RESEARCH REPORT
SWINE HEALTH

Title: Assessment of heteroclite-vectored cytokines as a means to increase efficiency of modified live PRRSV DIVA vaccine preparations - **NPB #12-157**

Investigator: Dr. Kay S Faaberg

Co-Investigator: Dr. Allyn Spear

Institution: USDA/ARS/NADC

Date Submitted: February 3, 2014

Industry Summary:

Many significant hurdles have complicated vaccine development for porcine reproductive and respiratory syndrome virus (PRRSV). This work utilized a naturally occurring byproduct of PRRSV replication called heteroclite RNA that has been shown to be packaged along with normal infectious virus. The first objective of this proposal was to engineer a heteroclite RNA to express proteins known to enhance the immune response. This modified RNA could then be used to augment a modified live vaccine (MLV). The second objective of this proposal was to assess the impact of these augmented MLV preparations in porcine alveolar macrophage (PAM) cells, the natural target cells for PRRSV replication.

The genes for the immune enhancing proteins were successfully cloned from swine tissues, and the PRRSV heteroclite RNAs were genetically modified to allow the insertion of the immune enhancing genes. Several trials were attempted using different methods to get infectious PRRSV MLV to take up the genetically modified heteroclite RNAs, however this proved to be very difficult. The MLV virus was able to generate its own heteroclites so rapidly that they overwhelmed the modified heteroclite RNAs, making the uptake very inefficient. Despite multiple experimental modifications, no protocol was ever able to achieve sufficient modified heteroclite uptake to allow testing in PAM cells. Continued efforts to develop a selection process to force uptake of heteroclite RNAs with immune enhancers are currently in progress. Should these current efforts succeed, this approach still has the potential to enhance the immune response to PRRSV MLV vaccines.

Kay Faaberg
kay.faaberg@ars.usda.gov
515 337-7259
Allyn Spear
allyn.spear@ars.usda.gov
515 337-6416

Keywords: PRRSV, Vaccine, Heteroclite, Immunity, Cytokine

Scientific Abstract:

Many significant hurdles have complicated vaccine development for porcine reproductive and respiratory syndrome virus (PRRSV). In an attempt to enhance the immune response to existing modified live virus (MLV) vaccines, this work utilized a naturally occurring form of subgenomic RNA produced by PRRSV during replication called heteroclite RNA. This heteroclite RNA was previously shown to be packaged with infectious virus, and demonstrated to be translation competent. cDNA clones of two distinct heteroclite RNAs were generated and modified to allow production of authentic heteroclite RNAs upon *in vitro* transcription. The porcine cytokines ILα7, ILα15, ILα18 and CD40L were cloned from porcine thymus/lymph node, and inserted into these modified heteroclite cDNA clones. Using a combination of infection and transfection approaches, attempts were made to co-rescue MLV preparations that contained significant levels of modified heteroclite that expressed one of the aforementioned cytokines. These attempts were unsuccessful, largely due to the unexpected efficiency with which the viral RNA generates its own endogenous heteroclite RNAs and the apparent preference shown by the virus for these endogenous heteroclites over exogenous modified heteroclite RNAs. Continued efforts are currently underway to develop a selection process to force uptake of modified heteroclite RNAs. Should these current efforts succeed, this approach still has the potential to enhance and potentially broaden the immune response to PRRSV MLV vaccines.

Introduction:

Current PRRS vaccines are under constant assault from new and different strains of PRRSV that break through the narrow, shallow immunity provided by current vaccination strategies. Because of this, PRRSV continues to cost the pork industry an estimated $664 million per year. This work sought to develop a completely novel strategy to boost the immune response to modified live virus (MLV) vaccine preparations that could be easily adapted to any live attenuated PRRSV vaccine. The use of immune enhancing proteins has been previously shown to increase PRRSV vaccine efficacy, however these approaches have used complicated, untargeted and/or expensive technologies to do so. This approach attempted to utilize a natural byproduct of PRRSV replication as a “Trojan horse” to deliver cytokine expression cassettes within an MLV preparation. Once developed, this augmented vaccine could be produced with minimal modification of current MLV production techniques, and has the potential to boost the immune response in and around the same cells that are being stimulated by the vaccine, thereby eliciting a deeper, broader immune response to PRRSV.

Objectives:

Objective 1a: Engineer modified heteroclite RNAs expressing pro-inflammatory/pro-memory cytokines. Use a previously obtained clone of a PRRSV heteroclite RNA (from strain VR-2332) as a backbone to express the pro-inflammatory/pro-memory cytokines IL-7, IL-12p70, IL-15, or IL-18.

Objective 1b: Rescue and amplify augmented vaccine stocks. Use simultaneous transfection of modified heteroclite RNA with DIVA-MLV RNA in MARC145 cells to generate primary augmented vaccine preparation. Before and after multiple amplification passages, assess vectored cytokine expression and genetic stability of vaccine stock.

Objective 2: Infect and profile cytokine responses of porcine alveolar macrophages and circulating lymphocytes. Use

augmented DIVA-MLV preparations to infect porcine alveolar macrophages and circulating lymphocytes to assess cytokine response profiles. Analyze this data to assess viability of augmented DIVA-MLV preparations for future vaccine trials.

Materials & Methods:

Generation of heteroclite RNA backbone

The S-2 and S-7 heteroclite cDNAs from the VR-2332 strain of PRRSV were amplified by PCR and cloned into the novel pCHAZ cloning vector, including the promoter for T7 RNA polymerase at the 5' ends. The 3' ends of both clones were further modified by ligating annealed dsDNA oligonucleotides which encoded the 3' end of the poly(A) tail and a *CspCI* restriction enzyme site for linearization leaving an authentic poly(A) end. These clones were subjected to site directed mutagenesis to insert an *SbfI* restriction site just downstream of the PRRSV RNA packaging signal in nsp1α, as well as a *BclI* restriction site further downstream in nsp1α. Each final clone was verified by overlapping DNA sequencing.

Cloning of modified heteroclite cDNAs

A 2A subcloning vector was created by inserting annealed dsDNA oligonucleotides which encoded the porcine teschovirus-1 (PTV-1) 2A peptide (GSGATNFSLLKQAGDVEENPGP) into the pCHAZ DNA vector. The coding sequences for IL-7, IL-15, IL-18, and CD40L were amplified by RT-PCR from total RNA obtained from porcine thymus and lymph node tissues, and subcloned into pGEM-T. Sequences were compared to published sequences and where necessary, site directed mutagenesis was used to repair cloned sequences to match those in GenBank. In addition, a nanoluciferase reporter gene was similarly amplified for use as a control expression cassette. Each cytokine and control was cloned into the 2A subcloning vector, downstream and in frame with the PTV-2A peptide. From these clones, the entire 2A-cytokine expression cassette was cloned into the modified heteroclite cDNA clones described above. Each final clone was verified by overlapping DNA sequencing, and large scale DNA stocks were generated using standard DNA maxi-preparation procedures.

Generation of in vitro RNA transcripts

Modified heteroclite cDNA clones were digested to completion with the *CspCI* restriction enzyme, and full length MLV infectious clone cDNA was digested to completion with the *PacI* restriction enzyme. Linearized DNAs were purified by phenol:chloroform extraction and ethanol precipitation. Purified linearized DNAs were quantitated and 1.0 ug per reaction was used as a template in the mMesssage mMachine T7 RNA transcription kit (Ambion/Life Technologies). The resultant RNA transcripts were purified using LiCl precipitation, and were visualized by glyoxal agarose electrophoresis to ensure appropriate RNA length and quality.

Heteroclite RNA rescue

Infection of MARC145 cells were performed at a range of MOI (as defined as TCID50/cell), from 0.01 to 10. Virus was added to cells and allowed to adhere at room temperature for 1 h with rocking, after which the inoculum was removed and replaced with fresh complete media. Infection were either performed 24 h pre-transfection or at 6 or 24 h post-transfection. Purified transcript RNAs were transfected into MARC145 cells using DMRIE-C transfection reagent (Life Technologies) according to manufacturer's instructions. Heteroclite RNA co-transfection with MLV RNA was performed at a range of mass ratios from 0.1:1 to 10^4:1, and transfections with no RNA and MLV RNA alone were performed as controls. Cell-free supernatants from experimentally infected/transfected wells were transferred to fresh MARC145 cells after 48-72 h and monitored for overt cytopathic effects (CPE) resulting from infection.

RT-PCR for viral and heteroclite RNA

Both cell lysates and cell-free supernatants were examined for the presence of various viral or heteroclite RNAs by RT-PCR. RNA was isolated from cells using TRIzol (Life Technologies), and from supernatants using the viral RNA extraction kit (Qiagen), both according to the manufacturer instructions. For detection of RNA specific to viral genome, primers within the nsp9 coding region were selected for amplification of a 600 bp sequence. These nsp9 sequences are not present in any known heteroclite or DI RNA, and would only detect full length viral RNA genome. For detection of S-2 or S-7 heteroclites, primers were selected that flank the heteroclite transcriptional junction, and PCR conditions were set such that only the shorter heteroclite specific products would be detected. For detection of the cytokine transgene, primers were selected within the cytokine gene, only producing a product in the presence of the specific cytokine modified heteroclite. All RT-PCR was performed using the One-Step RT-PCR kit (Qiagen), and products were visualized by agarose gel electrophoresis and ethidium bromide staining.

Results:

Objective 1a: Engineer modified heteroclite RNAs expressing pro-inflammatory/pro-memory cytokines. Two heteroclite RNA clones (S-2 and S-7) derived from strain VR-2332 were cloned into a new backbone to allow the genetic manipulation required for cytokine expression. These clones were edited to generate RNAs with authentic ends, which should allow more efficient rescue. Site directed mutagenesis was carried out on these clones to introduce restriction enzyme sites for insertion of cytokine genes downstream of the established packaging signal. The cytokines IL-7, IL-15, IL-18 and CD40L were amplified and cloned out of total porcine thymus/lymph node samples, and a reporter gene control (NanoLuc) was also amplified with cloning primers. Each cytokine and reporter gene was subcloned into an expression cassette downstream of the porcine teschovirus 2A peptide, which will self-cleave upon expression, releasing a free cytokine or reporter protein. This 2A-cytokine (or 2A-NanoLuc) cassette was then transferred into the modified heteroclite cDNA clones. A diagram of the resultant RNAs is shown below (Fig. 1).

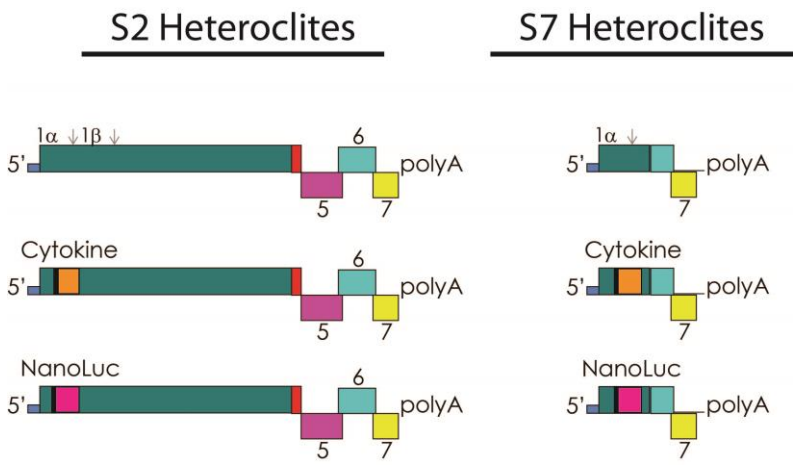

Figure 1. General schematic of heteroclite RNA constructs. S2 (left) and S7 (right) heteroclite RNAs are diagrammed. Unmodified heteroclite clones are shown at top, and heteroclite RNAs containing the cytokine (orange) or NanoLuc (pink) cassettes are shown in the middle and bottom, respectively. Each expression cassette contains an upstream 2A peptide, as indicated by the small black box.

Objective 1b: Rescue and amplify augmented vaccine stocks. The modified heteroclite cDNA clones described above were used to generate RNA transcripts *in vitro*. These *in vitro* transcripts were examined by denaturing agarose gel electrophoresis and shown to be of the correct size and without appreciable degredation (Fig. 2).

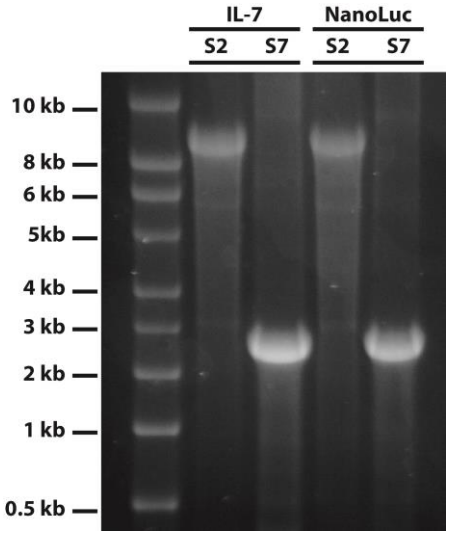

Figure 2. Denaturing agarose gel demonstrating correct size and integrity of *in vitro* transcribed RNA. The much larger S2 heteroclite and smaller S7 heteroclite RNAs are shown, modified by insertion of either the IL-7 gene (left) or NanoLuc control gene (right).

Multiple attempts were made to rescue these modified heteroclite RNAs through infection-transfection, transfection-infection, or co-transfection approaches in MARC145 cells. It was determined through diagnostic RT-PCR that the transfected heteroclite RNAs remained stable and retained their insert after 48 h in culture, however the low passage viral stock being used already contained high levels of endogenous heteroclite RNAs (Fig. 3). Further, the heteroclite RNAs and viral RNA remained detectable in the original cells for the duration of the experiment (Fig. 4); however, upon passage of supernatant to new cells, we were unable to detect any cytokine gene by RT-PCR (Fig. 5).

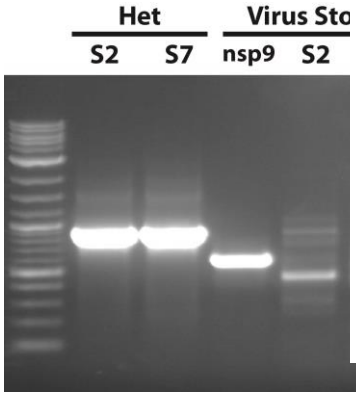

Figure 3. Diagnostic RT-PCR to examine heteroclite RNA stability and virally derived heteroclites. RT-PCR for the IL-7 gene was carried out on cell lysates at 48 h post-transfection ("Het"; Lanes 2 &3). The presence of this band indicates the continued presence of the IL-7 gene. RNA was isolated from the passage 3 stock used for rescue, and analyzed by RT-PCR for the viral nsp9 gene (nsp9; lane 4), as well as for the heteroclite junctions (S2 – lane 5; S7 – lane 6). The presence of multiple bands in the heteroclite junction lanes indicates a vast array of virally derived endogenous heteroclite RNAs already present in the virus population.

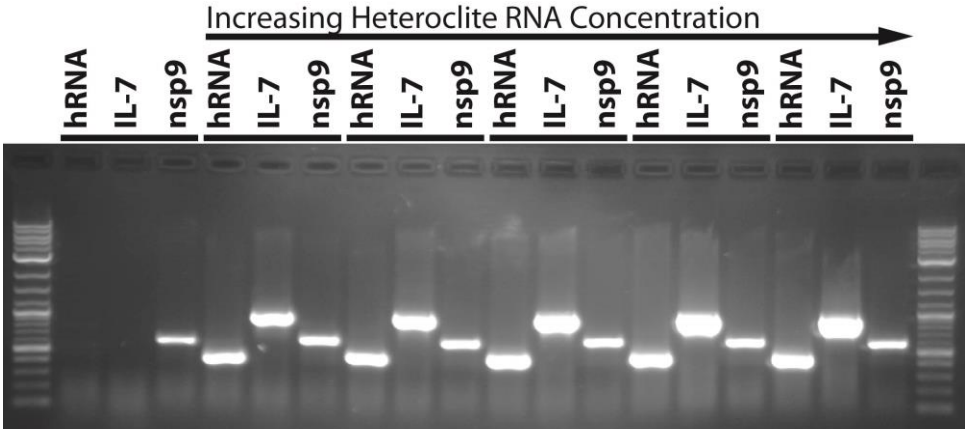

Figure 4. RT-PCR screen of cells co-transfected with full-length viral RNA and increasing concentrations of heteroclite RNA. RNA was extracted from cells 48 h post-transfection, and subjected to RT-PCR using primer pairs specific to the heteroclite junction ("hRNA"), the IL-7 gene ("IL-7"), or the viral nsp9 gene ("nsp9"). The nsp9 gene is only present in full-length viral RNA. Lanes 2-4 did not receive heteroclite RNA, and accordingly do not show bands for hRNA or IL-7.

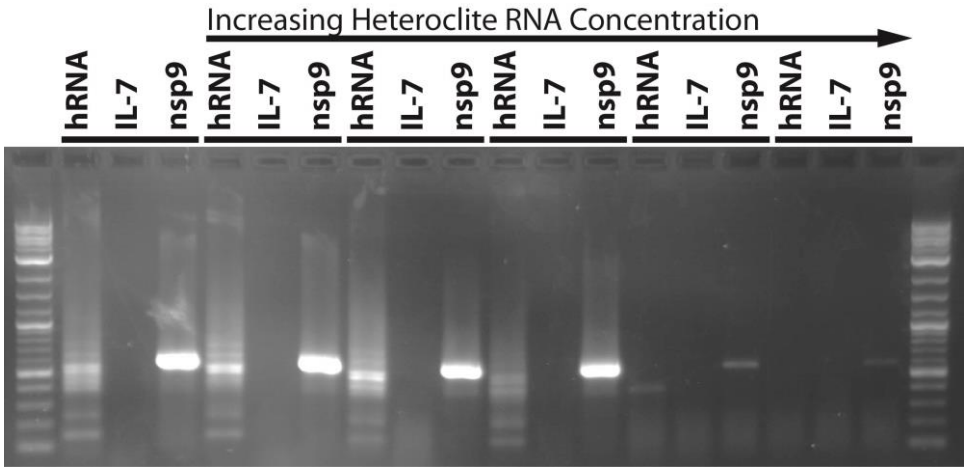

Figure 5. RT-PCR screen of cells infected with supernatants from cells transfected with increasing concentrations of heteroclite RNA. RNA was extracted from cells 96 h post-infection, and subjected to RT-PCR using primer pairs specific to the heteroclite junction ("hRNA"), the IL-7 gene ("IL-7"), or the viral nsp9 gene ("nsp9"). The detected hRNA bands are representative of the population of virally derived heteroclite RNAs, and do not contain the IL-7 gene, as there was no positive signal observed in those lanes.

Despite efforts to transfect a wide range of heteroclite RNA concentrations and transfections at 6 or 24 hours pre-infection, viable MLV containing modified heteroclite was undetectable. Based on the levels of endogenous heteroclite RNA in the low passage viral stock, it is likely that the modified exogenous heteroclites were excluded in favor of the unmodified virally generated heteroclites. To preempt these virally generated heteroclites, attempts were made to co-transfect both the modified heteroclite RNAs with full length RNA generated from an MLV infectious clone. Again, despite examining a wide range of heteroclite RNA concentrations, no appreciable uptake of these modified heteroclites was detected. Without an ability to selectively grow out the desired virus population, even if a minimal degree of modified heteroclite uptake had occurred, there was no mechanism to isolate and enrich for this population.

Objective 2: Infect and profile cytokine responses of porcine alveolar macrophages and circulating lymphocytes. The unsuccessful rescue of viral populations that included the cytokine-modified heteroclites prevented the infection of porcine alveolar macrophages and downstream investigation. Also, the funds were exhausted due to considerable time and extra reagent cost attempting to verify the presence of the heteroclites.

Discussion:

Physical generation of the DNA and RNA constructs proposed in objective 1a was accomplished successfully. This includes the cDNA cloning of two heteroclite RNAs, modification of these clones to allow generation of authentic RNA transcripts, and further modification to allow insertion of cytokine expression cassettes. Additionally, porcine cytokine cDNAs encoding IL-7, IL-15, IL-18 and CD40L were cloned directly from thymus/lymph node samples. These molecular reagents will facilitate future study of the role of heteroclite RNAs in PRRSV replication, and serve as foundational clones for continued work into the possible use of modified heteroclite RNAs in PRRSV MLV vaccine preparations.

After multiple and varied attempts to rescue modified heteroclite RNAs in combination with an MLV vaccine strain, it became evident that uptake of these modified heteroclites was miniscule and at times undetectable. Through these efforts, we were able to determine that even low passage viral stocks generated from rescued infectious clones possessed significant levels of endogenous heteroclite RNAs that likely overwhelmed the exogenous modified

heteroclite RNAs. Given this disequilibrium in favor of endogenous heteroclites, it is evident that some form of selective pressure will be required to enhance uptake of modified heteroclites and permit expansion of this selected population. This work has directly informed our current and future work in developing a new system for selective uptake of modified heteroclite RNAs, which then has the possibility of accomplishing the goals set forth in this project proposal.