RESEARCH REPORT
SWINE HEALTH

Title: Use of interferon alpha as an immunomodulator and metaphylactic therapeutic during PRRSV outbreaks
NPB #13-185

Investigator: Susan L. Brockmeier

Institution: USDA-ARS-National Animal Disease Center

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Industry Summary:

The host response to virus infection begins almost immediately with recognition of virus by host cells leading to the production of antiviral substances such as interferons. One interferon, interferon alpha (IFNα), plays a significant role in the antiviral immune response by stimulating the production of an antiviral state that inhibits viral replication in the host cells. In addition, IFNα plays a role in stimulating the adaptive immune response which is responsible for clearing the virus and preventing future infections. Compared to other viruses that infect the respiratory tract, such as swine influenza virus, porcine reproductive and respiratory virus (PRRSV) appears to induce little IFNα production in the pig. This might be one reason for the persistence of PRRSV in the host and the inadequate immune response to the virus and vaccines. Thus if we could increase the amount of IFNα present during PRRSV infection we may be able to inhibit the virus from replicating, causing disease, and spreading as well as improve the immune response to vaccination to prevent future infections. The objectives of this project were to 1) determine whether IFNα could be used as an adjuvant (a substance that enhances the body's immune response to a vaccine) with attenuated PRRSV vaccine; and 2) determine the effectiveness of metaphylactic use of IFNα during an outbreak of PRRSV. Metaphylactic use is defined as the timely mass medication of a group of animals to eliminate or minimize an outbreak of disease.

For objective 1, pigs were divided into 4 groups and given the following treatments: Group 1 received Ingelvac PRRS ATP vaccine only, group 2 received IFNα and Ingelvac PRRS ATP vaccine, group 3 received IFNα only, and group 4 did not receive either vaccine or IFNα for comparison. After vaccination blood was collected for virus detection and immune assays. The pigs in group 1, which were given the vaccine only, replicated the virus and developed a typical immune response to PRRSV vaccine. Conversely, no virus was recovered from the pigs in group 2 (given the vaccine with IFNα) after vaccination and these pigs did not seroconvert or develop an immune response after vaccination. Originally the pigs in groups 1-3 were to be challenged with PRRSV after vaccination to determine which group was best protected. Since the vaccine virus did not appear to replicate in pigs in group 2 and no measurable immune response was detected, the experimental design was changed and all pigs in groups 1-3 were given a second dose of the vaccine to determine if there would be a boost in the response to the vaccine in either group 1 or 2. The pigs in group 1 that had initially replicated the vaccine virus and developed an immune response did not respond to the second dose of vaccine. Pigs in group 2 and 3 responded in a similar manner replicating and developing an immune response to the subsequent dose of

vaccine virus demonstrating again that the pigs in group 2 behaved like naïve pigs that had never been exposed to vaccine. Although the presence of IFNα did not prove useful as an adjuvant when given simultaneously with a PRRSV attenuated vaccine under the conditions of this study, the results demonstrating the total inhibition of replication and transmission of the vaccine virus provides further evidence that IFNα has potential for metaphylactic use during an outbreak of PRRSV. It is possible that if the timing of administration of the IFNα is altered, perhaps if it were to be administered a day or two after the vaccine allowing an initial time for replication, or if the vaccine was administered intranasally, it would have the more desired adjuvant effect.

The second objective, determining the effectiveness of metaphylactic use of IFNα has yet to be completed. This experiment will consist of groups of pigs that consisted of both primary PRRSV infected pigs as well as contacts that will be treated with various combinations of IFNα. This experiment will mimic the early phases of an outbreak of PRRSV where some pigs in the herd have already been infected (primary challenged pigs) and some have yet to be exposed (contacts) and will examine the effectiveness of treatment with IFNα on controlling viral replication and disease, as well as transmission during an outbreak. The experiment will consist of pigs that are divided into 4 groups with seeder (directly infected) pigs and contact pigs. The seeders will be challenged with PRRSV. Twenty-four hours after challenge, contacts will be comingled with seeders. In group 1 neither seeders nor contacts will be treated with IFNα, in group 2 only seeders will be treated with IFNα but not contacts, in group 3 only contacts will be treated with IFNα and not seeders, and in group 4 both seeders and contacts will be treated with IFNα. IFNα will be given at the time of comingling and again 3 days later. Samples will be taken from both seeders and contacts to determine if the pigs replicate the virus, transmit the virus and develop an immune response to the virus. In previous experiments, we have shown that with one dose of IFNα pigs challenged with PRRSV had lower febrile responses, decreased lung lesions, delayed virus replication and decrease viral load in the sera. The results of the first objective demonstrating that IFNα can totally inhibit viral replication and transmission of a vaccine strain of PRRSV and preliminary data we have generated thus far indicates that IFNα may be very effective at minimizing disease impact and spread of PRRSV, one of the most devastating and costly diseases to the swine industry.

Susan L. Brockmeier, DVM, PhD
Research Veterinary Medical Officer
USDA-ARS-National Animal Disease Center
Building 20, Room 2529
1920 Dayton Avenue
Ames, IA 50010
susan.brockmeier@ars.usda.gov
Phone: 515-337-7221
Fax: 515-337-7428

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Scientific Abstract:

Type I interferons, such as interferon alpha (IFNα), contribute to innate antiviral immunity by promoting production of antiviral mediators and also play a role in the adaptive immune response. Porcine reproductive and respiratory syndrome (PRRS) is one of the most devastating and costly diseases to the swine industry world-wide and has been shown to induce a meager IFNα response. Previously we administered porcine IFNα using a replication-defective adenovirus vector (AD5-IFNα) and challenged with a moderately virulent PRRSV.

There was a better clinical outcome in pigs treated with IFNα, including lower febrile responses and decreased percentage of lung involvement. Viremia was delayed and there was a decrease in viral load in the sera of pigs treated with IFNα. In addition, there was an increase in the number of virus-specific IFNγ secreting cells, as well as an altered cytokine profile in the lung 14 days post-infection, indicating that the presence of IFNα at the time of infection can alter innate and adaptive immune responses to PRRSV. In this experiment we further explored the use of IFNα as an adjuvant given with attenuated PRRSV virus vaccine to determine if it would result in an enhanced immune response to the vaccine. One injection of the Ad5-IFNα given with the vaccine was able to totally abolish replication of the vaccine virus resulting in no detectible immune response. Although the IFNα did not end up having the desired adjuvant effect, the results are promising for the use of IFNα as a treatment for PRRSV infection. An additional study will examine its use as a metaphylactic treatment for an outbreak of PRRSV to both treat and prevent spread of the virus.

Introduction:

Porcine reproductive and respiratory syndrome virus (PRRSV) is one of the most important disease causing agents to the swine industry world-wide. As the name implies, PRRSV causes both reproductive and respiratory disease and is a major contributor to the porcine respiratory disease complex. PRRSV is an enveloped, single-stranded, positive-sense RNA virus belonging to the Arteriviridae family. In vivo, the virus replicates in macrophages, primarily alveolar macrophages, and has been shown to induce a delayed and inadequate adaptive immune response as well as persistent infections.

The course of infection includes an acute viremic stage, lasting approximately 1 month, followed by a chronic stage in which viremia resolves, but virus can still be isolated from secondary lymphoid tissues. Virus has been reported to persist in some animals for months, with and without shedding. During the course of disease, anti-PRRSV antibody can be detected by a week postinfection; however, neutralizing antibodies do not appear until 4 weeks postinfection or later. Cell-mediated immunity, measured as antigen-specific lymphocyte proliferation, is not detected until 4 weeks after infection. Antigen-specific gamma interferon (IFNγ) secreting T cells can be measured at 2 weeks postinfection, but this response is quite variable among animals and can take nearly 6 months to peak. Attenuated PRRSV vaccines have been shown to prevent morbidity associated with disease and provide protection when pigs are challenged with a homologous strain of the virus. However, vaccination does not prevent disease nor protect against heterologous strains of the virus. Overall, the early adaptive immune response to PRRSV is weak and results in delayed elimination of virus from the host.

The innate immune response to a virus begins almost immediately with recognition of conserved viral epitopes by host cells, such as double-stranded RNA (dsRNA). Type I interferon (IFNα/β) plays a significant role in the innate antiviral immune response by stimulating the production of antiviral mediators, such as Mx (myxovirus resistant; IFN-inducible GTPase) and PKR (double-stranded RNA dependent protein kinase), which inhibit viral replication. In addition, IFNα/β plays a role in the adaptive immune response by stimulating dendritic cell maturation and acting as a survival factor for activated T-cells.

Compared to other viruses that infect the respiratory epithelial cells, such as swine influenza virus or porcine respiratory coronavirus, PRRSV appears to induce only modest levels of IFNα and pro-inflammatory cytokines. Several mechanisms have been proposed as to how PRRSV inhibits type I IFN production, and multiple mechanisms may apply. PRRSV has been shown to inhibit dsRNA activation of interferon regulatory factor 3 (IRF3) via inactivation of IFNβ promoter stimulator 1 (IPS-1), an adaptor molecule in the retinoic acid-inducible gene 1 (RIG-1) pathway. Others have proposed that PRRSV interferes with modification of IκB, either through nsp2 OTU domain-mediated inhibition of polyubiquitination or nsp1α-mediated inhibition of phosphorylation, ultimately leading to impairment of NF-κB activity.

In 1986, IFNα was the first protein biotherapeutic approved by the FDA for use in humans. The biotherapeutic value of IFNα has also been investigated in livestock for potential uses as adjunctive therapies, adjuvants or as disease preventatives for various respiratory and enteric diseases of swine and cattle. A barrier to overcome for successful protein biotherapeutics is to devise delivery methods and forms of the protein that can be conveniently administered and have a sufficient duration of biological activity to provide a benefit against disease. Recently, it was shown that delivery of recombinant porcine IFNα via an adenovirus vector was capable of protecting pigs from foot-and-mouth disease virus (FMDV). A strategy based on a combination of delivery of IFNα and FMDV antigen for immediate innate protection while an adaptive immune response to FMDV is developing has been suggested as a control measure for outbreaks of FMD. The study proposed here is designed to determine whether porcine IFNα delivered via an adenovirus vector could be effective used metaphylactically (the timely mass medication of a group of animals to eliminate or minimize an expected outbreak of disease) and with simultaneous PRRSV vaccination.

Objectives:

1) Determine the efficacy of IFNα as an adjuvant with attenuated PRRSV vaccine. IFNα plays a role in the development of the adaptive immune response as well as the innate immune response, and there is little IFNα produced during PRRSV infection which might be one reason there is an inadequate adaptive response to the virus. In a previous experiment, there was an increase in the number of virus-specific IFN-γ secreting cells detected in the pigs receiving Ad5-pIFNα when challenged with PRRSV. In this experiment we examined the development of antibody titers and cell mediated responses when IFNα was present at the time of vaccination. We gave pigs 1 injection of the adenovirus/ IFNα vector since we theorized 2 injections may eliminate the replication of PRRSV in vivo, and we had shown effectiveness with 1 injection with PRRSV infection.

2) Determine the effectiveness of metaphylactic use of IFNα during an outbreak of PRRSV. Metaphylactic use is defined as the timely mass medication of a group of animals to eliminate or minimize an outbreak of disease. Since we have shown that a short duration of administration of IFNα improves the outcome of PRRSV infection, we want to test if longer duration of IFNα can eliminate or minimize and outbreak. Thus we will be testing the efficacy of delivery of IFNα for immediate innate protection to limit virus replication, disease, and transmission. In previous experiments, we have shown that pigs inoculated with Ad5-pIFNα and challenged with PRRSV had lower febrile responses, decreased lung lesions, delayed viremia and decrease viral load in the sera. There was endogenous production of IFNα through parenteral administration of the Ad5-pIFNα for 2 to 3 days. Thus, we would like to determine whether giving 2 injections of the adenovirus vector 3 days apart will further reduce or eliminate viral replication.

Materials & Methods:

Experiment 1: Determine the efficacy of IFNα as an adjuvant with attenuated PRRSV vaccine (Completed). Forty 4-week-old pigs were divided into 4 groups of 10 pigs each and given the following treatments: Group 1 received Ingelvac PRRS ATP vaccine only, group 2 received IFNα and Ingelvac PRRS ATP vaccine, group 3 received IFNα only, and group 4 were non-treated controls. A recombinant, replication-defective human adenovirus type 5 was used to deliver porcine IFNα (Ad5-pIFNα). On days 0, 1, 3, 5, 7, and 14 after vaccination blood was collected for virus isolation. On days 21 and 28 after vaccination blood was collected for peripheral blood mononuclear cell isolation to perform ELISpot assays and for serum isolation to determine antibody levels. Originally the pigs in groups 1-3 were to be challenged with virulent PRRSV isolate 6 weeks after vaccination, however, since no virus was recovered from the pigs in group 2 after vaccination and these pigs did not seroconvert or develop any measurable cell mediated immune response after vaccination, the experimental design was changed and all pigs in groups 1-3 were boosted with Ingelvac PRRS ATP on day 49

post 1st vaccination to determine if there would be an anamnestic response to the vaccine in either group 1 or 2. Blood was collected on day 49, 53, and 58 to collect serum for virus isolation and to test for antibody response and on days 49 and 58 for peripheral blood mononuclear cell isolation to perform ELISpot assays.

Experiment 2: Determine the effectiveness of metaphylactic use of IFNα and during an outbreak of PRRSV (Still to be completed). This experiment will consist of 4 groups of pigs that consisted of both primary PRRSV infected pigs as well as contacts that will be treated with various combinations of Ad5-pIFNα. This experiment will mimic the early phases of an outbreak of PRRSV where some pigs in the herd have already been infected (primary challenged pigs) and some have yet to be exposed (contacts) and will examine the effectiveness of treatment with IFNα on controlling viral replication and disease, as well as transmission during an outbreak. Because of the results of experiment 1 that showed that the Ad5-pIFNα inhibited replication of the vaccine virus we decided to alter the originally proposed experiment to just look at the effectiveness of metaphylactic use of IFNα during an outbreak of PRRSV. The revised experiment will consist of 48, 4-week-old pigs that are divided into 4 groups with 4 seeder (directly infected) pigs and 8 contact pigs each. The seeders will be challenged with 10^4 $CCID_{50}$ PRRSV SDSU73 intranasally. Twenty-four hours after challenge, contacts will be comingled with seeders. In group 1 neither seeders nor contacts will be treated with IFN, in group 2 only seeders will be treated with IFN but not contacts, in group 3 only contacts will be treated with IFN and not seeders, and in group 4 both seeders and contacts will be treated with IFN. Ad5-pIFNα will be given at the time of comingling and again 3 days later. One week after comingling the seeder pigs will be removed to separate isolation rooms. Seeders will be euthanized and necropsied on day 14 post challenge with PRRSV and contact pigs on day 21. Nasal swabs and blood samples will be collected on days 0, 1, 3, 5, 7, 10, 14, and 21 relative to challenge with PRRSV.

PRRSV isolation and detection. Viral titers in sera, nasal swabs, and lung lavage were measured by virus isolation. Virus isolation was performed by adding 100 µl of serum or lung lavage to a monolayer of MARC-145 cells in 1 well of a 24 well plate. After 2 hours media containing the sample was removed and replaced with fresh media. Each well was examined for cytopathic effect (CPE) and assessed as positive or negative after one week of culture.

Humoral immune response. Seroconversion to PRRSV was determined by an ELISA kit. ELISA S/P ratios were generated on collected serum samples by performing the HerdCheck® PRRS ELISA 2XR (IDEXX Laboratories) according to manufacturer's instructions.

Cell mediated immune response. To assess antigen-specific IFNγ responses, whole blood was collected into BD Vacutainer® CPT tubes with sodium citrate and the peripheral blood mononuclear cell (PBMC) fraction was collected according to the manufacturer's recommendations. PBMC's were washed once with RPMI-1640 (Invitrogen), passed through a 40 µm screen filter, washed a second time and enumerated. An ELISpot assay for IFNγ secreting cells was performed as previously described with slight modifications (Zuckermann). Briefly, 96-well membrane plates (MAIPS4510) were pre-wetted with 35% ethanol, washed, and coated overnight at 4° C with 6 µg/ml anti-pIFNγ (P2G10, BD Biosciences). The next day, the plate was washed and blocked with complete RPMI [RPMI-1640, 10% fetal bovine serum, 2mM L-glutamine, 1% antibiotic/antimycotic (Invitrogen), and gentamicin] for 2h at 37° C. The blocking medium was removed and 5x105 PBMCs were plated per well. Appropriate wells were treated with live homologous PRRSV at an MOI=1 (JA142 or SDSU73), control MARC medium, or phytohemagglutinin (PHA) added to a final concentration of 10 µg/ml (each treatment in triplicate) and the plates will be incubated for 18 h at 37° C in 5% CO2. After 18 h, plates were washed and incubated with anti-IFNγ detection antibody (0.5 µg/ml, P2C11, BD Biosciences) for 2 h at 37° C. Plates were washed and developed using ELISpot Blue Color Module (R&D Systems) according to the

manufacturer's recommendations. Plates were scanned and spots enumerated using CTL-ImmunoSpot® S5 UV Analyzer and ImmunoSpot software.

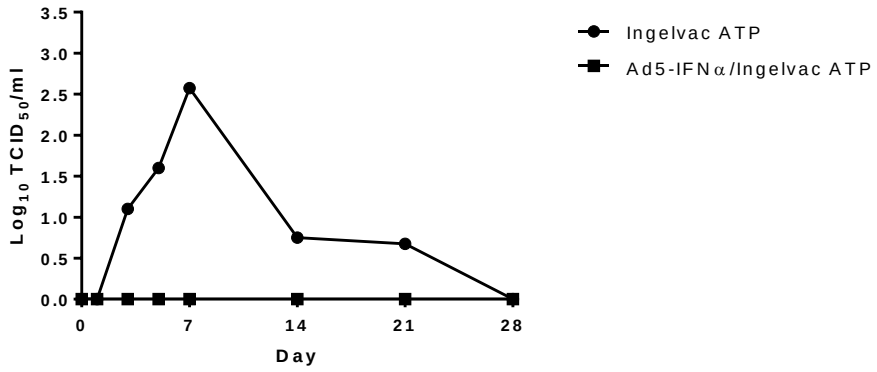
Results:
Experiment 1:

Table 1: Experimental Design

Group	Primary Vaccine	Boost
Group 1	**Ingelvac ATP**	**Ingelvac ATP**
Group 2	**Ad5-pIFNα + Ingelvac ATP**	**Ingelvac ATP**
Group 3	**Ad5-pIFNα**	**Ingelvac ATP**
Group 4	**None**	**None**

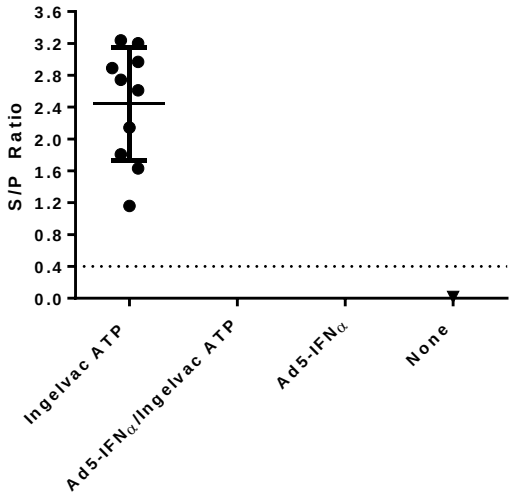
Viremia following primary vaccination. Virus was isolated from the sera of all pigs from group 1, which received Ingelvac ATP vaccine, from at least one blood sample taken between days 5 and 21 after primary vaccination. No virus was isolated from the sera from any of the pigs in group 2, which received Ingelvac ATP and Ad5-pIFNα, through day 28 after primary vaccination, an indication that the presence of pIFNα inhibited viral replication in these pigs. Figure 1 shows the viral titers from sera from pigs in groups 1 and 2.

Figure 1: Geometric mean serum virus titers through day 28 after primary vaccination.



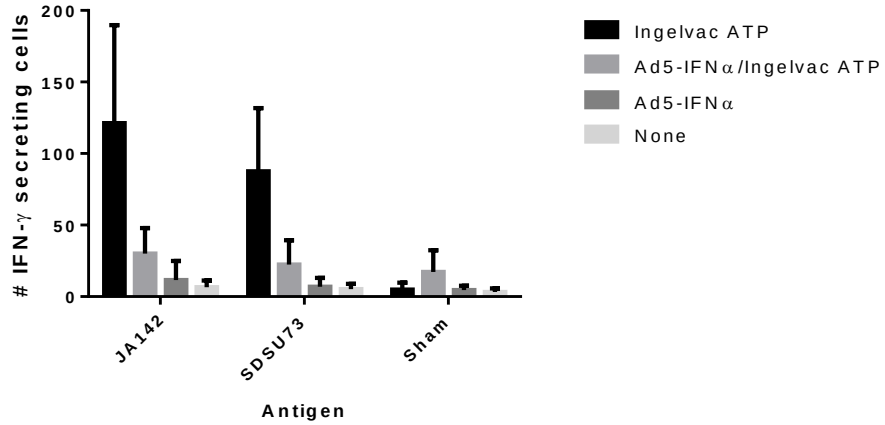
Seroconversion to PRRSV following primary vaccination. Using the recommended cut-off value of a S/P ratio of 0.400 measured by the HerdCheck® PRRS ELISA, all pigs in group 1, which received Ingelvac ATP vaccine, seroconverted to PRRSV as by day 28 after primary vaccination (Figure 2). No pigs in group 2, which received Ingelvac ATP and Ad5-pIFNα, had seroconverted by day 28 after primary vaccination (Figure 2), another indication that pIFNα inhibited viral replication.

Figure 2: S/P ratios of serum samples using the HerdCheck® PRRS ELISA on day 28 following primary vaccination.



Cell mediated immune response following primary vaccination. To determine the effects of Ad5-pIFNα administration on the cellular immune response to PRRSV vaccine, an IFN-γ ELISpot assay was used to enumerate the number of antigen-specific IFN-γ secreting cells (ISCs) circulating in the peripheral blood. On day 21 following primary vaccination, PRRSV-specific ISCs were detected in pigs from group 1, which received Ingelvac ATP vaccine (Figure 3). However, similar to the humoral response, no increase in ISCs was detected in pigs in group 2, which received Ingelvac ATP and Ad5-pIFNα (Figure 3), therefore there was no indication the immune system of these pigs responded to the vaccine.

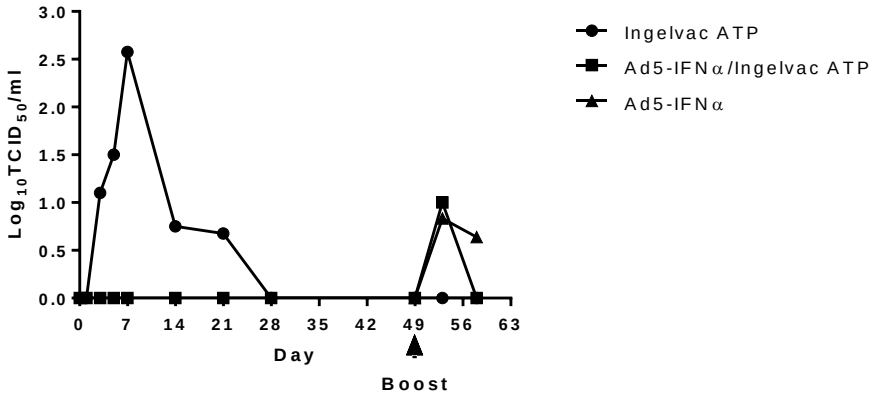
Figure 3: Number of PRRSV-specific IFN-γ secreting-cells on day 21 after primary vaccination with Ingelvac ATP with or without Ad5-IFNα. Cells were stimulated with either PRRSV JA142 (parent virus of Ingelvac ATP), PRRSV SDSU7, or media with no virus (sham).



Viremia following boost vaccination. After boost vaccination with Ingelvac ATP, no virus was isolated from the sera from pigs in group 1, which had already been given 1 dose of Ingelvac ATP 7 weeks previously. Pigs in group 1 had, in effect, a sterilizing immunity to the homologous vaccine virus. Virus was isolated from the sera from pigs in group 2 (originally given Ingelvac ATP and Ad5-pIFNα) and group 3 (originally given Ad5-pIFNα)

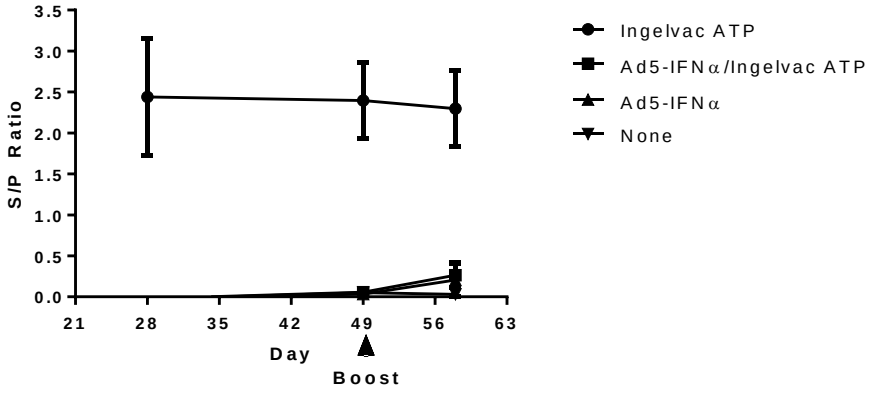
after boost vaccination with Ingelvac ATP. The comparable results between groups 2 and 3 indicate that the pigs previously given Ingelvac ATP and Ad5-pIFNα behaved similarly to naïve pigs. Figure 4 shows the viral titers from sera from pigs in groups 1-3.

Figure 4: Geometric mean serum virus titers after boost vaccination.



Antibody response to PRRSV following boost vaccination. The S/P ratio values for pigs in group 1 were similar or slightly lower on day 58 (9 days post boost) as compared to just prior to giving the boost vaccination, indicating no anamnestic response to the vaccine. The S/P values in all pigs in groups 2 and 3 were rising by 9 days post boost and 2 pigs in group 2 and 1 pig in group 3 had seroconverted using the recommended cut-off value of an S/P ratio of 0.400 measured by the HerdCheck® PRRS ELISA. There was no difference, however, between the values for group 2 and group 3 indicating that group 2 was behaving as naïve to the vaccine and had no anamnestic response that might indicate some prior exposure.

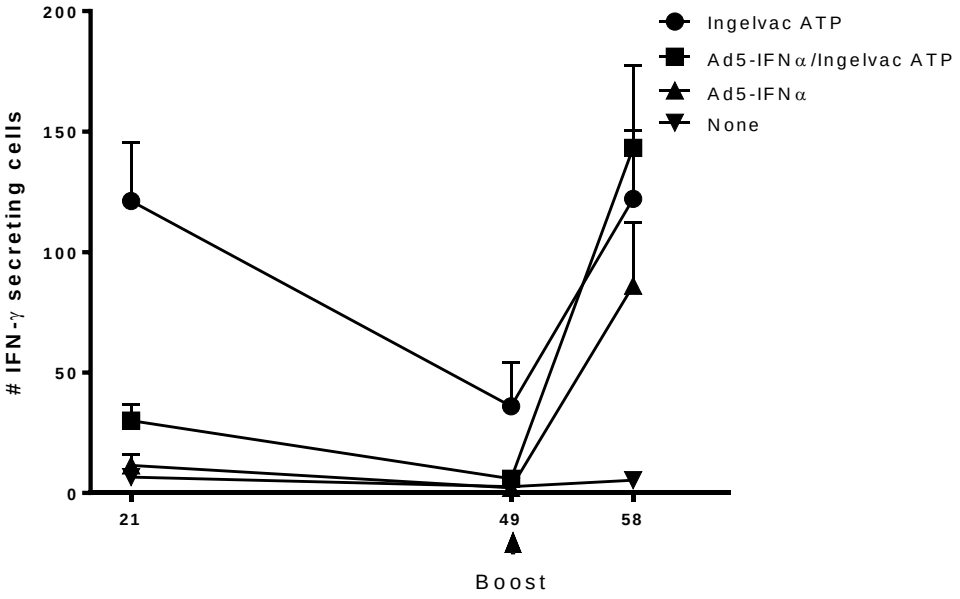
Figure 5: S/P ratios of serum samples using the HerdCheck® PRRS ELISA following boost vaccination.



Cell mediated immune response following boost vaccination. Nine days following boost vaccination, an increase in PRRSV-specific ISCs was detected in pigs from groups 1-3 (Figure 6). The magnitude of the response was similar for all three groups. Although the response from pigs in groups 1 and 2 might be expected this quickly indicating an anamnestic cell mediated response, the fact that pigs in group 3 responded similarly would argue against this explanation. Although the number of ISCs on day 49 had decreased in pigs from group 1 as compared to day 21, there was still an indication of a specific cell mediated immune response in

these pigs from the primary vaccine. There was no indication of a preexisting response on day 49 just prior to boost in the pigs in groups 2 and 3.

Figure 6: Number of PRRSV-specific IFN-γ secreting-cells after boost vaccination with Ingelvac ATP. Cells were stimulated with PRRSV JA142 (parent virus of Ingelvac ATP).



Discussion:

Although the presence of pIFNα did not prove useful as an adjuvant when given with a PRRSV attenuated vaccine under the conditions of this study, the results indicating the total inhibition of replication and transmission of the vaccine virus provides further evidence that pIFNα has potential for metaphylactic use during an outbreak of PRRSV. The differences seen in this experiment compared to our previous experiments, where pigs inoculated with Ad5-pIFNα and challenged with PRRSV had delayed viremia and decrease viral load but virus replication was still detected, may be due to a couple of reasons. One may be the differences in virulence and viral replication between an attenuated vaccine virus and a virulent field virus. The attenuated virus may be more sensitive to the effects of pIFNα. Secondly, in this experiment both the virus and Ad5-pIFNα were given intramuscularly as compared to previously when the Ad5-pIFNα was given ntramuscularly and the virus was given intranasally. The pIFNα levels may not be as high in the lung and this may allow for initial viral replication to occur in alveolar macrophages and overcome the systemic pIFNα levels that are only present for a few days when Ad5-pIFNα is administered once. It is possible that if the timing of administration of the pIFNα is altered, perhaps if it were to be administered a day or two after the vaccine allowing an initial time for replication, or if the vaccine was administered intranasally, it would have the more desired adjuvant effect.

We still plan to complete the second experiment, determining the effectiveness of metaphylactic use of IFNα, this spring. Initially both these experiments were to be completed simultaneously; however we lost some of our purchased pigs to existing infections and ended up with only enough pigs to complete the first objective. We are currently producing more of the Ad5-pIFNα and will have barn space open this spring to conduct the second experiment. We will update the final report when those results are generated.