that 100% of the animals in PRRSV-challenged groups were virus isolation positive at seven days post challenge. The Ingelvac® PRRS MLV-vaccinated group showed both a shorter duration of virus isolation positive animals (three out of the five sampling days) and fewer animals virus isolation positive between challenge and necropsy, with a peak of (70%) viremic animals three days post challenge.

Quantification of PRRSV RNA in each animal’s serum was achieved using real time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). None of the test groups in the study were found to have a significantly reduced post challenge level of PRRSV RNA in the sera when compared to the unvaccinated challenged group. It has been observed previously that the cell culture-based virus isolation and the qRT-PCR assays
indicate different levels of viremia (Johnson, Roof et al. 2004). It is important to remember that both assays provide valuable information as to the state of viremia post challenge. However, it is important to be cognizant of the differences between these two assays as the virus isolation assay detects only viable PRRSV, whereas the qRT-PCR detects both viable and non-viable PRRSV. The disparity in the two assays is evident in that the qRT-PCR indicated that every PRRSV-challenged pig on every post challenge sampling day tested had PRRSV RNA detected in their serum, whereas virus isolation results revealed many animals were negative for viable virus at the end of the study (data not shown).

The level of seroconversion to PRRSV was determined through the use of the IDEXX PRRS ELISA. It is important to note that this ELISA is designed to detect seroconversion to the nucleocapsid protein encoded by the viral ORF 7 gene. For this reason a measurable pre-challenge humoral immune response would not be expected for the groups vaccinated with the SELI prototypes, unless the ORF 7 gene expressed at levels equivalent to those seen in the animal during viral replication. It was interesting to note that by seven days post challenge the SELI vaccinated groups showed a group average S/P ratio above 0.4 (cut-off for a positive result) while the challenge control group average S/P ratio remained below 0.4. This shortened duration of time between virulent PRRSV exposure and seroconversion to a group average S/P ratio above 0.4 does suggest an anamnestic response to ORF 7 was induced by ORF 7 expression following the SELI DNA vaccinations. The level of ORF7 expression was very likely below the levels seen during a natural PRRSV infection or vaccination with a PRRS MLV vaccine. Since this study was designed to test the mode of delivery and promoter usage for the SELI concept, further testing is needed to determine the PRRSV-specific genes or regions of genes important in conferring protection. The use of
only relevant plasmids will reduce the overall number of plasmids in the preparations and allow for the inclusion levels of the remaining, most relevant, plasmids to be increased. If a higher inclusion level of only the relevant plasmids were used and if the expression of these plasmids were close to or equivalent to those seen during viral replication in the host, better protection could be seen. As for the Ingelvac® PRRS MLV group, a typical seroconversion curve was observed with an S/P ratio of about 2.0 reached by Day 21 and then maintained throughout the duration of the study.

In conclusion, PRRSV SELI DNA vaccines administered via electroporation and utilizing either the CMV or SPc5-12 promoter can reduce the level of lung lesions following virulent challenge, but it was apparent that the DNA vaccine prototypes evaluated in this study did not provide the same level of protection as was provided by Ingelvac® PRRS MLV. At this time, the costs for plasmid production, the practicality of the route of delivery, and the actual amount of DNA vaccine material needed for dosage of large animals in a production setting are major drawbacks to the use of DNA vaccines. The goal is lofty for a PRRS DNA vaccine but if the number of SELI plasmids can be reduced and the concentration of the remaining relevant plasmids can be increased then sufficient protection may be achievable. Therefore, further vaccination/challenge studies with selected SELI fragments would serve as an effective tool in the delineation of the relevant immunogenic regions of the PRRS virus.

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Table 1. PRRSV SELI Fragments.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Designated clone</th>
<th>Region of clone</th>
<th>Nucleotide span of clone (using Genbank U87392 as reference)</th>
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<td>190-1128</td>
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<td>1126-2082</td>
</tr>
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<td>ORF 1a</td>
<td>190-2082</td>
</tr>
<tr>
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</tr>
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</tr>
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<td>J</td>
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<td>23</td>
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Note: Gray highlighted clones were generated from genetic material purified from PRRSV isolate VR-2332. All remaining clones were generated from genetic material purified from PRRSV isolate 17198-6.

<sup>a</sup> Indicates the two clones that were not generated in the pVC1650 plasmid.
Table 2. PRRSV Cloning Primer Pairs.

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<tr>
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<td>ggtgaccggggt gtgttt</td>
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<td>ORF5 &amp; ORF6</td>
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</table>

Note: Gray highlighted portion of the primers indicates the ATG start codon for each of the fifteen SELI clones.
Table 3. Lung Involvement of Individual Animals by Treatment Group.

<table>
<thead>
<tr>
<th>Animal</th>
<th>pAVBNH SELI</th>
<th>pVC1650 SELI</th>
<th>Ingelvac® PRRSMLV</th>
<th>Challenge Controls</th>
<th>Negative Controls</th>
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<tr>
<td>1</td>
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<td>13.00^a</td>
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<tr>
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<td>10.00^a</td>
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<td>15.00^a</td>
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<td>19.00^a</td>
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<tr>
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<td>1.10</td>
<td>0.00</td>
<td>9.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

^a Indicates animals with lung involvement greater than or equal to ten percent, an arbitrary value chosen to illustrate differences between the groups.

Notice the pAVBNH DNA vaccinated group has only one animal or 10% of the animals in the group with involvement greater then ten percent while the challenge control group has 60% of the animals with involvement greater then ten percent.
Figure 1. Percent Lung Involvement by Treatment Group.

a Both the pAVBNH SELI group and the pVC1650 SELI group have significantly lower lung involvement than the challenge control group $P = 0.0126$ and $0.0494$ respectively.

b The Ingelvac® PRRS MLV group has significantly lower lung involvement than the pAVBNH SELI group, the pVC1650 SELI group, and the challenge control group.

c The challenge control group has significantly higher lung involvement than all of the test groups.

d The strict control group was not included in the statistical analysis.
Figure 2. Percent Virus Isolation Positive Animals by Treatment Group and Day-Post-Challenge (dpc).

The only group to show a significant reduction in the percentage of viremic animals after challenge when compared to the challenge control group was the Ingelvac® PRRS MLV group at 7 and 10 days post challenge (dpc) P = 0.0108 and 0.0198 respectively.
Figure 3. Real Time PRRSV RT-PCR by Treatment Group and Day-Post-Challenge (dpc).

a The only group to have animals positive for PRRSV genomic material on the day of challenge (study day 44 or 0dpc) was the Ingelvac® PRRS MLV group.

b Although not statically significant is was also observed that there was a greater than one log reduction in the level of PRRSV genomic material in the Ingelvac® PRRS MLV group as compared to all other challenged groups at the time of necropsy (study day 58 or 14dpc).
Figure 4. IDEXX PRRS ELISA Group Average S/P Ratio by Study Day.

a The Ingelvac® PRRS MLV group shows a typical IDEXX ELISA response to vaccination with seroconversion of all animals by study day 21.

b Although not statically significant it was also observed that both of the DNA vaccinated groups showed a group average S/P ratio above 0.4 (positive result) by study day 51 (7dpc) while the group average S/P ratio of the challenge control group remained below 0.4 (negative result).
References


CHAPTER 5. GENERAL CONCLUSIONS

In order to keep the development of PRRSV vaccines moving forward it is very important to remind all PRRSV vaccine researchers about a few very good suggestions, but one in particular could be considered the most important of all; always include a group vaccinated with a licensed commercial PRRSV vaccine in all study designs to serve as a direct comparison of the prototype vaccine being tested to something available in the marketplace today.

The notable in vivo differences between virulent and attenuated PRRSV isolates shed light on the relationship between the virulence of an isolate and its in vivo growth and replication. When administered at equivalent doses in pigs the more virulent isolates show \( \log_{10} \text{TCID}_{50}/\text{ml} \) titers and RT-PCR concentrations that are exponentially higher than the attenuated isolates. The virulent isolates induce a more rapid and intense humoral immune response. The virulent isolates negatively affect weight gain and induce higher death rates and more severe clinical signs as compared to the attenuated isolates. Attenuated and virulent PRRSV isolates induce remarkably different clinical signs, as well as immune responses that differ in intensity. We attribute these differences to the ability of the virus to replicate in vivo, a phenotypic characteristic that can be measured quantititatively in serum samples and may be developed for predicting the virulence of PRRSV isolates.

PRRSV SELI DNA vaccines administered via electroporation and utilizing either the CMV or SPc5-12 promoter can reduce the level of lung lesions following virulent challenge, but it was apparent that the DNA vaccine prototypes evaluated in this study did not provide the same level of protection as was provided by Ingelvac® PRRS MLV. At this time, the costs for plasmid production, the practicality of the route of delivery, and the actual amount
of DNA vaccine material needed for dosage of large animals in a production setting are major drawbacks to the use of DNA vaccines. The goal is lofty for a PRRS DNA vaccine but if the number of SELI plasmids can be reduced and the concentration of the remaining relevant plasmids can be increased then sufficient protection may be achievable. Therefore, further vaccination/challenge studies with selected SELI fragments would serve as an effective tool in the delineation of the relevant immunogenic regions of the PRRS virus.

While examining the tremendous body of work published on PRRSV protection strategies it was absolutely astonishing to find that so much has already been tested and learned about PRRSV since its classification, yet so little is truly known about protection from PRRSV. Simply put, PRRSV has been, is now, and will continue to be for at least the near future, one of the most significant and elusive pathogens researched in swine health.