RESEARCH REPORT
SWINE HEALTH

Title: Recombinant porcine interferon to control PRRSV and select other viral pathogens" – #19-217 IPPA

Investigator: Bradley Blitvich

Institution: Iowa State University

Date Submitted: 12/10/2020

Industry Summary:

Interferons (IFNs), particularly IFN-α and IFN-β, provide one of the first lines of defense against virus infections. Many viruses that affect swine, i.e. porcine reproductive and respiratory syndrome virus (PRRSV), Seneca Valley virus (SVV), porcine epidemic diarrhea virus (PEDV) and swine influenza virus (SIV), encode proteins that block IFN activity. These viruses have a devastating economic impact on the swine industry. Restoring the anti-viral state in pigs by IFN administration could greatly reduce the economic losses associated with viral disease outbreaks. IFN treatment has been successfully used for human healthcare; for example, IFN therapy is the most effective treatment against hepatitis C virus. We have recognized the lack of cost-effective IFN for use in the swine industry. The goals of this project were to produce inexpensive and industrially scalable porcine IFN using recombinant technology and to assess the ability of these IFNs to suppress the *in vitro* replication of PRRSV, SVV, PEDV and SIV in porcine cells. We successfully engineered bacterium to produce large quantities of porcine IFN-α and IFN-β but unfortunately observed no differences in the abilities of the aforementioned viruses to replicate in porcine cells treated with or without varying amounts of IFN.

Contact Information: Bradley J. Blitvich, Department of Veterinary Microbiology and Preventive Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA 50011, email: blitvich@iastate.edu, telephone: 970-294-9861

Key Findings:

- We successfully engineered bacterium to produce large quantities of porcine interferons
- The interferons did not affect the abilities of porcine reproductive and respiratory syndrome virus, Seneca Valley virus, porcine epidemic diarrhea virus and swine influenza virus to replicate in porcine cell lines
- Based on these findings, future studies in our laboratory will not test the abilities of these interferons to protect pigs from viral infection *in vivo*

Keywords:

Interferon, pig, virus, cell culture, immune response

Scientific Abstract:

Interferons (IFNs), particularly IFN-α and IFN-β, provide one of the first lines of defense against virus infections. Many viruses that affect swine, i.e. porcine reproductive and respiratory syndrome virus (PRRSV), Seneca Valley virus (SVV), porcine epidemic diarrhea virus (PEDV) and swine influenza virus (SIV), encode proteins that block IFN activity. These viruses have a devastating economic impact on the swine industry. Restoring the anti-viral state in pigs by IFN administration could greatly reduce the economic losses associated with viral disease outbreaks. The goals of this project were to produce inexpensive and industrially scalable porcine IFN using recombinant technology and to assess the ability of these IFNs to suppress the *in vitro* replication of PRRSV, SVV, PEDV and SIV in porcine cells. Recombinant IFN-α and IFN-β were produced using a *Bacillus megaterium* shuttle vector. First, however, it was necessary to modify the shuttle vector by cloning a cleavable self-aggregating (cSAT) module downstream of the xylose promoter (PxylA). The module consists of the coding sequence for a gyrA intein, a PT linker and a self-aggregating ELK16 tag. Next, the coding sequences of porcine IFN-α and IFN-β were codon optimized and cloned into the vector. The IFN coding sequences were introduced upstream of and in-frame to the cSAT module, resulting in the production of an IFN fusion protein. The IFN genes were be under the control of the inducible promoter PxylA, which could be activated by supplementation of the bacterial culture media with xylose. The shuttle vector was constructed in *E.coli* then transformed directly into *B. megaterium* by electroporation. Transformants were selected for tetracycline resistance. Expression of the fusion proteins was induced by providing xylose to the culture media. Aliquots of culture samples were collected at increasing optical densities until the optical density reached 1.5, at which point the bacteria were pelleted for protein purification. The aliquots were tested for the fusion protein by western blotting using tag-specific antibodies. These experiments revealed that the proteins were successfully purified. Bacterial pellets were sonicated and centrifuged to separate the soluble and insoluble fractions. Due to self-aggregating nature of the ELK16 tag in the cSAT module, the IFN fusion proteins accumulated in the insoluble fractions. IFN was released from the insoluble fractions by activating the self-cleaving ability of the GyrA intein using reducing buffer. The insoluble, cleaved cSAT module was removed from the soluble IFN by centrifugation followed by dialysis in a refolding buffer. The biological activity of purified porcine IFN-α and IFN-β was measured in Vero cells using the lytic vesicular stomatitis virus cytopathic assay. We compared the biological activity of our IFNs to commercially available recombinant human IFN-α and β (BPS BioSciences). Cells were incubated with serial twofold dilutions of each IFN starting with 100 pg/ml for 20 hr. Twenty-four hours after inoculation with VSV, cells were fixed and virus titers were calculated. VSV titers were approximately 1 x 10^8 pfu/ml in the absence of IFN. Virus titers were greatly reduced in the presence of all four IFNs that were tested. These findings demonstrate that the porcine IFNs that we produced are biologically active, although their ability to suppress VSV replication was approximately tenfold less as that observed with the commercially available human IFNs. Next, we assessed the ability of recombinant porcine IFNs to suppress the *in vitro* replication of PRRSV, SVV, PEDV and SIV in swine alveolar macrophages (MARC-145 cells). The cells were cultured in six-well plates then inoculated with virus in the presence or absence of IFN. All four viruses (PRRSV, SVV, PEDV and SIV) were used at a multiplicity of infection of 1.0 and the IFNs (porcine IFN-α and IFN-β) were tested both separately and together. Plaque assay experiments revealed that the ability of each virus to replicate in MARC-145 did not significantly differ when comparisons were performed between cultures with no IFNs, 2 units of rPor-IFN-α or rPor-IFN-β or 2 units of both rPor-IFN-α and rPor-IFN-β.

Introduction: An overview of the researchable question and its importance to producers.

Type I interferons: Type I interferons (IFNs), particularly IFN-α and IFN-β, are signaling proteins that have a critical role in regulating the activity of the vertebrate immune system. IFNs are potent inhibitors of viral replication but unfortunately many swine pathogens encode proteins that suppress IFN activity; for example, the NS1 proteins of PRRSV and SIV, 3Cpro of SVV and multiple nonstructural proteins of PEDV. Although these viruses use different molecular mechanisms to establish productive infections in their hosts, they converge in their behavior to suppress type I IFN pathways.

External supplementation of IFN to reduce viral replication: Limited work has been performed to determine whether IFN therapy provides an effective approach to reduce the disease burden of swine pathogens. Most research has focused on vaccine development. Although novel vaccine candidates are constantly being generated and evaluated, success has been limited. One fundamental problem is the rapid rate at which these viruses evolve due to antigenic drift, thus allowing vaccine escape variants to emerge. Therefore, alternate methods must be explored. Here, we tested the hypothesis that supplementation with recombinant porcine IFN-α and IFN-β significantly reduces the *in vitro* replicative abilities of PRRSV, SVV, PEDV and SIV. This hypothesis is based partially on the success of IFN treatment in human medicine. IFN-α administration is considered the most effective treatment against hepatitis C virus and IFNs are also used to treat other human viral infections. More pertinent to this study, recombinant swine IFN-β protects swine alveolar macrophages and MARC-145 cells from PRRSV infection. A more recent study demonstrated that the co-administration of recombinant IFN-α and the live-attenuated PRRSV vaccine abolished replication of the vaccine virus, providing additional evidence that IFN-α supplementation is an effective treatment for PRRSV infections.

The timely administration of IFN-α and/or β to infected animals could provide an effective approach to restore their anti-viral state and generate "IFN-induced herd immunity" in a vaccine-independent manner. Although porcine IFN-α and IFN-β have previously been produced and their abilities to protect animals against PRRSV have been tested, these studies were performed using an adenovirus delivery system. There are several major disadvantages associated with adenovirus-based delivery systems (1). First, adenoviral proteins are highly immunogenic which can results in an unwanted immune response against the adenovirus particle. Second, adenoviral vectors induce massive systemic inflammation. These two limitations reduce the number of administrations than an animal can safely receive. Finally, the large-scale production of adenoviral vectors requires the use of expensive bioreactors that are cost prohibitory for the swine industry.

Objectives:

The goals of this project were to produce inexpensive and industrially scalable porcine interferon using recombinant technology and to assess the ability of these interferons to suppress the *in vitro* replication of porcine reproductive and respiratory syndrome virus, Seneca Valley virus, porcine epidemic diarrhea virus and swine influenza virus.

Materials & Methods:

Engineering *B. megaterium* for IFN production: The coding sequences of Por-IFN-α and Por-IFN-β were codon optimized and cloned into a modified *B. megaterium* shuttle vector pMM1522 (Boca Scientific). The pMM1522 vector was modified by cloning a cleavable self-aggregating (cSAT) module downstream of the xylose promoter (PxylA). The cSAT module consists of the coding sequence for a gyrA intein, a PT linker (PTPPTTPTPPTTPTPTP) and a self-aggregating ELK16 tag (LELELKLKLELELKLK) The IFN coding sequences were introduced upstream of and in-frame to the cSAT module, resulting in the production of an IFN fusion protein. The IFN genes were be under the control of the inducible promoter PxylA, which is activated by supplementation of the bacterial

culture media with xylose. The shuttle vector was constructed in *E.coli* then transformed directly into *B. megaterium* by electroporation. Transformants will be selected for tetracycline resistance.

Expression and purification of IFN: *B. megaterium* transformants were grown in LB media in presence of tetracycline at 37°C to an optical density of 0.3 at 600nm, at which point we induced the expression of the rPor-IFN-α and rPor-IFN-β fusion proteins by providing 0.5% xylose to the culture media. Aliquots of culture samples were collected at increasing optical densities until the optical density reached 1.5, at which point the bacteria were pelleted for protein purification. The aliquots were tested for the fusion protein by western blotting using tag-specific antibodies. The fusion protein was purified upon determination of the bacterial density at which maximum protein accumulation has occurred. Bacterial pellets were sonicated and centrifuged at 15,000xg for 15 minutes at 4oC to separate the soluble and insoluble fractions. Due to self-aggregating nature of the ELK16 tag in the cSAT module, the IFN fusion protein accumulated in the insoluble fraction. Insoluble fractions were thoroughly washed and IFN was released by activating the self-cleaving ability of the GyrA intein using a reducing buffer that contains 40mM DTT. The insoluble, cleaved cSAT module was removed from the soluble IFN by centrifugation at 15,000x g for 15 minutes followed by dialysis of DTT in a refolding buffer. This method of purification is simple and extremely cost-effective because there is no need to perform expensive affinity/FPLC purification methods. *B. megaterium* does not produce endotoxins and therefore, the purified protein was endotoxin free. Protein concentrations were determined then assessed for purity by silver staining.

In vitro evaluation of IFN biological activity: The biological activity of purified rPor-IFN-α and rPor-IFN-β was measured in Vero cells using the lytic Vesicular Stomatitis virus (VSV) cytopathic assay. We compared the biological activity of our IFNs to commercially available recombinant human IFN-α and β (BPS BioSciences). Cells were incubated with serial dilutions of each IFN for 20 hr. Cells incubated in the absence of IFN were used as negative control. Cells were washed, inoculated with VSV at a multiplicity of infection of 1.0 and incubated at 37°C for 24 hr. Cells were fixed with crystal violet and plaques were counted the following day.

Evaluation of the ability of IFN to suppress viral replication: The ability of porcine IFN-α and β to inhibit the *in vitro* replication of select viruses (PRRSV, PEDV, SVV and SIV) was determined by plaque reduction assay. Assays were performed using swine alveolar macrophages, MARC-145 cells. Briefly, 100 plaque-forming units of virus was mixed with various concentrations of serially diluted IFN and inoculated onto confluent cell monolayers. Cells were incubated for 1 to 5 days. Plaque reduction assays were performed using an agar overlay, with cells fixed using 5% (v/v) formaldehyde and plaques visualized by crystal violet staining (0.015% w/v).

Results:

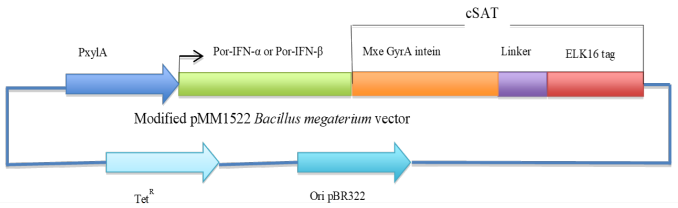
Objective 1: Produce inexpensive and industrially scalable porcine interferon using recombinant technology

The coding sequences of porcine IFN-α and IFN-β were downloaded from the Genbank database (Genbank Accession Nos. M28623.1 and M86762.1, respectively) and synthesized as codon-optimized genes (Bio Basic Inc., Markham, ON, Canada). The resulting lyophilized products were reconstituted in distilled H20, amplified by reverse transcription-polymerase chain reaction using several pairs of overlapping primers and analyzed by Sanger sequencing for sequence confirmation (data not shown). The aforementioned genes were successfully cloned into a *Bacillus megaterium* shuttle vector pMM1522 (Boca Scientific Inc., Westwood, MA) by making use of unique restriction enzyme sites located at the cloning site. However, before performing these cloning experiments, it was necessary to modify the shuttle vector. Briefly, we inserted a cleavable self-aggregating (cSAT) module downstream of the xylose promoter (PxylA). The cSAT module consists of the coding

sequence for a gyrA intein, a PT linker (PTPPTTPTPPTTPTPTP) and a self-aggregating ELK16 tag (LELELKLKLELELKLK). The IFN coding sequences were introduced upstream of and in-frame to the cSAT module (**Figure 1**). IFN genes are under the control of an inducible promoter PxylA. The shuttle vector was constructed in *E.coli* then transformed directly into *B. megaterium* by electroporation.

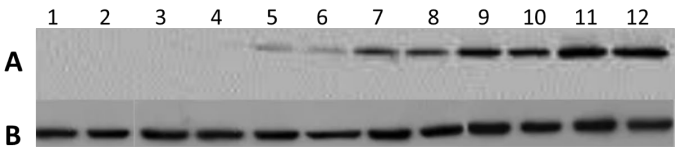
B. megaterium transformants were grown in Lysogeny broth in presence of tetracycline at 37oC to an optical density of 0.3 at 600 nm. Expression of the IFN-α and IFN-β fusion proteins was induced by providing 0.5% xylose to the culture media. Aliquots of culture samples were collected at increasing optical densities until the optical density reached 1.5, at which point the bacteria was pelleted for protein purification. The aliquots have been tested for the fusion protein by western blotting using a tag-specific antibody. IFN-α and IFN-β fusion proteins were successfully detected (**Figure 2** and results not shown, respectively).

Figure 1: Schematic of the newly created *B. megaterium* plasmid



Porcine IFN-α and IFN-β coding sequences were cloned into a pMM1522 vector upstream of a cleavable self-aggregating tag (cSAT) which consists of an intein derived from the gyrA gene of *Mycobacterium xenopi*, a linker (PTPPTTPTPPTTPTPTP) and a ELK16 tag (LELELKLKLELELKLK). The IFN genes and cSAT are under the control of xylose inducible promoter (PxylA). The origin of replication (ori pBR322) and tetracycline resistance are denoted. The black arrow indicates the IFN translation start site.

Figure 2. Testing bacterial cultures for porcine IFN-α fusion protein by Western blot



Samples were collected from a bacterial cultures at 12 different optical densities and centrifuged. Cell pellets were resuspensed in lysis buffer, with an aliquot of each sample resolved by SDS polyacrylamide gel electrophoresis and analyzed by western blot using **(A)** an antibody specific to the fusion tag or **(B)** an antibody specific to α-actin to ensure equal sample loading

The biological activity of purified rPor-IFN-α and rPor-IFN-β was measured in Vero cells using the lytic vesicular stomatitis virus cytopathic assay. We compared the biological activity of our IFNs to commercially available recombinant human IFN-α and β (BPS BioSciences). Cells were incubated with serial twofold dilutions of each IFN starting with 100 pg/ml for 20 hr. Twenty-four hours after inoculation with VSV at multiplicity of infection of 0.1, cells were fixed with crystal violet and virus titers were calculated. VSV titers were approximately 1 x 10^8 pfu/ml in the absence of IFN. Virus titers were greatly reduced in the presence of all four IFNs that were tested: rPor-IFN-α, rPor-IFN-β, human IFN-α and human IFN-β (**Table 1**). These findings demonstrate that the porcine IFNs that we produced are biologically active, although their ability to suppress VSV replication was approximately tenfold less as that observed with the commercially available human IFNS.

Table 1. Vesicular stomatitis virus titers in Vero cells treated with or without interferon

IFN concentration (units)	Vesicular stomatitis virus titer (plaque forming units per milliliter)			
	human IFN-α	human IFN-β	rPor-IFN-α	rPor-IFN-β
0	1.6 x 10^8	1.6 x 10^8	1.6 x 10^8	1.6 x 10^8
0.5	3.6 x 10^6	3.7 x 10^6	8.3 x 10^7	7.7 x 10^7
1.0	4.9 x 10^5	2.3 x 10^5	3.2 x 10^6	4.7 x 10^6
2.0	2.7 x 10^4	3.6 x 10^4	4.2 x 10^5	2.8 x 10^5

Objective 2: Determine the ability of recombinant porcine IFNs to suppress the *in vitro* replication of PRRSV, SVV, PEDV and SIV in porcine cells

Swine alveolar macrophages (MARC-145 cells) in six-well plates were inoculated with virus in the presence or absence of IFN. Viruses used in these experiments were PRRSV, SVV, PEDV and SIV and all were used at a multiplicity of infection of 1.0. The IFNs used in these experiments were rPor-IFN-α and rPor-IFN-β, and these were tested both separately and together. Two units of each IFN

was used. Plaque assay experiments revealed that the ability of each virus to replicate in MARC-145 did not significantly differ when comparisons were performed between cultures with no IFNs, 2 units of rPor-IFN-α or rPor-IFN-β or 2 units of both rPor-IFN-α and rPor-IFN-β (**Table 2**).

Table 2. Assessing the ability of recombinant porcine interferons to suppress the *in vitro* replication of common porcine viruses

Virus	Interferon	Virus titer (pfu/ml)		
		Days post virus infection		
		1	3	5
PRRSV	none	2.7×10^3	4.3×10^5	5.0×10^5
	rPor-IFN-α	2.6×10^3	2.9×10^5	4.8×10^5
	rPor-IFN- β	2.7×10^3	4.4×10^5	4.9×10^5
	Both	2.5×10^3	3.9×10^5	5.3×10^5
SSV	none	NT	8.9×10^5	9.9×10^4
	rPor-IFN-α	NT	9.5×10^5	1.2×10^5
	rPor-IFN- β	NT	8.8×10^5	9.5×10^4
	Both	NT	8.3×10^5	9.0×10^4
PEDV	none	NT	3.4×10^4	1.0×10^6
	rPor-IFN-α	NT	3.8×10^4	8.9×10^5
	rPor-IFN- β	NT	3.0×10^4	9.1×10^5
	Both	NT	2.9×10^4	9.0×10^5
SIV	none	1.2×10^2	3.1×10^6	5.0×10^7
	rPor-IFN-α	1.3×10^2	2.9×10^6	4.8×10^7
	rPor-IFN- β	1.2×10^2	3.2×10^6	4.9×10^7
	Both	1.3×10^2	3.0×10^6	5.3×10^7

Discussion:

We successfully engineered bacterium to produce large quantities of porcine IFNs. Unfortunately, the IFNs did not affect the *in vitro* replicative abilities of the selected porcine viruses. Nevertheless, the IFN production system that we developed could have other applications not immediately related to the overall scope of this study.