Evaluation of replicon particle vaccines for porcine reproductive and respiratory syndrome virus

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Evaluation of replicon particle vaccines for porcine reproductive and respiratory syndrome virus

by

Mark Alan Mogler

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

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CHAPTER 1: GENERAL INTRODUCTION

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is one of the most important diseases of domestic swine, due to both the large economic and animal welfare impacts it causes worldwide. The etiologic agent is a virus (PRRSV), which is a member of the family Arteriviridae. PRRSV has proven itself to be a difficult target for control and eradication efforts within the swine industry since emerging in the late 1980s. Vaccine development efforts have relied chiefly upon attenuated viruses due to the ineffectiveness of inactivated and subunit vaccines, but heterologous protection from attenuated vaccines is quite variable. Control efforts are further hampered by the lack of diagnostic assays that can differentiate vaccinated from infected animals. Therefore, it is desirable to develop an improved generation of PRRS vaccines to address these and other shortcomings.

Early investigations into the antigenic determinants of the PRRSV-neutralizing antibody response identified the major neutralizing epitope in the ectodomain of GP5 (2, 3, 5). An association between N-linked glycosylation of GP5 and the antibody response to this epitope has also been described (1). Work done in preparation for the series of studies presented in this dissertation suggested that the use of two N-glycan mutant GP5 proteins might be beneficial for the induction of broadly neutralizing antibody responses (D.L. Harris, unpublished data). Studies presented in this dissertation, specifically in Chapters 4 and 5, make use of these N-glycan GP5 proteins as part of a candidate PRRS vaccine.
Alphavirus-derived replicon particle (RP) vaccines have been in pre-clinical development for well over a decade, and have demonstrated efficacy and safety in a wide range of disease and animal models (4). Recently, an RP platform derived from Venezuelan equine encephalitis virus (strain, TC-83) has been developed for use in veterinary vaccines. The key attributes of RP technology are that they are propagation-defective, single-cycle RNA virus vectors, and are capable of eliciting potent humoral and cellular immune responses to a wide variety of antigens. The use of RP allow for native expression of viral antigens, in contrast to many subunit protein expression systems, which may be crucial for the development of improved PRRS vaccines. The propagation-defective nature of the RP addresses one of the chief concerns with attenuated PRRS vaccines, which is the risk of reversion to virulence.

This dissertation describes a body of work based on the application of RP technology to the development of control measures for PRRS. The first manuscript of this dissertation describes the use of an RP influenza vaccine to reduce viral load in young pigs challenged with PRRSV. This manuscript highlights the utility of RP as a platform for evaluating both the innate and adaptive immune responses to PRRSV infection. The second manuscript describes the evaluation of two candidate RP PRRS vaccines in pregnant gilts, with a focus on farrowing and litter performance. This manuscript represents one of very few attempts at evaluating vectored PRRS vaccines in this model. The third manuscript presents a series of three experiments conducted with young pigs, wherein various candidate RP PRRS vaccines were evaluated in challenge studies. The second and third manuscripts represent the only known systemic evaluation of multiple candidate RP PRRS vaccines.
**Dissertation organization**

This dissertation is organized in manuscript format. Chapter 1 includes a short introduction to PRRSV and RP, describes the content of the included manuscripts, and outlines the format of this dissertation. Chapter 2 provides a literature review of PRRSV natural history, immunology, and vaccinology. Chapters 3, 4, and 5 are manuscripts describing the research and results obtained by the primary research author, Mark Mogler, and co-authors. Chapter 6 provides general conclusions of the research, brief discussion of future work, and is followed by a brief acknowledgements section.

**References**


CHAPTER 2: LITERATURE REVIEW, PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS

Introduction

Porcine respiratory and reproductive syndrome (PRRS) is the most economically significant disease of swine, costing US producers over $600 million annually (47, 95). The reproductive form of the disease causes fever, abortion, infertility, and low piglet viability, while the respiratory form causes fever, pneumonia, anorexia, and susceptibility to sequelae (115). The etiologic agent of PRRS is an enveloped virus (PRRSV) containing a single-stranded, positive-sense, RNA genome, which is a member of the Arterivirus genus in the order Nidovirales (10). Due to its interesting virological and immunological properties, PRRSV presents a complex challenge for researchers and swine producers alike.

The order Nidovirales

The prefix ‘nido-’ is derived from the Latin ‘nidus’ for ‘nest’, and the initial organization of the Nidovirales order was based upon the common 5’ leader sequence on all nested subgenomic mRNA species. However, this characteristic has been shown to be less conserved and is no longer considered a hallmark of the order (17). The characterization of the multidomain replicase gene has revealed two main distinguishing genetic markers, the multinuclear zinc-binding domain (ZBD) and the uridylate-specific endoridonuclease (NendoU) (42, 43). Other domains are generally conserved among the nidovirus replicase, but are shared with other virus orders.
The order *Nidovirales* can be divided into four main groups: coronaviruses, toroviruses, roniviruses, and arteriviruses. Of these groups, the arteriviruses have the smallest genomes at approximately 15 kilobases (10). The remaining so-called ‘large nidoviruses’ possess genomes of 25-32 kilobases, easily the largest known RNA genomes (41-43). One common characteristic of the large nidoviruses is the presence of a 3’-to-5’ exoribonuclease (ExoN) in the replicase machinery, which supplies a proofreading function that likely contributes to the exceptional genome size of these viruses (43, 124).

**PRRSV biology**

The other members of the *Arterivirus* genus are equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV), and simian hemorrhagic fever virus (SHFV). Of these, LDV is most closely related to PRRSV, and EAV is the most phylogenetically distant (10). The approximately 15.5 kilobase RNA genome of PRRSV contains a 5’ leader sequence, ten known open reading frames (ORFs), and a 3’ untranslated, polyadenylated tail region. The two most 5’-proximal ORFs (1a and 1ab) occupy approximately three-quarters of the genome and encode two replicase polyproteins (pp1a and pp1ab). These two precursor polyproteins are extensively processed into at least twelve nonstructural proteins (nsp 1-12) by three viral proteases (nsp1, nsp2 and nsp4) (1, 92, 138, 156, 166). A ribosomal frameshift mediates the translation of pp1ab. The 3’ end of the viral genome contains a set of eight smaller genes (ORFs) that partially overlap with each other. These genes encode the structural proteins of the virus and are expressed from subgenomic mRNAs (125). The expression of all eight genes in the genomic 3’ end (ORFs 2a, 2b, 3, 4, 5a, 5, 6, and 7) is required for the production of infectious progeny virions (39, 53, 154, 155).
A model for PRRSV attachment, internalization, and uncoating was developed from multiple investigations using porcine alveolar macrophages (PAMs) and other cell types (135). Attachment of the virion to the host cell is mediated by heparin sulfate (26, 28, 31). This interaction is relatively unstable, but the close contact allows for more stable binding of porcine sialoadhesin to the viral GP5/M heterodimer, and uptake of the virion is through clathrin-mediated endocytosis (26, 28-30, 32, 136, 141, 146). In the early endosome, the scavenger receptor CD163 interacts with GP2a and GP4 to permit genome release into the cytoplasm (8, 19, 20, 140, 141, 151). Acidification of the endosome and the cellular protease cathepsin E are also required for genome release (65). The possibility of additional host factors or alternative entry pathways cannot be dismissed at this time.

The replication cycle of PRRSV begins when the positive-sense RNA genome enters the host cell. Translation of the replicase polyproteins from ORF1a and ORF1ab is followed by self-processing of the polyproteins into replicase subunits (125). The replicase complex produces seven nested replicative intermediate RNA strands. These replicative intermediates are then used as template for transcription of the positive-sense genomic mRNA and six positive-sense, nested, subgenomic mRNAs (33, 123, 125). In PRRSV, as in all arteriviruses, the mRNAs are 3’ co-terminal and also share a 5’ leader sequence. The addition of the 5’ leader sequence is accomplished by a negative-sense strand “body jump”, which in turn is regulated by base-pairing at the leader-body junction sites (11, 33, 83, 84, 125, 162).
Viral particles are assembled by nucleocapsid budding into the lumen of the smooth endoplasmic reticulum and/or Golgi, and viral particles are released by exocytosis (22, 125). Membrane proteins, including the GP5/M heterodimer, are required for viral particle assembly (154, 155). Defective interfering particles have not been described for arteriviruses. The viral particle is spherical and is approximately 50 nm in diameter (23, 34).

**History**

In the late 1980s, swine producers in the United States and Europe began reporting the existence of a previously unknown reproductive syndrome, variously termed: Mystery Swine Disease; Blue Ear Disease; Swine Infertility and Respiratory Syndrome; and Porcine Endemic Abortion and Respiratory Syndrome (3, 14, 86, 108, 130, 152, 153). The causative agent of PRRS was isolated in the early 1990s in both Europe and North America (14, 153). The prototype European (Type 1) strain is Lelystad virus (LV), while the prototype North American (Type 2) strain is ATCC VR2332. There is approximately 60% nucleotide homology between LV and VR2332, indicating their probable distinct evolutionary history (58, 81, 82, 129). PRRSV is a principal component of the so-called ‘porcine respiratory disease complex’ (PRDC), with porcine circovirus 2, *Mycoplasma hyopneumoniae*, swine influenza virus, *Pasteurella multocida*, and *Haemophilus parasuis* (45, 59, 96, 131).

The origin of the nidoviruses generally, and PRRSV specifically, remains unclear (105). The nidoviruses are hypothesized to have evolved from a large RNA virus that acquired genes encoding antecedents of their unique replicase machinery (43). This newfound ability to
support stable genome expansion allowed the nidoviruses to adapt to their respective niches while independently evolving their unique host interaction strategies.

The arteriviruses are relatively host-restricted, in that they generally are very limited in host range. Indeed, the extremely limited host cell tropism (excepting EAV) points towards a deep co-evolutionary relationship with the traditional host species (125). This raises the question of how arteriviruses ‘jump’ between species to establish a divergent lineage. It has been hypothesized that the origin of PRRSV can be traced to a common ancestor with LDV, and that the interaction between LDV-infected mice and swine allowed for a proto-PRRSV to establish itself within swine, although direct evidence for this hypothesis is limited (105).

One of the hallmarks of PRRSV evolution is the wide diversity of strains. In some instances, there is evidence of two distinct strains recombining to form an entirely new lineage of PRRSV (68). In other cases, new lineages have been reported following the establishment of vaccine-like strains in geographic areas (77). These reports highlight some of the challenges imposed by the mutable nature of the PRRSV genome. Type 1 (European-like) PRRSV strains form three distinct subtypes (88). The current diversity of Type 2 PRRSV strains can be organized into nine lineages by ORF5 nucleotide homology (121).

**Viral proteins**

The twelve cleavage products of pp1a and pp1ab, encoded by ORF1a and ORF 1ab, are non-structural proteins with functions in both replication and regulation of host response (38). Proteolytic activity has been identified for nsp1 and nsp2 (cysteine protease), as well as nsp4
(serine protease) (38, 122, 125, 127, 128). The replicase complex is formed by nsp9-12, and is associated with cell membranes via interactions with nsp2, nsp3, and nsp5 (126, 137, 139).

The minor structural proteins account for less than 5% of the virion by mass, and include GP2a, GP2b (E), GP3, GP4, and ORF5a protein (22, 39, 53, 90, 157). Alternate reading frames within their respective subgenomic mRNAs encode both the E and ORF5a proteins. The E protein has ion-channel properties, and is likely associated with viral genome release upon cell entry (65). The GP2a/GP3/GP4 heterotrimer has been implicated in the interaction with CD163, and is a major determinant of cell tropism (19, 132). There are reports of GP3 existing as a secreted protein, although there appears to be strain differences in this property (22, 40, 79, 142). Virus neutralizing (VN) antibodies have been associated with linear epitopes on GP3 (50, 147). A VN epitope has also been identified on GP4 of Type 1 PRRSV (85). In contrast to the VN epitope on GP5, which is located in a highly conserved region, the VN epitope on GP4 is associated with a hypervariable region and is very susceptible to immunoselection (16, 85, 99, 107).

The nucleocapsid (N) protein, encoded by ORF7, is the most abundant viral protein by mass, accounting for up to 40% of viral protein (22). The N protein exists as both a monomer and disulfide-linked homodimer, and is the target of monoclonal antibodies widely used as diagnostic reagents (78). The main functions of the N protein are to encapsidate the viral genome and associate with the viral envelope proteins during budding. The N protein may also function to interfere with the host immune response by blocking the activation of a type I interferon response via IRF3 (116).
The unglycosylated M (membrane) protein is encoded by ORF6, and is thought to play a key role in viral budding. The M protein forms a disulfide-linked heterodimer with GP5 through a cysteine residue in the N-terminal ectodomain (21, 37, 80).

The major glycoprotein (GP5) is encoded by ORF5, and exists as both a monomer and a heterodimer (GP5/M) in the virion. The sequence of ORF5 is the basis of most phylogenetic analyses of PRRSV diversity (121). The GP5/M heterodimer associates with heparin sulfate and sialoadhesin during cell attachment (135). The GP5/M heterodimer also interacts with the GP2/GP3/GP4 heterotrimer (19). In EAV, the ORF5 protein carries the main epitopes involved in virus neutralization (2). The major neutralization epitopes of PRRSV (both Type 1 and 2) have been mapped to the GP5 protein N-terminal ectodomain (9, 27, 99, 104, 106, 150, 158, 163). However, immunization of pigs with peptides corresponding to the putative neutralizing region does not induce VN antibodies, and further work is needed to resolve the issue (73).

**Epidemiology**

Transmission of PRRSV occurs primarily by close contact of infected and naïve pigs, as well as by *in utero* infection of fetuses (115). The density of swine production facilities in certain regions raises the concern that PRRSV may be transmitted by aerosols over long distances, and infectious virus has been recovered from air samples up to 9.1 km from experimentally infected pigs (24, 100). Field trials comparing incidence of PRRS in HEPA filter-equipped and unfiltered barns show significant reductions in outbreaks (25). In these studies, some
filter-equipped barns experienced PRRS clinical disease, but evidence suggested possible introduction of PRRSV by mechanical means. Herd closure has been shown to eliminate the virus from production sites, in conjunction with strict biosecurity measures (15). Interest in large-scale surveillance of swine herds for pathogens, including PRRSV, has driven the search for alternatives to serum-based diagnostics. The development of oral fluid-based diagnostics allows for relatively easy and inexpensive sample collection from swine. Oral fluids have been primarily used to detect antibodies, although some studies have tested for viral RNA (12, 61, 74, 109, 114).

**Immune responses to PRRSV**

The initial innate cellular immune response to PRRSV infection is weak, and was initially thought to be due to suppression of the classical interferon response (89). Recent work suggests that the situation is more complex, and that suppression of interferon, as measured by *in vitro* systems, may have little relevance to *in vivo* disease progression (87). Genome-wide association studies have identified a potential role for interferon-induced genes in host resistance to PRRSV infection (4).

The initial non-neutralizing antibody response to PRRSV infection is primarily directed against the nucleocapsid (N) protein. These antibodies appear in as little as five days post-infection, and form the basis for the commercially available diagnostic ELISA (54). Levels of IgG directed against PRRSV peak several weeks after infection, and slowly decline over the course of 6-12 months (93). Development of neutralizing antibodies is typically delayed several weeks, although neutralizing titers have been reported within nine days post-infection.
Passive transfer of neutralizing antibodies into pregnant gilts provides sterilizing immunity, but only partial immunity is achieved in young pig passive transfer studies (75, 98). In early investigations, PRRSV was reported to cause antibody-dependent enhancement (ADE) (160, 161). Vaccination studies conducted since those early reports have not reported ADE. Passive immunization studies with serum containing high levels of neutralizing antibody do not support the interpretation of ADE because there is dose-dependent relationship between antibody and inhibition of infection (75).

The cell-mediated immune response to PRRSV is thought to be responsible for ultimate clearance of the virus, because virus can be recovered from lungs and lymphoid tissue in the presence of VN antibodies (13, 64, 87, 148). However, the correlates of an effective cell-mediated immune response are poorly understood at this time. Stimulation of lymphocytes with PRRSV antigens yields interferon-gamma production, and is thought to be important in ascertaining the relative strength of anti-PRRSV cell-mediated immunity (89). More work is needed to resolve the questions surrounding anti-PRRSV immunity, especially the cellular response and the role of neutralizing antibodies (73, 87).

**Vaccinology**

Current approaches to PRRSV control by vaccination can be divided broadly into three categories: infectious viruses, inactivated viruses, and recombinant proteins. There are currently three commercially available attenuated PRRSV vaccine strains licensed for sale in the U.S. Attenuated vaccines have good efficacy against homologous strains, but variable efficacy against heterologous strains (63, 87, 89). Inactivated vaccines have generally poor
efficacy, especially against heterologous strains (60). A widespread current practice involves the intentional exposure of replacement breeding animals to virulent field viruses, which raises serious logistical and ethical concerns (87).

Multiple investigators have evaluated recombinant vaccine candidates under laboratory conditions, with mixed results. Viral vectors are increasingly being used as experimental vaccine platforms, and include both DNA and RNA vectors (18).

Recent work with naked DNA vaccines shows some level of protection when expressing a GP3-GP5 fusion in combination with interferon alpha and gamma (35). Three published studies from Chinese researchers describe the development of a pseudorabies (PRV) vector expressing PRRSV antigens (52, 113, 133). In these studies, expression of either GP5 or GP5/M induced specific antibody and cell-mediated immune responses to both PRV and PRRSV. Challenged animals had reduced viremia or clinical signs compared to unvaccinated controls. Recombinant adenovirus (rAd) vectors have been used to express a GP5-M fusion in mice, and induced neutralizing antibodies and specific lymphocyte responses, but was not evaluated in pigs (49). Additional constructs expressing either a GP3-GP5 or GMCSF-GP3-GP5 fusion were evaluated in both mice and pigs, where they induced specific immune responses and reduced viremia (51, 72, 149). Poxviruses, such as fowlpox (FPV) and modified vaccine virus Ankara (MVA), have been evaluated in a number of other disease models. The use of FPV to co-express a GP3-GP5 fusion with IL-18 reduced viremia in a piglet challenge model (120). Vaccination of mice with MVA expressing GP5-M induced neutralizing antibodies, but swine challenge studies were not reported (165).
RNA virus vectors have not been used extensively in PRRSV vaccinology research, although recently interest in this approach has increased. Transmissible gastroenteritis virus (TGEV) is a coronavirus, and thus distantly related evolutionarily to PRRSV. An inactivated vaccine consisting of recombinant TGEV expressing PRRSV GP5 and M was used to immunize pigs. Vaccinated animals had improved anamnestic antibody responses after challenge, and some reduction in viremia was also observed (18).

**Immune modulators**

The use of rAd vectors to express type I interferon, in cell cultures or in vivo, shows promise as a means to control PRRSV infection by reducing viremia and lung pathology (6, 7, 46, 101). Short (20-25 bases) RNA inverted repeats (shRNA) can stimulate sequence-specific antiviral responses. Investigators used rAd vectors to express PRRSV shRNA in vivo, thereby stimulating an RNAi effect, and reduced viremia and clinical signs were observed (48, 69-71). The *in vivo* effect was only demonstrated at 24 hours pre-challenge, however. Peptide-conjugated phosphorodiamidate morpholino oligomers (PPMO) are nucleoside analogs with a modified backbone that resist nuclease degradation, and they also contain an arginine-rich peptide that promotes uptake by cells. PPMO bind to complementary mRNA sequence and prevent translation by steric hindrance. Synthetic PPMO were administered swine via aerosol inoculation before and after PRRSV challenge (44, 97, 102, 103, 164). Reduced viremia and clinical signs were observed in treated pigs compared to placebo.
**Alphavirus replicon particles**

Alphavirus-derived replicon particles (RP) are propagation-defective RNA virus vectors, which have been developed as a platform for veterinary vaccines (5, 36, 143-145). The RP platform has a proven record of safety and efficacy in a wide range of human and animals disease models (55-57, 62, 94, 110-112, 145). Vaccination with RP induces potent cellular and humoral immunity, and can stimulate innate immune responses that confer protection to heterologous viruses (66, 67, 91, 117-119, 134). This dissertation will present results from several studies that evaluated candidate RP PRRS vaccines in both pregnant gilt and young pig challenge models. These studies represent the first reports of RP used for PRRS vaccinology.

**Conclusions**

Since it emerged in the late 1980s, PRRS has caused significant harm to the swine industry worldwide. The biology of PRRSV has hindered vaccination and control efforts, and improvements will require significant advances in vaccine technology. Alphavirus-derived RP have proven efficacy in many disease models, allow for differentiation of vaccinated and infected animals, and represent a novel approach to PRRSV vaccinology.
Figures

Figure 1. The PRRSV genome organization.

Figure 2. PRRSV discontinuous mRNA transcription (from Snijder et al, 1998).

Figure 3. PRRSV replication strategy (from Snijder et al, 1998).
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CHAPTER 3. ALPHAVIRUS REPLICON PARTICLE ADMINISTRATION PRIOR TO PRRSV CHALLENGE REDUCES VIREMIA

A paper to be submitted to Clinical and Vaccine Immunology

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Abstract

Porcine reproductive and respiratory syndrome (PRRS) causes significant economic, food production, and animal welfare problems for the world swine industry. The immune factors responsible for prevention of PRRS virus (PRRSV) infection are poorly understood. Further exploration of the factors responsible for PRRSV’s evasion of host defenses may contribute to improved vaccine design. We hypothesized that vaccination of pigs with a replicon particle (RP) derived from Venezuelan equine encephalitis virus (strain, TC-83) would interfere with the natural progression of PRRSV viremia due to the robust innate immune responses induced by the replicon system immediately after RP injection. To test this, we vaccinated young pigs with an RP vaccine against swine influenza virus and challenged with PRRSV at 24 or 72 hours post-vaccination. Compared to placebo, RP-vaccinated pigs had reduced viremia at various time points post-challenge, as well as a reduction in total viral load. The use of RP to modulate the immune system may prove useful for further study of PRRSV and other pathogens.
Introduction

Porcine reproductive and respiratory syndrome (PRRS) is one of the most economically significant diseases affecting the swine industry (14, 35). The causative agent is a virus (PRRSV), which belongs to the Arterivirus genus in the order Nidovirales (5, 27, 28, 32). PRRSV causes abortion, stillbirth, and poor piglet viability in breeding animals; in growing pigs, the virus causes pneumonia, anorexia, and immune suppression (40). Currently available vaccines include attenuated live viruses, inactivated whole viruses, and recombinant protein (33). Problems with these vaccines include a risk of reversion to virulence, incomplete protection, and lack of differential serological assays. This is due in part to the extensive genetic and antigenic diversity of PRRSV, as well as the lack of a clearly identified antigenic determinant from which to design an efficacious vaccine. Additionally, the key determinant(s) of a protective anti-PRRSV immune response are not well understood. Advancements in PRRS vaccinology will necessitate a more complete understanding of the immunological factors required for protection from disease (33).

Early research indicated that PRRSV infection impaired the innate immune response to infection, leading to prolonged disease and an ineffective adaptive immune response. For example, PRRSV interferes with portions of the interferon-signaling pathway, potentially delaying the onset of an effective antiviral state in host cells (21, 25, 26, 29). Further work has revealed a more complex model for the interaction of PRRSV with the host innate responses. For example, strain differences in the in vitro interferon phenotype suggest that PRRSV may not necessarily require the suppression of the innate immune response to cause disease (21, 24). PRRSV infection does not impact the ability of pigs to mount humoral
responses to heterologous antigens, implying that the many relevant immune pathways are still functioning (31). However, modulation of antiviral responses with recombinant interferon has shown promise as a means to interfere with PRRSV infection in several studies (3, 4, 13, 36). Understanding of this effect may lead to the development of improved vaccines and control measures for PRRS.

Alphavirus-derived replicon particles (RP) are propagation-defective RNA viral vectors that have been developed as a vaccine platform. An alphavirus RP derived from Venezuelan equine encephalitis virus (VEEV) strain TC-83 shows significant promise as a vaccine platform for use in veterinary medicine, as has been demonstrated with swine influenza (2, 11, 45, 47). Extensive work in humans and other hosts has demonstrated the safety and efficacy of alphavirus-derived RP vaccines in a wide variety of disease models, including Ebola virus and botulinum neurotoxin (12, 18, 20, 38, 46). RP vaccination results in the stimulation of potent humoral and cellular immune responses, in part due to the innate immune pathways stimulated by the intrinsic properties of alphavirus-derived RP (10, 19, 22, 23, 34, 41-44). The systemic antiviral state induced by an RP can even confer protection from heterologous viral challenge (19). We reasoned that this inherent property of RP could impact the course of PRRSV infection, even in the absence of expression of PRRSV-derived genes from the vector. To test this hypothesis, two animal experiments were conducted using an RP expressing antigen derived from swine influenza virus followed by PRRSV challenge.
Materials and methods

Animals

Crossbred pigs were obtained at approximately three weeks of age from a commercial herd (Wilson’s Prairie View Farm, Burlington, WI) with no history of PRRSV or influenza by serology or clinical signs. Pigs were randomized into groups and allowed to acclimate for one week. Animals were identified using numbered ear tags and groups were comingled in a shared pen space.

Replicon particle vaccine

The open reading frame encoding swine influenza virus H3N2 cluster 4 hemagglutinin (H3) was cloned into the alphavirus replicon vector as previously described (2, 15, 45, 46). Replicon and helper plasmids were used as template to produce RNA transcripts in vitro. Purified replicon RNA and helper RNAs were mixed with Vero cells and transfected via electroporation (16). Cells were incubated eighteen hours in serum-free media. RP were harvested by affinity chromatography, sterile filtered, and formulated in RP diluent (PBS + 5% sucrose + 1% normal swine serum). The RP were tested for the presence of replication competent virus by serial blind passage on Vero cells, and titrated by immunofluorescence assay on Vero cells. Prior to treatment of animals, RP were formulated to a titer of 5.00x10^8 RP/ml (45, 47). The placebo formulation contained only RP diluent.

Virus

The challenge strain of PRRSV (HLV349) was isolated from a clinical specimen in 2008 and propagated on MARC-145 cells for two passages prior to use in this study. The challenge
virus inoculum was prepared by diluting stock virus in PBS to a final titer of $1.00 \times 10^5$ TCID$_{50}$/ml. Titer of the PRRSV challenge inoculum was confirmed by titration on MARC-145 cells (39).

**Challenge studies**

Experiment 1

On day -1, each group (n= 6 pigs/group) was vaccinated with 2.0 ml of either RP or placebo by intramuscular injection in the neck (Table 1). The total RP dose in Group A was $1 \times 10^9$. At 24 hours post-vaccination (day 0), all animals were challenged with virulent PRRSV by intranasal administration of 2.0 ml virus inoculum, for a total dose of $2.00 \times 10^5$ TCID$_{50}$.

Blood samples were collected by jugular venipuncture on days (-7), 3, 5, 7, 10, 14, 17, and 21. The serum fraction was collected and stored at -80°C prior to use in diagnostic assays. Animals were humanely euthanized on day 21 of the study. All animal protocols were reviewed and approved by the Harrisvaccines Institutional Animal Care and Use Committee (Protocol # 081810-01).

Experiment 2

On day -3 or day -1, each group (n= 6 pigs/group) was vaccinated with 2.0 ml of either RP or placebo by intramuscular injection in the neck (Table 2). The total RP dose in Groups A and B was $1 \times 10^9$. At 72 or 24 hours post-treatment (day 0), all animals were challenged in the same manner as Experiment 1. Blood samples were collected by jugular venipuncture on days (-7), 1, 4, 7, 12, 14, 18, and 21. The serum fraction was collected and stored at -80°C prior to use in diagnostic assays. Animals were humanely euthanized on day 21 of the study.
**Assays**

Viral RNA was extracted from 140 µl of serum samples using the QIAmp Viral RNA Mini kit (QIAGEN, Valencia, CA) according to manufacturer’s instructions, and eluted in 60 µl of the supplied buffer. Extracted viral RNA was used in a quantitative real-time, reverse transcriptase PCR (qPCR) (Applied Biosystems, Carlsbad, CA) assay using the supplied RNA to construct a standard curve. The qPCR assay was run on a C1000 thermocycler with a CFX96 real-time unit, and data were analyzed using CFX Manager™ software (Bio-Rad, Hercules, CA). All RNA samples were run in duplicate reactions, and the mean, log-transformed RNA copy number per 1 µl of sample RNA was used for statistical analysis. The qPCR results were also used to calculate the area under the curve (AUC) for each animal. Briefly, the average value for each pair of consecutive time measurements was multiplied by the interval, and the values were summed to obtain the AUC. In addition, homologous virus neutralization was assayed by fluorescent focus neutralization (FFN) using previously described methods (48). PRRSV ELISA (HerdChek X3, IDEXX Laboratories, Westbrook, ME) was conducted according to the manufacturer’s directions.

**Statistical analysis**

Analysis of variance (ANOVA) was used to analyze log$_2$-transformed FFN titers, AUC values, ELISA S/P ratios, and log$_{10}$ transformed qPCR RNA copy number. Analyses were performed using JMP software (SAS, Cary, NC) Statistical significance was set at p<0.05.
Results

PRRSV ELISA

All challenged animals became seropositive (S/P ratio ≥0.4) prior to day 21 in all experiments. There were no differences between groups in the number of animals that were seropositive at any time point in Experiment 1 and Experiment 2.

qPCR

In Experiment 1, the RP-vaccinated group had statistically significantly lower mean qPCR titers for PRRSV on days 10, 14 and 17, compared to the placebo group (Figure 1). In Experiment 2, the RP group vaccinated on day (-1) had significantly lower qPCR titers than the placebo group by ANOVA on day 14 (Figure 2).

In Experiment 1, animals in the RP-vaccinated group had a significantly lower viral load, as determined by AUC, when compared to placebo animals. In Experiment 2, the animals vaccinated on day (-3) had significantly lower viral load than placebo (p<0.05).

FFN

No statistically significant differences by ANOVA were observed in FFN titers at necropsy (day 21) from any study.
Discussion

These results indicate that H3 RP administered 24 hours prior to PRRSV challenge significantly reduces both the magnitude of viremia and total viral load in serum as measured by qPCR. The observed differences in the magnitude of viremia occurred between ten and seventeen days post-challenge, rather than occurring in the early viremic period, as was hypothesized. These data suggest that the mechanism responsible for the reduced viremia is not sufficiently robust to prevent the initial viremia associated with PRRSV infection. This contrasts with the protective response observed in pigs that have recovered from PRRSV infection (including attenuated strains) prior to re-challenge (33). The RP vaccination did not stimulate more rapid production of PRRSV-neutralizing antibodies following challenge, suggesting an alternative mechanism of viremia reduction. Detailed immunological data, such as mRNA expression levels of various cytokines, were not collected in the reported studies, limiting conclusions about potential mechanisms. Further examination of the role of various cytokines in anti-PRRSV immunity would be prudent.

There are numerous examples of RP inducing a protective antiviral state in heterologous virus challenges. For example, null RP (no heterologous gene) administration protected against homologous VEEV challenge and heterologous influenza challenge (19). However, protection was not seen with heterologous vesicular stomatitis virus challenge, suggesting that the innate immune stimulation afforded by RP is dependent upon the nature of the virus challenge and relevant protective responses required for protection.
The studies presented here utilized RP derived from TC-83, an attenuated form of VEEV. The current study also used RP containing a heterologous gene, while earlier work, reported by Konopka et al, was conducted with RP lacking a heterologous gene (19). Another potential difference arises from the fact that different strains of VEEV-derived RP have varying efficiencies of titration on cells in culture as determined by immunofluorescence. These differences have been associated with varying affinity for cellular heparan sulphate moieties imparted by the glycoproteins of the different strains of VEEV-derived RP (17). RP derived from the V3000 VEEV glycoproteins have a low heparan sulphate affinity relative to RP derived from the TC-83 VEEV glycoproteins; RP derived from these VEEV strains will show significantly different infectious titers (and associated genome to infectious unit ratios) on the same cell line due to this difference (17). It has been shown previously that animals dosed with TC-83 and V3000 derived RP based on equal genome equivalents induce similar immune responses while dosing animals with the same RP based on infectious titers induce significantly different immune responses. That is, V3000 derived RP demonstrate a 100-fold higher genome to infectious unit ratio when compared to TC-83 RP and it appears that all of the V3000 derived RP genomes not measured in the in vitro titration assay are functional in vivo. This characteristic could result in large differences in the amount of RP used to vaccinate animals and this could influence the magnitude of the innate immune response induced in a dose-dependent manner. Knowledge of these differences will ensure that study of the induction of the innate immune system by alphavirus replicon systems can be compared across studies.
Studies conducted by the PRRS Host Genetics Consortium have identified a quantitative trait locus that correlates with decreased viral load after PRRSV challenge (1). The region of the swine genome identified in this work contains a member of the interferon-stimulated gene family. Use of adenovirus vectors expressing porcine interferon-alpha has shown that PRRSV viremia is delayed by administration of this vector prior to challenge (3, 4).

Adenovirus-vectored interferon has also shown efficacy in foot and mouth disease virus challenge models using both swine and cattle (6-9, 30, 37). Induction of an antiviral state, in this case by vectored expression of interferon, seems to be a promising approach to enhancing the efficacy of PPRSV vaccines. Further studies are needed to evaluate the swine cytokine response to RP administration, and the use of alphavirus replicons for this purpose may prove useful.

In the reported studies, a single administration of RP was conducted, which may have limited the effectiveness of this approach. Future work should examine the relationship between administration strategy and various immunological and virological outcomes to further develop a comprehensive, mechanistic understanding of the interaction between innate immunity and PPRSV infection.

Acknowledgements

The authors would like to thank Jill Gander, Kay Kimpston-Burkgren, and Kara Burrack for laboratory technical assistance, and Pamela Whitson, Ashley Baert, and the Harrisvaccines staff for animal care and handling.
Figures

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**Table 1.** Experiment 1 design. Pigs received either RP expressing hemagglutinin of H3N2 influenza (H3) or placebo. Vaccination was at 24 hours pre-challenge with PRRSV.

<table>
<thead>
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<th>RP dose</th>
<th>Treatment</th>
<th>Challenge</th>
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**Table 2.** Experiment 2 design. Pigs received either RP expressing hemagglutinin of H3N2 influenza (H3) or placebo. Vaccination was at 72 hours (Group A) or 24 hours (Groups B and C) pre-challenge with PRRSV.
Figure 1. Experiment 1 quantitative real-time RT-PCR for PRRSV RNA (qPCR) results.

The qPCR assay was conducted using RNA extracts from sera in Experiment 1. Group A (n=6 pigs) was vaccinated with RP on day (-1), and group B was vaccinated with placebo on day (-1). No PRRSV RNA was detected in samples from day 0 post-challenge. Group A has significantly lower mean RNA copies per 1 µl at 10, 14, and 17 days post-challenge compared to Group B when compared by ANOVA (p=0.006, p=0.005, p=0.004, respectively). Data are expressed as group mean log-transformed RNA copy number per 1 µl of RNA extract. Bars represent +/- one standard error of the mean. Different letter superscripts between groups represent statistical significance within each timepoint.
Figure 2. Experiment 2 quantitative real-time RT-PCR for PRRSV RNA (qPCR). The qPCR assay was conducted using RNA extracts from sera in Experiment 2. Group A (n=6 pigs) was vaccinated with RP on day (-3), group B (n=6 pigs) was vaccinated with RP on day (-1), and Group C (n=6 pigs) was vaccinated with placebo on day (-1). No PRRSV RNA was detected in samples from day 0 post-challenge. Group B had significantly lower mean RNA copies per 1 µl on day 14 post-challenge, when compared to Group C when compared by ANOVA (p=0.032). Data are expressed as group mean log-transformed RNA copy number per 1 µl of RNA extract. Bars represent +/- one standard error of the mean. Different letter superscripts between groups represent statistical significance within each timepoint.
Figure 3. Viral load in experimental animals for Experiment 1 and Experiment 2. Area under the curve (AUC) was calculated from qPCR output (RNA copies per 1 µl). The AUC was determined by calculating the mean of two consecutive measurements (e.g. days 3 and 7 post challenge), multiplying the result by the length of the interval (e.g. 4 days), repeating this process for all time points, and summing the values. Figure 3A displays AUC data for Experiment 1. Group A (n=6 pigs) was vaccinated with RP on day (-1), and group B (n=6 pigs) was vaccinated with placebo on day (-1). Data are the group mean AUC, and bars represent +/- one standard error of the mean. Group A has a significantly lower AUC when compared to Group B by ANOVA (p<0.05). Different letter superscripts between groups represent statistical significance within each timepoint. Figure 3B displays AUC data for Experiment 2. Group A (n=6 pigs) was vaccinated with RP on day (-3), group B (n=6 pigs) was vaccinated with RP on day (-1), and Group C (n=6 pigs) was vaccinated with placebo on day (-1). Data are the group mean AUC, and bars represent +/- one standard error of the mean. Group A has a significantly lower AUC when compared to Group C by ANOVA (p<0.05). Different letter superscripts between groups represent statistical significance within each timepoint.
References


CHAPTER 4: REPLICON PARTICLE PRRS VACCINE DOES NOT PROTECT AGAINST CHALLENGE IN A PREGNANT GILT MODEL

A paper to be submitted to Vaccine

Mark A. Mogler, Ryan L. Vander Veen, J. Dustin Loy, D. L. Hank Harris, Kurt I. Kamrud

Abstract
Porcine reproductive and respiratory syndrome (PRRS) is one of the most economically significant diseases of domestic swine worldwide. Currently available control measures have had limited success in controlling PRRS within some production systems, but improved solutions are needed. The use of an alphavirus-derived replicon particle (RP) vaccine platform has shown promise as a potential tool for the development of novel PRRS vaccine candidates. First-parity gilts were vaccinated with RP-based PRRS or influenza vaccines, challenged with virulent PRRS virus, and observed for farrowing performance and litter mortality. When compared to non-challenged controls, all challenged gilts had significantly worse farrowing performance and increased litter mortality. Neither of the candidate PRRS vaccines provided statistically significant improvements in either farrowing performance or litter mortality, when compared to the influenza vaccine. Gilts that received PRRS vaccine candidates developed antigen-specific antibody responses, but failed to develop detectable virus neutralizing antibody titers, suggesting a possible reason for vaccine failure.
Introduction

Porcine reproductive and respiratory syndrome (PRRS) is responsible for significant economic losses in the global swine industry (5, 11). PRRS is caused by an enveloped, positive-sense, ssRNA virus (PRRSV), which is a member of the Arterivirus genus in the order Nidovirales (1). Other members of this genus include simian hemorrhagic fever virus, equine arteritis virus, and lactate dehydrogenase-elevating virus. In pregnant swine, PRRS is characterized by anorexia, fever, reproductive failure (late term abortion, stillbirths, mummified fetuses, etc), and occasionally, death (15). Infected neonates suffer from high pre-weaning mortality, sometimes reaching 100%, which may be influenced by co-infection with other swine pathogens (e.g. Haemophilus parasuis).

Prevention of PRRS in breeding herds has been accomplished by herd closure and exclusion of the pathogen, but many herds suffer from recurrent outbreaks despite control measures (2). One control measure utilizes intentional exposure of PRRSV-naïve replacement breeding stock to virulent virus present on the production site. This is intended to provide some level of homologous immunity to the prevalent strain(s) of PRRSV, but the practice raises serious ethical and logistical concerns (9). Prevention of disease through vaccination yields inconsistent results, due to the variable efficacy of attenuated PRRSV against heterologous strains (10, 12). Inactivated PRRSV, subunit vaccines, and vectored vaccines have shown relatively poor results when evaluated under experimental conditions (3, 10). The relevant antigenic determinants and underlying immune correlates of protection are poorly understood at this time. Most, if not all, currently available PRRS vaccines do not allow for the
differentiation of infected and vaccinated animals (DIVA), further hindering control efforts. An improved PRRS vaccine would be of great utility in future control and eradication efforts.

Alphavirus-derived replicon particles (RP) are propagation-defective RNA viral vectors, and have been in use as a vaccine platform for over a decade (14, 16). An alphavirus RP derived from Venezuelan equine encephalitis virus (VEEV) strain TC-83 has been developed, allowing for improved safety and simplicity in manufacturing. The safety and efficacy of the TC-83-derived RP platform is well-established in various models of both human and animal disease, including swine influenza (17).

The use of RP to express various open reading frames of PRRSV shows promise as a means to develop a vectored PRRS vaccine compatible with DIVA strategies. Our group has developed RP that express the GP5 and M proteins of PRRSV from the same vector. We have also developed a variety of RP that express individual PRRSV proteins, including GP3, GP4, GP5, and M. It was hypothesized that immunization of swine with RP expressing various PRRSV structural components would induce protective immune responses against virulent, heterologous PRRSV challenge. To test this hypothesis, an experiment was conducted to evaluate the effect of two candidate RP vaccines on litter performance in a pregnant gilt-PRRSV challenge model.
Materials and Methods

Animal experiment

First-parity gilts (n=24) were obtained from a commercial herd with no serological evidence or clinical history of PRRSV or swine influenza infection. Gilts were bred by artificial insemination and confirmed pregnant prior to shipment. The bred gilts all had expected farrowing dates within a three-day range. Animals were randomized into four groups of six gilts, with three groups sharing a room, and the fourth in a separate room. Gilts were housed in individual farrowing crates under BSL-2 conditions for the duration of the study.

Animals arrived at the experimental facilities on approximately day 43 of gestation. Vaccine administration occurred on days 50 and 71 of gestation. On day 90 of gestation (19 days post-booster vaccination), the three groups sharing a space were challenged with $1\times10^3$ TCID$_{50}$ of PRRSV strain MN184c by intramuscular injection in the neck. The fourth group was not challenged with PRRSV. Blood was collected from gilts at 4, 11, and 17 days post-challenge.

Following PRRSV challenge, gilts were observed for abortions and stillbirths. All litters were delivered without artificial induction of labor, and total number of piglets born alive was determined at 24 hours post-farrowing. Litters were reared without cross fostering for two weeks post-farrowing and observed for mortality. At termination of the study, all remaining animals were euthanized. The study protocols were reviewed and approved by the Harrisvaccines Institutional Animal Care and Use Committee (Protocol # 050110-06).
Replicon particle vaccines

Open reading frames encoding various PRRSV or swine influenza genes were cloned into the alphavirus replicon vector as previously described (7). Replicon and helper RNAs were produced by in vitro transcription, mixed with Vero cells, and transfected via electroporation prior to overnight incubation (7). Following incubation, RP were harvested by affinity chromatography and formulated with diluent (PBS + 5% sucrose + 1% swine serum). Prior to use, RP were tested for replication competent virus by serial blind passage on Vero cells (17). The titer of each RP was determined by an immunofluorescence assay on Vero cells.

Each component RP to be used in the vaccine candidates was confirmed to express the appropriate protein product by Western blot analysis of RP-infected Vero cell lysates with anti-PRRSV convalescent swine serum (data not shown).

Group A received a PRRS vaccine candidate containing a mixture of two RP expressing both GP5 and M from the same replicon RNA. The two constructs differed in the strain of virus from which they derived the GP5 sequence. Each component RP was provided at 1x10^9 per dose, or 2x10^9 total RP per dose.

Group B received a PRRS vaccine candidate containing a mixture of five different RP, each expressing one of the following genes: GP3, GP4, GP5, and M. Two of the constructs expressed GP5, but they differed in their parental strain. Each component RP was provided at 1x10^9 per dose, or 5x10^9 total RP per dose.
Group C received a control vaccine containing RP expressing the hemagglutinin gene derived from a swine H3N2 influenza strain. The titer of RP was $1 \times 10^9$ per dose. Group D did not receive any injection, and served as a non-vaccinated, non-challenged control group.

**Challenge virus**

The PRRSV strain MN184c was kindly supplied by Dr. Darwin Reicks (Swine Vet Center, Saint Peter, MN). This strain is heterologous to all parental strains used to develop the candidate RP vaccines. The challenge material was titrated on MARC-145 cells to determine the infectious titer, and diluted in PBS to a final titer of $5 \times 10^2$ TCID$_{50}$/ml prior to challenge. Gilts were challenged with 2.0 ml IM, for a total challenge dose of $1 \times 10^3$ TCID$_{50}$ PRRSV.

**Assays**

Anti-PRRSV ELISA (HerdChek X3, IDEXX Laboratories, Westbrook, ME) was conducted according to the manufacturer’s instructions. Sera were tested for anti-GP5 and anti-M antibody responses by Western blotting. Briefly, purified virus was run on SDS-PAGE and transferred to PVDF membrane. Strips of membrane were incubated with swine serum and developed using anti-swine peroxidase-conjugated antibody with TMB substrate. Bands corresponding to GP5 and M were identified based on molecular weight and comparison to positive and negative control samples. Sera were also tested for virus neutralization by two methods. The fluorescent focus neutralization assay was conducted as previously described (19). Sera were tested for neutralizing antibody using porcine alveolar macrophages (PAMs) as previously described (18). Homologous hemagglutination inhibition (HI) was conducted according to standard protocols at the University of Minnesota Veterinary Diagnostic Lab.
**Statistical tests**

Analysis of variance (ANOVA) was used to analyze log$_2$-transformed virus neutralization titers, log$_2$-transformed HI titers, and piglet numbers at various time points. Statistical significance was set at p<0.05.

**Results**

**Immune response**

No gilts seroconverted to PRRSV nucleocapsid protein prior to challenge, based on the HerdChek X3 ELISA. All challenged gilts seroconverted by this assay by 17 days post-challenge, while non-challenged gilts remained seronegative until study termination (Table 2). On the day of challenge, no gilts in Groups C or D had detectable anti-PRRSV GP5 and M antibodies by Western blot. In Group A, all gilts had detectable anti-PRRSV GP5 and M antibody responses. In Group B, five of six gilts had detectable anti-PRRSV GP5 and M antibodies. Groups A and B both were vaccinated with RP that expressed GP5 and M, while Groups C and D were not.

All gilts in Group C developed HI antibody titers to homologous H3N2 influenza virus by the day of challenge, with a geometric mean titer of 1:275. No gilts in Groups A, B, or D developed detectable HI antibody titers at any point in the study.

No gilts developed detectable (≥ 1:4) PRRSV-neutralizing antibody titers prior to challenge, as measured by both the PAM VN assay and the FFN assay.
**Litter performance**

There were no statistically significant differences between groups in the average total litter size (both live and dead births combined). Average live births per litter in Groups A, B, and C (challenged groups) were significantly lower than Group D (non-challenged group) (p<0.005) (Figure 1). At one week post-farrowing, Group B had significantly lower percent litter survival than both Groups C and D (p<0.05) (Figure 2). At two weeks post-farrowing, Groups A, B, and C had significantly lower percent litter survival than Group D (p<0.005) (Figure 2). The total numbers of litters per group containing at least one piglet by two weeks post-farrowing were: four in Group A; two in Group B; four in Group C; and six in Group D. The total numbers of live piglets at two weeks post-farrowing per group were: ten in Group A; four in Group B; fifteen in Group C; and 53 in Group D.

**Discussion**

These results indicate that two PRRS vaccine candidates evaluated here did not induce a protective response in pregnant gilts, as measured by litter performance. However, the challenge model appears to be effective at inducing significant clinical disease in all challenged animals. The severity of disease induced here may have masked marginal effects of vaccination. Additionally, the heterologous nature of the vaccine antigen and challenge virus may have contributed to the lack of protection, as has been demonstrated previously (8).

Prior work by Osorio et al demonstrated that passive transfer of PRRSV-neutralizing antibodies to pregnant gilts protected offspring against PRRSV challenge (13). The failure of
the RP candidate vaccines to induce neutralizing antibodies is consistent with the observed outcomes in litter mortality. The antigens required to induce significant levels of PRRSV-neutralizing antibodies remains an unresolved question. This study used either a combination of GP5 and M, or a combination of GP3, GP4, GP5, and M. It is possible that one or more of the antigens was not expressed in the required conformation in vivo. It may also be the case that the precise manner of expression of these antigens is critical, and that further refinement of the RP platform is needed. For example, it has been demonstrated that the three minor glycoproteins (GP2, GP3, and GP4) form a heterotrimer, and that this complex is required for proper protein trafficking within the host cell (4). The RP vaccines in this study did not include GP2 as an antigen, and it is unlikely that RP expressing different proteins would co-infect the same cell in vivo. Future work should evaluate new techniques to express all required components of the GP2-GP3-GP4 heterotrimer in a way that is conducive to proper trafficking and immune stimulation.

Prior work by our group has demonstrated that the candidate vaccines evaluated here induce specific cell-mediated immune responses, as measured by an interferon-gamma ELISPOT assay (Mogler, unpublished data). It is likely that the gilts that received the PRRS vaccine candidates would have mounted similar responses, but the assay was not conducted during this experiment. Regardless, the induction of such a response in pregnant animals may not be sufficient to mediate protection from PRRSV without an accompanying neutralizing antibody response.
The lack of anti-nucleocapsid antibodies prior to challenge, as determined by ELISA, highlights the compatibility of the RP platform with DIVA vaccine approaches. Given the inability to determine whether an individual animal is truly free of replication-competent PRRSV by serological tests complicates PRRS control efforts. The use of vectored vaccines that are DIVA compatible would greatly improve the clarity of PRRS status descriptions for herds, which is currently based on evaluation of disease severity (e.g. “stable”, “unstable”, etc) (6).

These results provide further evidence that more work is needed to determine both the antigen(s) necessary for stimulating an protective immune response, and the immunological correlate(s) of protection.

**Acknowledgements**

The authors would like to thank Jill Gander, Kay Kimpston-Burkgren, and Kara Burchack for laboratory technical assistance, and Pamela Whitson, Ashley Baert, and the Harrisvaccines staff for animal care and handling. The influenza hemagglutination inhibition assay was performed under the capable supervision of Dr. Marie Gramer at the University of Minnesota. This work was support in part by a grant from the National Pork Board and by internal funding at Harrisvaccines.
Figures

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**Table 1.** Animal experiment design. Groups A and B received candidate PRRS vaccines, Group C received an RP influenza vaccine (H3-RP), and Groups A, B, and C were challenged with PRRSV. Group D was not vaccinated and was not challenged with PRRSV.

Group A received a combination of two RP, each expressing GP5 and M. Group B received a combination of RP, each expressing a single gene (GP3, GP4, GP5#1, GP5#2, or M).

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**Table 2.** Immunological outcomes by group presented as number positive over total animals per group. Groups A and B received candidate PRRS vaccines, Group C received an RP influenza vaccine, and Groups A, B, and C were challenged with PRRSV. Group D was not vaccinated and was not challenged with PRRSV.
Figure 1. Average live piglets per litter. The number of live births per litter was determined by counting live piglets at 24 hours post-farrowing. Groups A and B received candidate PRRS vaccines, Group C received an RP influenza vaccine, and Groups A, B, and C were challenged with PRRSV. Group D was not vaccinated and was not challenged with PRRSV. There were no statistically significant differences observed between groups in total litter size. Groups A, B, and C each had significantly lower average live births per litter when compared to Group D (p<0.005). Error bars represent +/- one standard error of the mean, and groups were compared by ANOVA. Different letter superscripts denote statistically significant differences between groups.
**Figure 2.** Litter survival at one and two weeks post-farrowing. The percentage of piglets remaining alive at one and two weeks post-farrowing was calculated by dividing the number alive at a time point by the original number born alive per litter (“1 week” and “2 weeks”). Groups A and B received candidate PRRS vaccines, Group C received an RP influenza vaccine, and Groups A, B, and C were challenged with PRRSV. Group D was not vaccinated and was not challenged with PRRSV. At one week post-farrowing, Group B survival was significantly lower than that of Groups C and D (p<0.05). At two weeks post-farrowing, Groups A, B, and C each had significantly lower survival when compared to Group D (p<0.005). Error bars represent the standard error of the mean, and group comparisons were by ANOVA. Different letter superscripts denote statistically significant differences between groups within each timepoint.
References


CHAPTER 5: REPLICON PARTICLE PRRS VACCINE REDUCES VIREMIA IN A YOUNG PIG MODEL

A paper to be submitted to Vaccine

Mark A. Mogler, Ryan L. Vander Veen, J. Dustin Loy, D. L. Hank Harris, Kurt I. Kamrud

Abstract

Porcine reproductive and respiratory syndrome (PRRS) is one of the most economically significant diseases of domestic swine. Young pigs suffer from fever, anorexia, and pneumonia, and have reduced productivity. The disease is caused by an RNA virus (PRRSV), which is a member of the genus Arterivirus. Vaccines have variable efficacy and some have a risk of reversion to virulence under field conditions, highlighting the need for improved vaccine technology. Alphavirus-derived replicon particle (RP) vaccines in swine provide a novel approach to PRRS vaccine development. Several RP-based candidate PRRS vaccines were evaluated in young pig PRRS challenge studies. These RP vaccines expressed various combinations of PRRSV structural proteins, including GP3, GP4, GP5, and M. Vaccinated pigs developed specific humoral and cellular immune responses prior to challenge, although neutralizing antibodies were not detected. Some vaccine candidates reduced viremia and/or viral load, as measured by quantitative RT-PCR and virus titration. These results demonstrate that vaccination with RP-based PRRS vaccines can reduce viremia in young pigs. Therefore, it is possible that RP vaccine efficacy as a function of antigen expression could be further optimized to reduce both viremia and disease.
Introduction

Porcine reproductive and respiratory syndrome (PRRS) causes large economic losses for swine producers through both poor reproductive performance and diminished productivity of finishing pigs (11, 20). The disease is caused by an enveloped RNA virus (PRRSV), which belongs to the *Arterivirus* genus in the order *Nidovirales* (3). In young pigs, PRRS is characterized by fever, anorexia, pneumonia, and enhanced susceptibility to co-infection by other swine pathogens (8, 28, 29, 31). Piglets can be infected *in utero* or as neonates, but can also be infected through close contact as growing pigs (28).

The immune response to PRRSV infection is characterized by the delayed appearance of virus neutralizing antibodies, although non-neutralizing antibodies can be detected as early as five days post-infection (17). The initial viremia lasts for several weeks to months, but virus can be detected in lymphoid tissue for much longer, with occasional reappearance of viremia after initial clearance (1, 38). Immunity to reinfection is generally very strong, although exceptions have been observed (13, 14, 30). Immunity to a heterologous virus strain is less robust, although systems for determining immunologically cross-protective groups of PRRSV have not been systematically tested (19). Currently available vaccines, which are most commonly attenuated live virus or inactivated virus, have variable efficacy (21). This may be due, in part, to strain differences, host genetics, additional pathogens, and management practices. The use of attenuated live PRRSV also poses the risk of reversion to virulence, and is not compatible with strategies to differentiate infected animals from vaccinated animals (DIVA).
Experimental efforts to determine the antigen(s) required to induce protective immunity have met with little success. The structural proteins of PRRSV are the obvious targets of vaccine research. Much of the work has investigated GP5 and/or M, since these two components comprise a large proportion of the viral particle by mass, and were initially implicated in cell attachment (5, 7, 33). GP5 has been described as a target of neutralizing antibodies, and a neutralizing epitope in the N-terminal region of GP5 has been identified (2, 6, 23-25, 37, 40, 41). The minor glycoproteins (GP2, GP3, and GP4) have recently been implicated as targets of neutralizing antibodies as well (4, 36). Passive transfer of neutralizing antibodies to pregnant gilts completely protects both the gilts and their offspring from PRRSV challenge (22). However, passive transfer of the same neutralizing antibody preparation to young piglets gives partial protection from PRRSV challenge; in these piglets, lymphoid tissues are infected, but there is an absence of viremia (16).

Alphavirus-derived replicon particles (RP) derived from Venezuelan equine encephalitis virus (VEEV) are propagation-defective RNA virus vectors (26). The safety and efficacy of RP as a vaccine platform is well established in various models of human and animal disease, including both viral and bacterial pathogens (34). Recently, RP derived from attenuated VEEV (strain, TC-83) have been developed as a veterinary vaccine platform (35). The use of TC-83-derived RP allows for manufacturing under BSL-2 conditions, and adds to the inherent safety of the RP approach. The successful development of TC-83-derived RP as an influenza vaccine in swine highlights the potential of the platform as a means to develop
vaccines for other swine pathogens. To this end, RP were created that expressed various structural proteins of PRRSV.

It was hypothesized that immunization with RP expressing PRRSV-derived genes would induce immune responses that afforded protection from PRRSV challenge. To test this hypothesis, a series of experiments were conducted to evaluate the efficacy of several candidate RP vaccines in a young pig PRRSV challenge model. The logical starting point for this screen was the GP5 antigen. Our preliminary data revealed broad neutralizing antibody production (Mogler and Harris, unpublished data) in pigs serially infected with PRRSV strains HLV013 and HLV093. GP5 from both of these strains were provided to animals in combination, using the RP delivery system, to mimic the protection afforded by the parental strains. Furthermore, data from a previous study showed differences in immunogenicity of PRRSV strains (as measured by neutralizing antibody and peptide ELISA) when strains differ in glycosylation of GP5 (9). This glycosylation of GP5 can be successfully reproduced in an RP expression system (Mogler, unpublished data). For the pig immunization trials presented herein, the GP5 component was either derived from a PRRSV strain with four predicted glycosylation sites (HLV349), or a combination of two GP5 variants lacking a predicted glycosylation site at either amino acid residue 34 or 44 (HLV013 and HLV093, respectively). The GP3 and GP4 components were each derived from HLV349.
Materials and Methods

Replicon particle vaccine

Open reading frames encoding various PRRSV or swine influenza genes were cloned into the alphavirus replicon vector as previously described (12). Replicon and helper RNAs were produced by in vitro transcription, mixed with Vero cells, and electroporated prior to overnight incubation (12). Following incubation, RP were harvested by affinity chromatography and formulated with diluent (PBS + 5% sucrose + 1% swine serum). Prior to use, RP were tested for replication competent virus production by serial blind passage on Vero cells (35). The titer of each RP was determined by immunofluorescence assay on Vero cells. Placebo formulations contained only the diluent material.

Parental virus strains used in replicon vectors

A triple-reassortant H3N2 cluster 4 swine influenza virus isolated from a clinical case in 2008 was sequenced, and the hemagglutinin gene sequence was used to in the construction of a replicon vector (H3-RP) (35). Sequences of open reading frames 3, 4,5, and/or 6 derived from PRRSV strains HLV013, HLV093, and HLV349 were de novo synthesized and used to construct replicon vectors for evaluation as vaccine candidates.

Challenge virus

The challenge strain of PRRSV (HLV349) was isolated from a clinical specimen in 2008 and propagated on MARC-145 cells for two passages prior to use in this study. The challenge virus inoculum was prepared by diluting stock virus in phosphate buffered saline (pH 7.4) to
a final titer of $1 \times 10^5$ TCID$_{50}$/ml. Titer of the challenge inoculum was confirmed by titration on MARC-145 cells.

*Animal experiments*

All animals were obtained from a high-health status commercial herd, with no clinical or serological history of PRRSV or influenza infection, at approximately three weeks of age. All groups were challenged intranasally with 2 mL of material from the same stock of virus, for a total challenge dose of $2 \times 10^5$ TCID$_{50}$ PRRSV. At necropsy, a board-certified veterinary pathologist blinded to group treatments scored macroscopic lung lesions according to the methods described by Halbur *et al* (10). The Iowa State University or Harrisvaccines Institutional Animal Care and Use Committees approved all animal study protocols.

**Experiment 1**

Pigs were randomized into groups of ten and allowed to acclimate for one week prior to the study’s initiation. Pigs were vaccinated on day 0 and day 21 of the study with various combinations of RP vaccine candidates or placebo (Table 1). Group A received RP expressing GP5 and M from HLV349; Group B received RP expressing GP5 from HLV013 and HLV093, and M from HLV349; Group C received sham vaccine without RP. Challenge occurred on day 35. Blood samples were collected on days 35, 42, and 49. Pigs were humanely euthanized on day 49, and lung tissues were collected for histopathological examination.
Experiment 2

Pigs were randomized into groups of ten or eleven and allowed to acclimate for one week prior to the study’s initiation. Pigs were vaccinated on day 0 and day 21 of the study with various combinations of RP vaccine candidates or placebo (Table 2). Group A received RP expressing GP3 and GP4 from HLV349; Group B received RP expressing GP5 from HLV013 and HLV093, and M from HLV349; Group C received RP expressing GP3, GP4, and M from HLV349 and GP5 from HLV013 and HLV093; Group D received sham vaccine without RP. Challenge occurred on day 42. Blood samples were collected on day 42, 48, 51 and 56. Pigs were humanely euthanized on day 56, and lung tissues were collected for histopathological examination. The post-challenge period was extended to 21 days in order to collect additional serum samples for viremia analysis.

Experiment 3

Pigs were randomized into groups of eight and allowed to acclimate for one week prior to the study’s initiation. Pigs were vaccinated on day 0 and day 27 of the study with various combinations of RP vaccine candidates or placebo (Table 3). Group A received RP expressing GP5 from HLV349; Group B received RP expressing M from HLV349; Group C received RP expressing GP5 and M from HLV349; Group D received RP expressing GP3, GP4, and M from HLV349 and GP5 from HLV013 and HLV093; Group E received RP expressing GP3 from HLV349; Group F received H3-RP. Group F served as the negative vaccination control, in contrast to the placebo groups in prior experiments. The timing of the second vaccination was changed to day 28 in Experiment 3 to allow for the possibility of enhanced affinity maturation of the antibody population. Challenge occurred on day 49.
Blood samples were collected on day 49, 56, 63 and 70. Pigs were humanely euthanized on day 70, and lung tissues were collected for histopathological examination. As in Experiment 2, the post-challenge period was extended to 21 days for additional viremia analysis.

**Assays**

Viral RNA was extracted from serum samples using the QIAmp Viral RNA Mini kit (Qiagen, Valencia, CA), and processed according to manufacturer’s directions. Extracted RNA samples were used in a quantitative real-time, reverse transcriptase PCR (qPCR) (Applied Biosystems, Carlsbad, CA) assay using the supplied RNA standards to construct a standard curve. Alternatively, RNA samples were used in a similar assay (Tetracore, Rockville, MD) with the standard curve created with *in vitro* transcripts kindly provided by Dr. Jane Christopher-Hennings, South Dakota State University. All qPCR samples were run in duplicate, and the mean, log-transformed RNA copy number per 1 µl was used for further analysis. The qPCR data were used to calculate area under the curve (AUC) from day of challenge until study termination.

Virus titration from serum samples was conducted in triplicate on MARC-145 cell monolayers, and the TCID$_{50}$/mL of each sample was determined by measuring cytopathic effect for one week (27). Sensitivity of the virus titration assay was determined to be >3.20x10$^1$ TCID$_{50}$/ml (data not shown). Virus titration data were used to calculate the AUC from day of challenge until study termination. Serum PRRSV neutralization titers were determined by fluorescent focus neutralization assay, as previously described (39). PRRSV ELISA (HerdChek 2XR or HerdChek X3, IDEXX Laboratories, Westbrook, ME) was
conducted according to manufacturer’s directions. Histopathological scoring of lung tissue was according methods described by Halbur et al (10).

Statistical analysis
Analysis of variance (ANOVA) was used to analyze AUC values, ELISA S/P ratios, log$_2$ transformed FFN titers, log$_{10}$ transformed serum virus TCID$_{50}$, and log$_{10}$ transformed viral RNA copy number. Microscopic and macroscopic lung lesions were compared by t-test. Statistical significance was set at p<0.05.

Results
Experiment 1
Animals were vaccinated with a two-dose regimen on day 0 and day 21 of the study with various combinations of RP vaccine candidates or a sham vaccine, then challenged on day 35. Group A received RP expressing GP5 and M from HLV349; Group B received RP expressing GP5 from HLV013 and HLV093, and M from HLV349; Group C received sham vaccine without RP (Table 1).

All Group A (10/10) and a majority of Group B (7/10) animals were seropositive by Western blot for anti-GP5 and anti-M antibodies as determined by Western blot. No animals from Group C were seropositive by this assay. Prior to challenge, no animals seroconverted to PRRSV nucleocapsid (N) protein as measured by IDEXX HerdChek ELISA. All pigs became seropositive by study termination. Group B mean S/P ratios were significantly lower than Group C at study termination (p=0.003), but no other statistically significant differences
were observed. No virus neutralizing antibody titers were detected in any groups on the day of challenge.

No significant differences were observed between groups in macroscopic or microscopic lung lesions, and lung pathology was mild in all pigs. No significant differences were observed between groups in qPCR titers at 7 or 14 days post-challenge. Serum virus titers on MARC-145 cells from Groups A and B were significantly lower at 14 days post-challenge versus Group C (p=0.0003 and p=0.03, respectively) (Figure 1). Virus titration AUC was significantly lower for Group A, when compared to Group C (p=0.0005), but no other differences were observed (Figure 2). Group B ELISPOT titer was significantly higher versus Group C on day of challenge (p=0.01), although not significantly different post-challenge (Figure 3). The qPCR AUC was significantly lower in Group B when compared to Group C (p=0.036), but no other statistically significant differences were observed (Figure 4).

Experiment 2

Animals were vaccinated with a two-dose regimen on day 0 and day 21 of the study with various combinations of RP vaccine candidates or a sham vaccine, then challenged on day 42. Group A received RP expressing GP3 and GP4 from HLV349; Group B received RP expressing GP5 from HLV013 and HLV093, and M from HLV349; Group C received RP expressing GP3, GP4, and M from HLV349 and GP5 from HLV013 and HLV093; Group D received sham vaccine without RP (Table 2).
No animals seroconverted to PRRSV N protein prior to challenge, and all pigs seroconverted by study termination. There were no statistically significant differences between group mean S/P ratios at any time post-challenge. No virus neutralizing antibody titers were detected in any animals pre- or post-challenge. No statistically significant differences between groups were observed in either macroscopic or microscopic lung lesions, and lung lesions were mild in all pigs.

No significant differences in qPCR titers at any time post-challenge, and no differences were observed in qPCR AUC. Group C virus titration was significantly lower than Group D at 6 days post-challenge (p=0.036); groups A, B, and C were all significantly lower than Group D at 9 days post-challenge (p=0.004, p=0.002, p=0.005, respectively) (Figure 5). The area under the curve for virus titration was significantly lower for all vaccinated groups (A, B, and C) when compared to Group D (p=0.013, p=0.0006, p=0.005, respectively) (Figure 6). Groups B and C had significantly higher ELISPOT titers on the day of challenge than Group D (p=0.032 and p=0.001, respectively), but no significant differences were observed post-challenge (Figure 7).

Experiment 3
Animals were vaccinated with a two-dose regimen on day 0 and day 27 of the study with various combinations of RP vaccine candidates or a sham vaccine, then challenged on day 48. Group A received RP expressing GP5 from HLV349; Group B received RP expressing M from HLV349; Group C received RP expressing GP5 and M from HLV349; Group D received RP expressing GP3, GP4, and M from HLV349 and GP5 from HLV013 and
HLV093; Group E received RP expressing GP3 from HLV349; Group F received H3-RP (Table 3).

No animals seroconverted to PRRSV N protein prior to challenge, and all pigs seroconverted by study termination. Group A had a significantly lower mean S/P ratio than Group F at 14 and 21 days post-challenge (p=0.048 and p=0.03, respectively), but no other groups had group mean S/P ratios significantly different from Group F. No virus neutralizing antibody titers were detected in any animals before or after challenge. No differences in macroscopic lung lesions were observed, and pathology was classified as mild in all animals.

There were no differences in qPCR titers or qPCR AUC in any group when compared to Group F. Groups B and C had significantly lower virus titers on MARC-145 cells at 7 days post-challenge when compared to Group F (p=0.003 and p=0.001, respectively) (Figure 8); no significant differences were observed in virus titration AUC.

**Discussion**

These results demonstrate that reductions in PRRSV serum virus titer are achievable with a two-dose regimen of replicon particles expressing various combinations of GP3, GP4, GP5, and/or M (Figures 1, 5, and 8). There was also an observed reduction in total viral load, as determined by area under the curve of viremia measures (Figures 2, 4, and 6). The level of viremia in vaccinated animals was reduced by 99% compared to controls in some cases (Figures 1 and 5). The mechanism(s) underlying these phenomena are unknown, although it appears that neutralizing antibodies are not responsible, since there was no detectable
PRRSV neutralizing antibody response prior to challenge in any experiments. Animals that received RP expressing GP5 and M developed specific antibodies by Western blot, suggesting a possible mechanism for the observed effect. In two experiments, the testing of PBMCs with an anti-PRRSV interferon-gamma ELISPOT assay clearly demonstrated the induction of cellular immune responses (Figures 3 and 7). It is conceivable that both humoral and cellular immunity are critical to effect clinically significant protection from PRRSV infection. To this end, future work to refine the antigenic composition of candidate replicon particle vaccines may result in potent induction of both types of immunity.

Recent work has demonstrated an effect on viremia similar to that reported here, although that experiment used peptides corresponding to the GP5 and/or M ectodomains as immunogens (15). This highlights the ability of non-neutralizing antibodies raised against GP5 and/or M to limit viremia, and complicates the interpretation of data from other challenge studies. There is a contradiction between previous reports of neutralizing epitopes located on GP5 (discussed in Introduction), and recent work suggesting that the immune response to GP5 is not protective. Further work is needed to resolve this discrepancy.

In the studies reported here, there were four groups of pigs vaccinated with RP expressing only GP5 and M in combination. Each of those groups had a statistically significant decrease in viremia and/or viral load. Two additional groups received RP expressing GP5 and M in combination with GP3 and GP4, and one of those groups had a statistically significant reduction in viremia and/or viral load. Other groups that had reductions in viremia received RP expressing GP3 and GP4 (one group) or RP expressing M only (one group). No
significant effect on viremia was seen in groups receiving RP expressing GP3 only (one group) or GP5 only (one group). These results are summarized in Table 4. The implication of these results is that the strongest effect on vaccination was seen with RP expressing GP5 and M in combination. Further work is needed to determine the relative importance of each component, and the strain differences that impact vaccine efficacy.

It remains possible that the neutralizing antibody response to replication-competent PRRSV is different in unknown, critical aspects from the response to inactivated virus, subunit proteins/peptides, and vectored antigens. Novel approaches to antigen delivery, including advanced adjuvants and vectored vaccines, will be critical to solving this problem. The lack of neutralizing antibody responses pre- and post-challenge in these studies implies that the candidate vaccines did not prime the animals for a more rapid and/or robust neutralizing antibody response. It is possible that the vaccine candidates reported here are unable to induce a sufficient protective immune response in PRRSV-naïve animals. Future work should examine the effects of RP vaccination in pigs previously infected with either wild-type or attenuated PRRSV strains. The potential differences in immune response in such an experiment may provide insights regarding the relevance of various subunit antigens to both the humoral and cellular immune responses.

In each of the reported experiments, intranasal challenge with PRRSV failed to induce macroscopic lung lesions consistent with “severe” cases reported by other researchers and veterinarians (10, 18, 32, 42). This could be due to many factors, including: the specific source of animals for these trials, the specific strain of PRRSV being tested, the type and
abundance of other pathogens at the time of challenge, and the challenge protocol itself. The qPCR and virus titration data indicate that all pigs were infected and maintaining viral titers in serum within one week of challenge (Figures 1, 5, and 8). Likewise, the IDEXX HerdChek ELISA results do not identify any obvious delay in seroconversion consistent with inadequate challenge. Prior work with this challenge strain observed that challenge doses of $2 \times 10^5 \text{TCID}_{50}$, delivered intranasally in 2 mL, resulted in uniform viremia within 24-72 hours post-challenge (Mogler, unpublished data). Throughout all experiments, animals that were vaccinated with replicon particles did not seroconvert to PRRSV N protein prior to PRRSV challenge, demonstrating the compatibility of the system with DIVA approaches to PRRSV control and eradication.

Ultimately, the results presented here highlight the challenges involved in PRRSV vaccinology, including the variable efficacy and incomplete protection afforded by vectored subunit vaccines. That said, RP vaccination is a promising avenue to pursue for PRRSV control.

**Acknowledgements**

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Table 1. Group treatments for Experiment 1. All animals received the indicated vaccination on day 0 and day 21 by intramuscular injection in the neck. Antigens are described based upon the parental strain of PRRSV from which each gene (GP5 or M) was derived. The RP/dose refers to the number of RP expressing the indicated gene contained in each vaccine dose. Group C received placebo without RP.

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**Figure 1.** Serum virus titration results for Experiment 1. Virus titration from sera collected at 7 and 14 days post-challenge (dpc) was conducted on MARC-145 cells to determine PRRSV TCID₅₀/mL. Statistical comparisons were done by ANOVA. Group A received RP expressing GP5 and M from HLV349; Group B received RP expressing GP5 from HLV013 and HLV093, and M from HLV349; Group C received sham vaccine without RP. Groups A and B had significantly lower viremia than Group C at 14 days post-challenge (p=0.0003 and p=0.03, respectively). Error bars denote +/- one standard error of the mean. Different letter superscripts denote statistically significant differences between groups.
Figure 2. Area under the curve for Experiment 1 serum virus titration. Virus titration data were used to calculate total area under the curve (AUC), and group means were compared by ANOVA. Group A received RP expressing GP5 and M from HLV349; Group B received RP expressing GP5 from HLV013 and HLV093, and M from HLV349; Group C received sham vaccine without RP. Group A had significantly lower viral load than Group C (p=0.0005). Error bars denote +/- one standard error of the mean. Different letter superscripts denote statistically significant differences between groups.
Figure 3. Interferon-gamma production for Experiment 1. An interferon-γ ELISPOT assay was conducted using PBMCs collected on the day of challenge using purified PRRSV as the recall antigen. Results are displayed as group mean spot-forming units per 1x10⁶ PBMC. Group A received RP expressing GP5 and M from HLV349; Group B received RP expressing GP5 from HLV013 and HLV093, and M from HLV349; Group C received sham vaccine without RP. Group B had a significantly higher response than Group C when compared by ANOVA (p=0.01). Error bars denote +/- one standard error of the mean. Different letter superscripts denote statistically significant differences between groups.
Figure 4. Area under the curve for Experiment 1 qPCR. The qPCR data were used to calculate total area under the curve (AUC), and group means were compared by ANOVA. Group A received RP expressing GP5 and M from HLV349; Group B received RP expressing GP5 from HLV013 and HLV093, and M from HLV349; Group C received sham vaccine without RP. Group B had significantly lower viral load than Group C (p=0.036). Error bars denote +/- one standard error of the mean. Different letter superscripts denote statistically significant differences between groups.
Table 2. Group treatments for Experiment 2. All animals received the indicated vaccination on day 0 and day 21 by intramuscular injection in the neck. Antigens are described based upon the parental strain of PRRSV from which each gene (GP3, GP4, GP5 or M) was derived. The RP/dose refers to the number of RP expressing the indicated gene contained in each vaccine dose. Group D received placebo without RP.

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Figure 5. Serum virus titration results for Experiment 2. Virus titration from sera collected at 6, 9 and 14 days post-challenge (dpc) was conducted on MARC-145 cells to determine PRRSV TCID₅₀/mL. Statistical comparisons were by ANOVA. Group A received RP expressing GP3 and GP4 from HLV349; Group B received RP expressing GP5 from HLV013 and HLV093, and M from HLV349; Group C received RP expressing GP3, GP4, and M from HLV349 and GP5 from HLV013 and HLV093; Group D received sham vaccine without RP. Group C had significantly lower viremia than Group D at 6 days post-challenge (p=0.036). Groups A, B and C had significantly lower viremia compared to Group D at 9 days post-challenge (p=0.004, p=0.002, p=0.005, respectively). Error bars denote +/- one standard error of the mean. Different letter superscripts denote statistically significant differences between groups.
Figure 6. Area under the curve for Experiment 2 serum virus titration. Virus titration data were used to calculate total area under the curve (AUC), and group means were compared by ANOVA. Group A received RP expressing GP3 and GP4 from HLV349; Group B received RP expressing GP5 from HLV013 and HLV093, and M from HLV349; Group C received RP expressing GP3, GP4, and M from HLV349 and GP5 from HLV013 and HLV093; Group D received sham vaccine without RP. Groups A, B, and C had significantly lower viral load than Group D (p=0.013, p=0.0006, p=0.005, respectively). Error bars denote +/- one standard error of the mean. Different letter superscripts denote statistically significant differences between groups.
Figure 7. Interferon-gamma production for Experiment 2. An interferon-γ ELISPOT assay was conducted using PBMCs collected on the day of challenge using purified PRRSV as the recall antigen. Results are displayed as group mean spot-forming units per 1x10⁶ PBMC. Group A received RP expressing GP3 and GP4 from HLV349; Group B received RP expressing GP5 from HLV013 and HLV093, and M from HLV349; Group C received RP expressing GP3, GP4, and M from HLV349 and GP5 from HLV013 and HLV093; Group D received sham vaccine without RP. Groups B and C had significantly higher responses than Group D when compared by ANOVA (p=0.032 and p=0.001, respectively). Error bars denote +/- one standard error of the mean. Different letter superscripts denote statistically significant differences between groups.
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**Table 3.** Group treatments for Experiment 3. All animals received the indicated vaccination on day 0 and day 28 by intramuscular injection in the neck. Antigens are described based upon the parental strain of PRRSV from which each gene (GP3, GP4, GP5 or M) was derived (except in the case of Group F). Group F received an RP expressing the hemagglutinin gene of H3N2 swine influenza. The RP/dose refers to the number of RP expressing the indicated gene contained in each vaccine dose.
Figure 8. Serum virus titration results for Experiment 3. Virus titration from sera collected at 7, 14 and 21 days post-challenge (dpc) was conducted on MARC-145 cells to determine PRRSV TCID$_{50}$/mL. Statistical comparisons were by ANOVA. Group A received RP expressing GP5 from HLV349; Group B received RP expressing M from HLV349; Group C received RP expressing GP5 and M from HLV349; Group D received RP expressing GP3, GP4, and M from HLV349 and GP5 from HLV013 and HLV093; Group E received RP expressing GP3 from HLV349; Group F received H3-RP (derived from H3N2 influenza). Groups B and C had significantly lower viremia at 7 days post-challenge when compared to Group F by ANOVA (p=0.003 and p=0.001, respectively). Error bars denote +/- one standard error of the mean. Different letter superscripts denote statistically significant differences between groups.
<table>
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<tr>
<th>Experiment &amp; Group ID</th>
<th>n=</th>
<th>PRRSV neut. antibody</th>
<th>lung score difference</th>
<th>qPCR titer</th>
<th>qPCR AUC</th>
<th>serum virus titer (dpc)</th>
<th>serum virus AUC</th>
<th>ELISPOT IFN-γ</th>
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Table 4. Summary table for Experiments 1, 2, and 3. Column headings indicate the experiment and group ID (e.g., Experiment 1, group C = 1C), as well as various assays conducted in the experiment. The terms “yes” or “no” correspond to whether the indicated group’s result was statistically significant when compared to the control group for that study. “PRRSV neut. antibody” refers to the detection of neutralizing antibodies by FFN assay; “lung score differences” refers to both microscopic and macroscopic lung lesions; “qPCR titer” refers to differences in viremia by PRRSV qPCR assay; “qPCR AUC” refers to the area under the curve of qPCR data; “serum virus titer (dpc)” refers to differences in serum virus titration, and the number in parentheses indicates the day post-challenge where differences were detected; “serum virus AUC” refers to area under the curve of serum virus titration data; “ELISPOT IFN-γ” refers to the interferon-gamma ELISpot titer. “nd” means no data available.
References


CHAPTER 6: GENERAL CONCLUSIONS

The results presented here demonstrate that the alphavirus replicon particle (RP) vaccine platform is a powerful tool for the investigation of both innate and adaptive immunity to PRRSV infection. The use of RP vaccines in young pig PRRSV challenge studies provided significant reductions in viremia and viral load, although protection was not seen in pregnant gilts. Specific humoral and cellular immune responses to PRRSV GP3, GP4, GP5, and M were induced by RP vaccination when used in a prime-boost vaccination strategy. These studies represent the first reports of RP used for PRRS vaccinology.

In Chapter 3, it was shown that administration of an RP influenza vaccine (H3-RP) 24 hours prior to PRRSV challenge resulted in decreased viremia and decreased viral load in young pigs. These data are consistent with the reported references of strong non-specific immune stimulation by RP, even when the vector is expressing heterologous or null genes (4, 8). It is likely that innate immune factors and cytokine responses were responsible for the observed reductions in viremia. Further exploration of these effects may lead to a better understanding of both PRRSV and RP.

The H3-RP vaccinates had significantly reduced viremia between 10 and 17 days post-challenge, suggesting that improved adaptive immunity may have been partly responsible for the mechanism of viral clearance. There was no significant increase in PRRSV-neutralizing antibodies among animals vaccinated with H3-RP compared to controls by the end of the study (21 days post-challenge), which suggests that the innate immune stimulation caused by
RP does not result in virus-neutralizing antibody responses. It could be that other types of adaptive immune responses were responsible for the effect, but those were not directly measured. The work presented in Chapter 3 is the first reported use of heterologous RP vaccines to reduce viremia in a PRRSV challenge model. The subsequent chapters report the first uses of RP to immunize both breeding animals and young pigs against PRRSV antigens.

In Chapter 4, pregnant gilts were immunized with two candidate RP PRRS vaccines and challenged with virulent virus. The RP PRRS vaccines expressed either the GP5/M heterodimer, or a combination of GP3, GP4, GP5, and M. As a control, one group of gilts received the same H3-RP vaccine that was described in Chapter 3. Neither of the RP PRRS vaccine groups had significantly improved litter outcomes compared to the control group. Furthermore, the three PRRSV-challenged groups all had significantly increased litter mortality when compared to non-challenged controls. No vaccinated animals developed virus-neutralizing antibodies prior to challenge, but all animals were successfully challenged with PRRSV based on seroconversion to N protein.

Despite the lack of efficacy, the RP proved to be safe when administered to pregnant swine, which is encouraging for future developmental efforts. The RP vaccine is also compatible with strategies for differentiating infected from vaccinated animals (DIVA), which is a major advantage of RP (and other subunit or vectored) vaccines for future PRRSV control and elimination efforts.
Chapter 5 presents the results of three candidate RP PRRS vaccine evaluation studies in young pigs. In these studies, the RP PRRS vaccines expressed combinations of GP3, GP4, GP5, or M. Reductions in viremia and/or viral load were observed in each study, suggesting that the RP vaccines provided partial protection from challenge.

None of the vaccinated animals in the Chapter 5 studies developed PRRSV-neutralizing antibodies prior to challenge, as in the pregnant gilt study. However, vaccinated animals developed significant PRRSV-specific cellular immune responses, based on an interferon-\(\gamma\) ELISPOT assay. No vaccinated animals seroconverted to N protein prior to challenge, again demonstrating the compatibility of RP with DIVA strategies. Clinical disease in all trials was mild, and no significant differences were observed in lung pathology. The lack of significant lung pathology in the young pig challenge trials is interesting, given the published record of significant clinical disease in similar challenge models (3). However, the viremia observed in serum samples collected in all young pig studies was remarkably consistent across studies. Perhaps the source of experimental animals used for these studies played a role, as there were never noticeable lesions associated with bacterial co-infection at necropsy. Reduction of PRRSV viral load has been associated with improved weight gain in genomic mapping studies, although causality has not been proven at this time (1).

The failure of the candidate PRRS vaccines to elicit a neutralizing antibody response in either pregnant gilts or young pigs was unexpected. Neutralizing epitopes have been described in the ectodomain regions of GP5, GP4, and GP3, and each of these antigens were expressed by RP in the described studies (2, 6, 7, 9-12). Expression analysis was performed for each RP
tested in the described studies by infecting Vero cells with RP, harvesting cell lysates, and conducting Western blot with various reagents. Results of those Western blots were consistent with the interpretation that the RP candidates were capable of expressing the desired protein. Immunogenicity of the GP5 and M components was confirmed by seroconversion of vaccinates to viral GP5 and M, as measured by Western blot. Unfortunately, technical difficulties with the assay prevent analysis of the GP3 and G4 components.

It appears likely that the non-neutralizing antibody generated against GP5 and/or M was able, in part, to reduce viremia in young pigs, at least in some instances. This observation is consistent with recent reports that demonstrate viremia reduction due to non-neutralizing anti-GP5 antibodies (5). Data collected by our group, but not presented here, are also consistent with this interpretation. Published reports by other investigators demonstrate a similar magnitude of viremia reduction, although there are occasional reports of concurrent neutralizing antibody responses in those studies. Regardless, the overall theme of PRRS vaccine development efforts with inactivated, subunit, and vectored vaccines is that of reduced viremia and/or clinical signs, without the near-sterilizing protection afforded by attenuated, homologous vaccine. This points to a profound difference in the nature of the immune response to native PRRSV infection versus inactivated or vectored vaccines.

The body of work presented here raises the question of whether it is possible to elicit meaningful neutralizing antibody titers by immunization with vectored vaccines. It appears that further work will be needed to determine the appropriate antigenic requirements for
inducing such a response. One strategy for elucidating the relevant antigens may involve combining proteomics-based approaches to antigen discovery, rather than simply using inference from already defined, whole viral proteins. Another strategy may require the expression of non-native antigens in order to expose the relevant epitopes to the immune system in a manner compatible with vector-based delivery. Whatever the strategy used to determine these protective antigens, the work presented here make a strong case that RP would be useful as a delivery mechanism.

REFERENCES


strain VR-2332 is located in the middle of the GP5 ectodomain. Archives of virology 147:2327-2347.


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“The first principle is that you must not fool yourself, and you are the easiest person to fool.” -Richard Feynman (1918-1988)

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