RESEARCH REPORT
SWINE HEALTH

Title: Molecular Identification of Type I Interferon Antagonistic Components of PRRSV Proteins – **NPB# #09-239**

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Industry Summary
Basic knowledge of PRRSV infections and clues to successful PRRSV control have led to the discovery of type 1 interferon inhibition by several viral strains, which is purported to be instrumental in the dampening of the immune response. This study aimed to detect and dissect the type 1 interferon inhibition by the individual proteins of Type 2 strain MN184. The experimental approaches for identifying PRRSV type I interferon antagonist(s) utilized expressed MN184 proteins and a panel of type I interferon assays composed of a bioassay, an immunoassay and quantitative RT-PCR. The individual MN184 proteins were successfully expressed in MARC-145 cells. However, the assay results suggested that no inhibition of type 1 interferon by PRRSV strain MN184 could be detected using our devised MARC-145 cell assay, although some PRRSV nonstructural proteins, nsp3 and nsp7-11, induced a moderate amount of one class of type 1 interferon.

Keywords
PRRSV strain MN184, type 1 interferon [alpha (α) and beta (β)], bioassay on Marc-145 cells, quantitative RT-PCR (qRT-PCR), ELISA

Scientific Abstract
We set out to define the regions of PRRSV Type 2 strain MN184 responsible for inhibiting type 1 interferon using cloned PRRSV proteins expressed in MARC-145 cells, which supports virus replication. The individual genes for each nonstructural and structural proteins of MN184 proteins was best implemented by the use of the eukaryotic expression vector pCI, modified to include a Flag tag. The original bioassay used NDV modified to express green fluorescent protein that stimulates type 1 interferon in Marc-145 cells. Thus it was used an indicator for IFN inhibition by PRRSV. We found that this bioassay system was extremely difficult to optimize for reliable and consistent results. Our final bioassay eliminated the NDV-GFP indicator step, since the chosen ELISA system measured IFN-β protein when present in small amounts. A commercially prepared qRT-PCR test was implemented to assess the level of cellular mRNA for IFN-β. The NS1 gene of pandemic influenza was to be used as our positive control for inhibition. However, the pandemic flu NS1 gene was found not to inhibit type 1 interferon substantially using this assay, as recently confirmed by others. Additionally, our results suggested that MARC-145 cell IFN-β inhibition by MN184 proteins was not detected. Rather, we saw that strain MN184 nsps 3, 7-11 actually modestly induced IFN-β protein into the cell supernatant. We have yet to establish the level and protein product sizes of our

expressed PRRSV proteins. The reasons for our failure to detect type 1 IFN inhibition may lie in the strain of PRRSV tested, the type of cells utilized, the methods used to detect interferon, and the IFN chosen for ELISA or mRNA detection. However, the MN184 protein expression system developed through this funding will be used to further define the host response to PRRSV infection.

Introduction

PRRSV, one of the most important swine pathogens, is challenging the swine industry worldwide. Currently, there is no effective method to eradicate or control PRRSV infections in swine populations due in part to the lack of knowledge concerning protective immunity to the virus. Type I interferons (IFN-α/β) are cytokines secreted by nucleated cells upon virus infections, are one of the important components of the host innate immune response and also plays an important role in the initiation of an adaptive immune response. Viral antagonism against type I interferon has been characterized as one of the important virulence determinants of many viruses. PRRSV has recently been shown to down-regulate type I interferon production both *in vivo* and *in vitro* for some PRRSV strains, but the mechanism involved in type I interferon antagonism in PRRSV is largely unknown and results to date have mainly been assessed using cells which do not support PRRSV replication or by a synthetic reporter assay. To date, type 1 interferon antagonism has been reported for PRRSV strains VR-2332, PA8, FL12, and SD01-08, BJ-4, VR2385, Ingelvac PRRS MLV, and others {Yoo, 2010 #3629}, {Sun, 2010 #3635;Song, 2010 #3622;Shi, 2010 #3627;} {Beura, 2010 #3642}{Patel, 2010 #3625}. One study using Marc-145 cells describes a study in which PRRSV Type 2 strain PA8 was shown to inhibit interferon alpha (IFN-α) in infected cells stimulated with poly(I:C) through the use of a paramyxovirus that induces interferon (Kim, 2010 #3636). We proposed to establish a similar bioassay but assessing PRRSV Type 2 strain MN184, and then to express individual MN184 proteins to identify specific type I interferon antagonism domains. Identification of the MN184 viral protein(s) responsible for type I interferon antagonism would lead to a better understanding of anti-PRRSV immunity and the development of effective vaccines against more recent PRRSV infections.

This study intended to dissect the molecular components of PRRSV strain MN184 involved in type I interferon antagonism, which would increase our understanding of how PRRSV interacts with host immune system. This knowledge is also critical in designing novel PRRSV vaccines and developing new strategies for PRRSV eradication.

Objectives

The objective of this study was to identify the PRRSV protein(s) involved type I interferon antagonism using an in vitro screening system established at the NADC. The putative type I interferon antagonist protein(s) of PRRSV Type 2 strain MN184 identified in this study were to be further analyzed by mutagenesis and evaluated for type I interferon antagonism in mutant recombinant virus constructed from wild type strain.

Objective 1. To pinpoint PRRSV encoded protein(s) involved in type I interferon antagonism using a bioassay, ELISA and real-time RT-PCR.

Objective 2. To map the active region(s) of the putative type I interferon antagonist(s) in PRRSV through site-directed mutagenesis.

Objective 3. To construct recombinant PRRSV strains with attenuated type I interferon antagonism by introducing mutations into the active region(s) of the putative type I interferon antagonist protein(s).

Materials and Methods

Vectors: In order to clone individual PRRSV strain MN184, several vectors were initially compared. The chosen vector was pCI from Promega, which uses the cytomegalovirus promoter to accomplish mammalian expression. pCI was modified to include a Kosak sequence (GCCACC) as well a "FLAG-tag" (5'-GATTACAAGGATGACGATGACAAG-3' coding for DYKDDDDK) at the amino terminal or carboxy terminal end of the inserted PRRSV nucleotides.

Bioassay: Seed wells of a 24-well cell culture plates with 0.5 ml of $3x10^5$ Marc-145 cells/ml. In polypropylene tubes, PRRSV-expressing plasmids (0.8 μg/well) were diluted in 50μl OptiMEM/well of 24 well plate. In separate polypropylene tubes , 2 μl Lipofectamine™ 2000 (per well of 24 well plate) is diluted in 50μl OptiMEM and incubated for 5 minutes at room temperature. After the 5 minute incubation, the diluted DNA is combine with the diluted Lipofectamine™ 2000

(1μg:3μl ratio; total volume = 100 μl). Mix gently and incubate for 20 minutes at room temperature.
Add the 100 μl of complexes to each well containing cells and medium. Mix
gently by rocking the plate back and forth. Incubate cells at 37°C in a CO_2 incubator for 40 hours. If poly(I:C) is used (250 ηg), it is prepared in a like manner as described above and transfected into the same cells 24 hours after PRRSV-plasmid transfection. At 40 hours post PRRSV plasmid transfection, the supernatants were harvested for analysis by an interferon-β ELISA kit (VeriKine-HS™ Human Interferon-Beta Serum ELISA Kit; PBL InterferonSource). Intracellular mRNA was harvested from the transfected cells.

Interferon-β Real-Time RT-PCR. A gene specific quantitative RT-PCR (qRT-PCR) assay was utilized to amplify and detect a Human IFN-β (Hs01077958_s1) product in conjunction with a similar assay to amplify and detect the endogenous control gene for eukaryotic 18S rRNA (4319413E,), as developed by the manufacturer (Applied Biosystems).

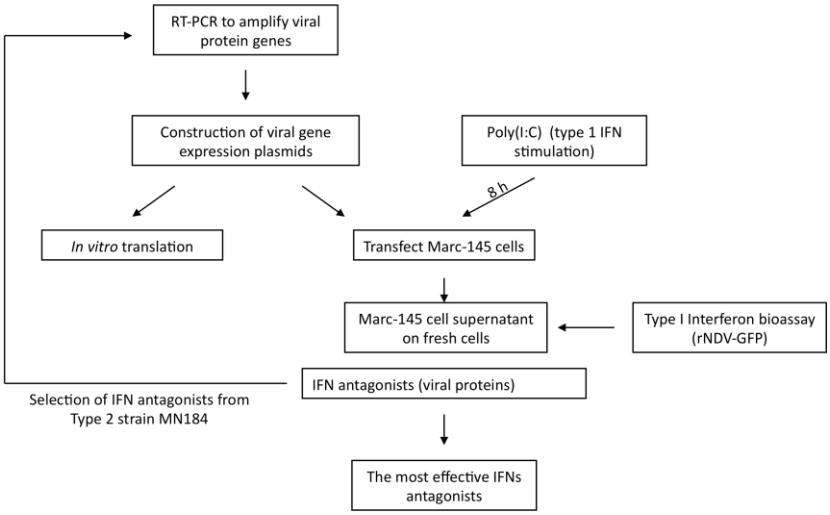
Interferon-β ELISA. *Verikine-HS™* Human IFN-β Serum ELISA kits were utilized to assess the amount of IFN-β protein present in 50 μl of extracellular medium of transfected cells.

Results

Objective 1. To pinpoint PRRSV encoded protein(s) involved in type I interferon antagonism using a bioassay, ELISA and real-time RT-PCR.

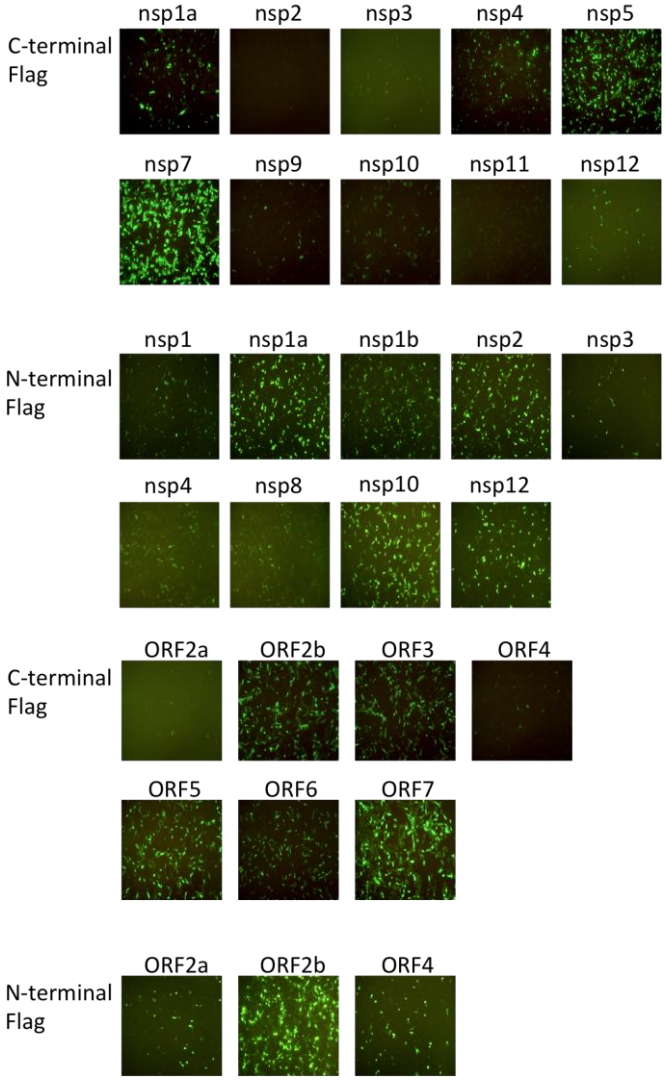
A schematic of the proposed work for Objective 1, as originally conceived, is shown in

Figure 1



1. Individual MN184 proteins, both structural and nonstructural, were cloned into the mammalian expression vector pCI (Fig. 2). Most proteins were expressed well in Marc-145 cells, using either the N- or C-terminal FLAG tag (Figure 2). Since ORFs 2a, 3, 4, 5 have cleaved N-terminal signal sequences, the likelihood that the immunofluoresence seen from an N-terminal Flag-tag represents the MN184 glycoprotein is uncertain.

Figure 2. Vector pCI promoted visual expression of PRRSV strain MN184 proteins



2. Development of a type 1 interferon bioassay. We have concentrated our efforts on developing a bioassay using rNDV expressing green fluorescent protein (GFP). rNDV-GFP is sensitive to type 1 interferon and provides a marker for potential PRRSV anti-interferon activity. We began to evaluate the assay using virus itself, assuming that the assay would be easily developed and this was an objective in the prior funded proposal.

   a. First, we determined the timing and dosage of polyinosine-polycytidylic acid [poly(I:C)], a synthetic analog of double-stranded RNA (dsRNA) which is recognized by toll-like receptor 3 (TLR3) and activates the type 1 interferon (IFN) response. Results based on a bioassay without PRRSV infection indicated optimal Type 1 IFN production was a 12 hr poly(I:C) stimulation in the presence of Lipofectamine 2000 at the dosages from 62 to 1000 ng/well.

   b. We also determined the $TCID_{50}$ at which all cells would be infected (focus forming units; FFU) at 8-16 h post infection for a number of PRRSV strains (Table 1).

Table 1

PRRSV Strains	CID_{50}/0.1ml	FU/0.1ml	CID_{50}/FFU
2332	3.16E+07	2.50E+06	13
	2.15E+07	3.50E+05	61
184	3.16E+07	3.50E+06	9
N184	4.63E+06	3.00E+06	2
	3.16E+09	TNTC	
V	1.00E+08	1.90E+07	5
N184ORF1/MLV	1.00E+07	5.00E+05	20
VORF1/MN184	3.16E+08	5.00E+06	53

Next, we assayed several PRRSV strains to see if any strain could counteract type 1 IFN production of Marc-145 cells stimulated by 100 ng poly(I:C) complexed with Liptofectamine 2000. This experiment was repeated, but the results were not conclusive, as the virus strains did not display an inhibition titer after poly(I:C) stimulation that was stronger (lower dilution) than control medium only and also gave inconsistent results (Tables 2 and 3).

Table 2: IFN bioassay using rNDV-GFP (exp 04-28-10)

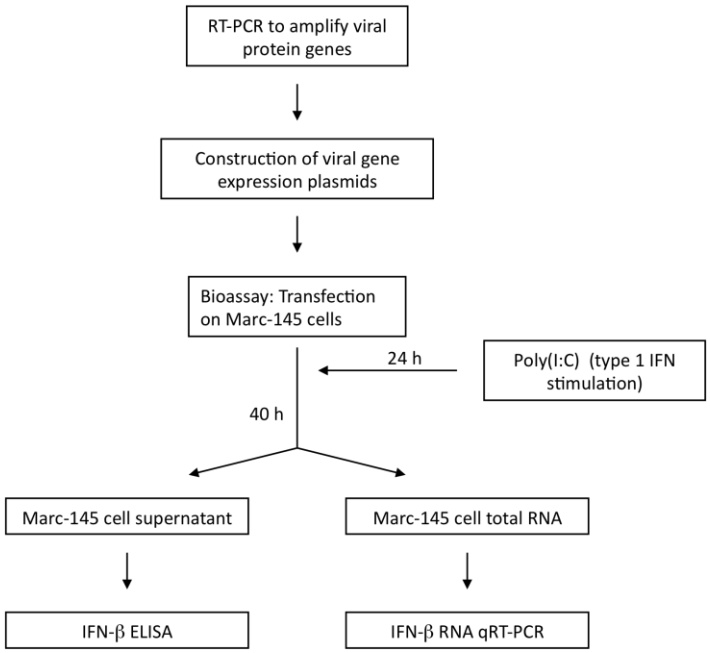
	rNDV-GFP inhibition titer	
PRRSV	MEM	Poly(I:C)
2332	< 1:1	1:27
7	< 1:1	1:81
N184	< 1:1	1:9
N184	< 1:1	1:27
V	< 1:1	1:27
LV	< 1:1	1:27
N184ORF1/MLV	< 1:1	1:27
LVORF1/MN184	< 1:1	1:27
M	< 1:1	1:27

ole 3: IFN bioassay using rNDV-GFP (exp 06-06-10)

	rNDV-GFP inhibition titer	
PRRSV	MEM	Poly(I:C)
2332	< 1:1	1:27
7	< 1:1	1:27
N184	< 1:1	1:3, 9
N184	< 1:1	1:9
V	< 1:1	1:9
LV	< 1:1	1:9
N184ORF1/MLV	< 1:1	1:3
LVORF1/MN184	< 1:1	1:9, 27
M	< 1:1	1:9

c. Our final bioassay, as outlined in the Materials and Methods and outlined in Figure 3, omitted the use of NDV-GFP, as results obtained were unclear. Instead, we simplified the assay, and used the swine influenza pandemic H1N1 (A/Mexico/4108/2009) NS1 gene as a positive control. However, very recent evidence indicated that the pandemic H1N1 NS1 gene is unable to block general host interferon gene expression {Hale, 2010 #3756}, and access to the NS1 gene of influenza strains that have been shown to globally inhibit interferon was not readily available in order to include a true positive control in the present study.

Figure 3



3. Titration to determine timing of 0.8 μg/well PRRSV plasmid transfection into Marc-145 cells and subsequent poly(I:C) transfection (250 ηg/well). As shown in Figure 4, in the absence of poly(I:C), 40 hours was chosen for cell incubation with transfected plasmid prior to analysis by ELISA. If poly(I:C) is to be used after the initial test plasmid transfection, the ELISA results suggested that the Marc-145 cells could be readily transfected with poly(I:C) any time after 10 hours, and 16 hours was chosen. Analysis of INF-β mRNA expression (Figure 5) indicated that a similar time point (40 hours) was amenable for diagnostic assay.

Figure 4

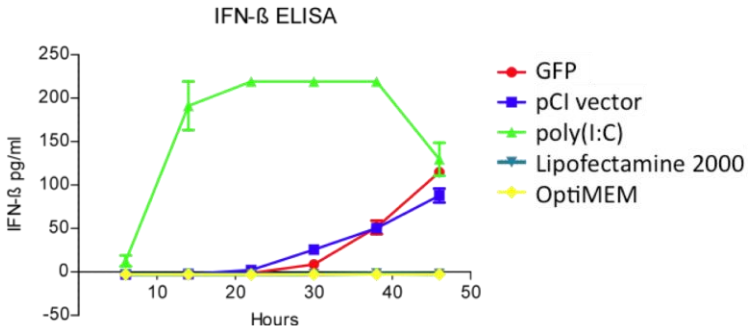
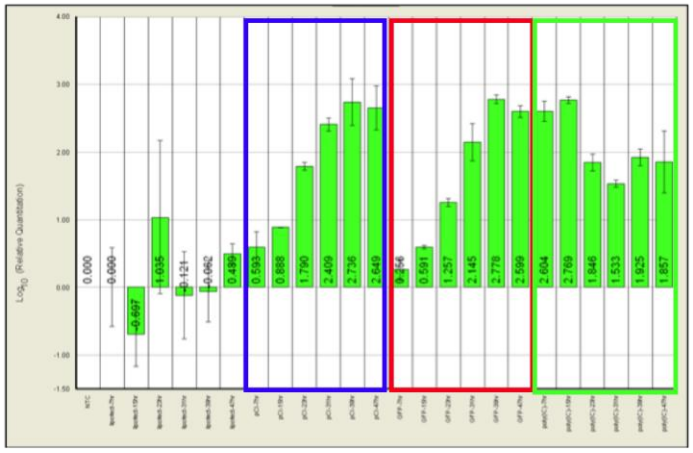
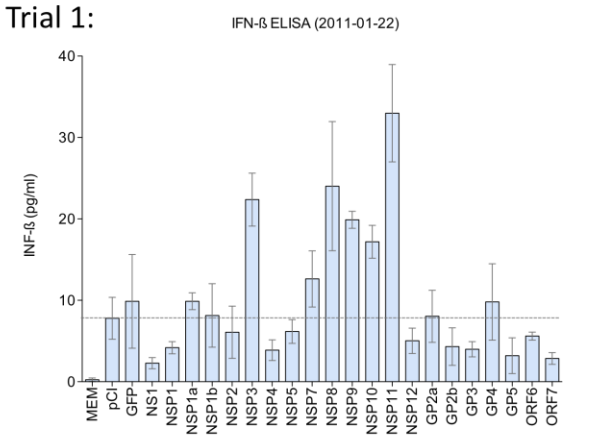


Figure 5

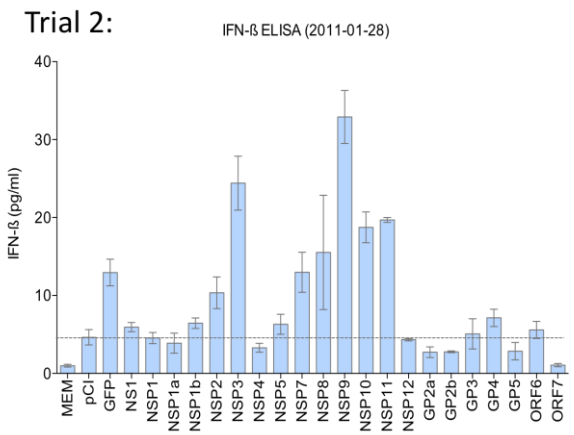


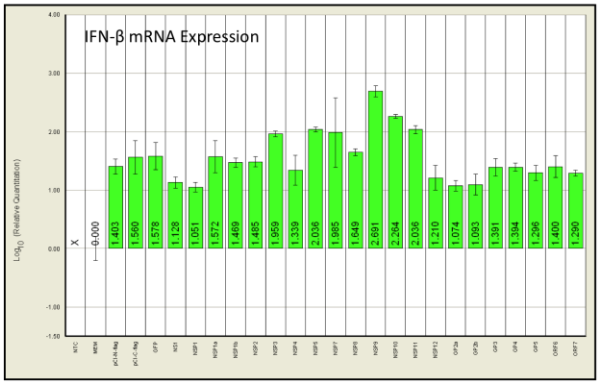
4. Replicate trials examining the ability of individual proteins of PRRSV strain MN184 to inhibit type 1 IFN (IFN-β).
   a. Trials 1-3 show the effect of the individual proteins on the expression of IFN-β on supernatant protein expression (ELISA, top panel) and on mRNA expression when normalized to expression of the 18S ribosome (qRT-PCR, bottom panel in Trials 1 and 2). All Y-axes were adjusted to equivalent values simplify the analysis when comparing results between trials. The expression vector (pCI, pCI-N-flag, pCI-C-flag) was used in all constructs, and thus was the background level of IFN-β expression. Note that mRNA expression was altered between transfection with different proteins, but the most it varied between the two trials was only 1 log or less. Importantly, no inhibition by PRRSV strain MN184 proteins was seen using this assay. IFN-β protein levels in the cell supernatant indicated that little or no inhibition was detected, and the amount of inhibition sometimes detected was to small (a few picograms/ml) to reliably conclude an effect was seen. However, some MN184 proteins appeared to induce IFN-β protein levels, particularly nsp3 and nsp7-11. The level of MN184 protein expressed in the Marc-145 cells may vary, so firm conclusions concerning the effect of strain MN184 proteins on IFN-β await of the level and size of expressed PRRSV polypeptides by Western blot analysis using anti-FLAG antibodies.
   b. Trial 4 shows the effect of poly(I:C) transfection 24 hours after transfection with the MN184 proteins, then isolating the mRNA 15 h later. There appeared to be a dampening effect on all IFN-β mRNA expression by the addition of poly(I:C), a result not anticipated. However, the level of IFN-β protein expression surprisingly suggested that although there was no inhibition evidenced, we again detected the ability of nsp3 and nsp8-11 to dramatically stimulate the secretion of type 1 interferon into the extracellular supernatant.

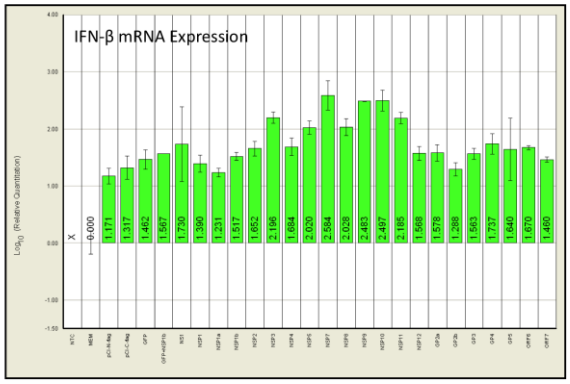
Trial 1:



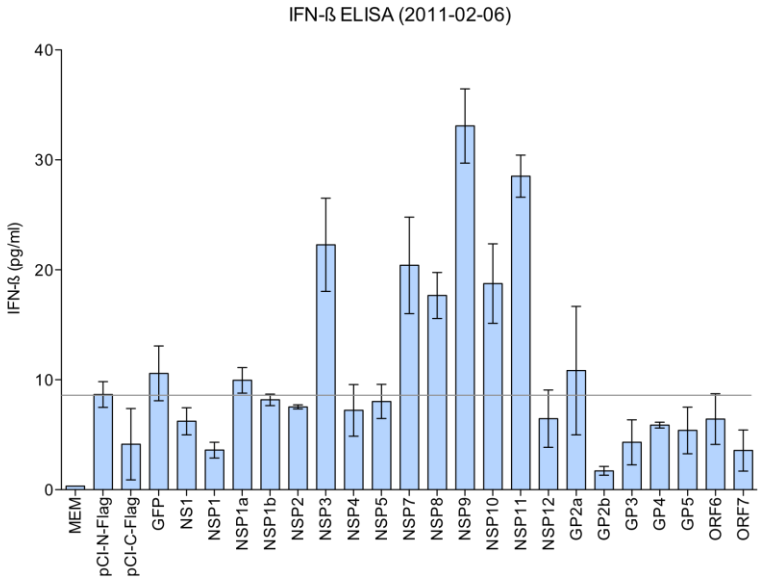
Trial 2:



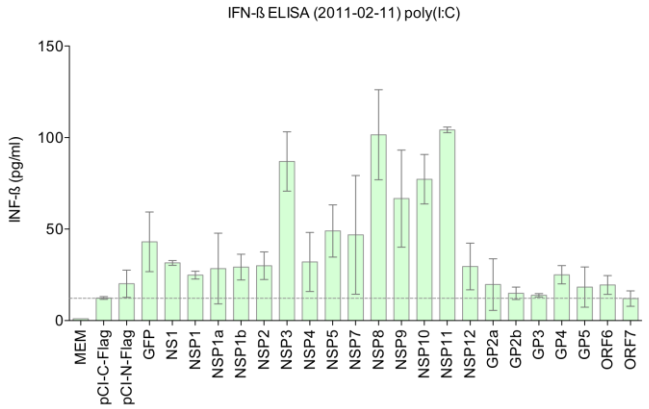


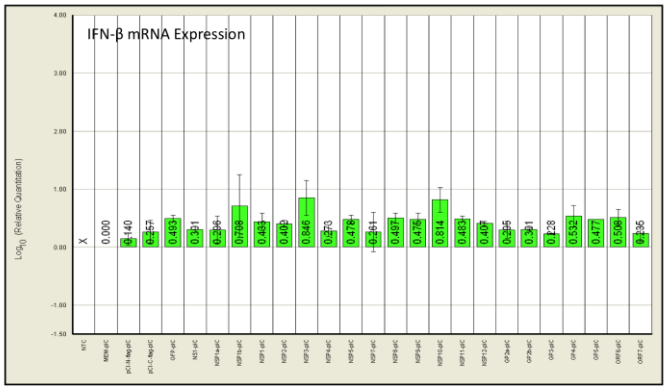


Trial 3:



Trial 4:





Objective 2. To map the active region(s) of the putative type I interferon antagonist(s) in PRRSV through site-directed mutagenesis.

Due to the difficulty in establishing the bioassay and the failure to detect Type 2 strain MN184 genes that inhibit INF-β, this objective could not be completed.

Objective 3. To construct recombinant PRRSV strains with attenuated type I interferon antagonism by introducing mutations into the active region(s) of the putative type I interferon antagonist protein(s).
Due to the difficulty in establishing the bioassay and the failure to detect Type 2 strain MN184 genes that inhibit INF-β, this objective could not be completed.

Discussion:
We set out to define the regions of PRRSV Type 2 strain MN184 responsible for inhibiting type 1 interferon in a cell line supporting virus replication.
We successfully cloned the individual genes for each nonstructural and structural proteins of MN184 (Fig. 2), and found that the visualization of nonstructural and E proteins was best implemented by the use of the eukaryotic expression vector pCI, modified to include an N-terminal Flag tag. A C-terminal flag tag was best for expression of the glycoproteins and the membrane and nucleocapsid proteins.
The original experimental bioassay used NDV modified to express green fluorescent protein (NDV-GFP), which stimulates type 1 interferon in Marc-145 cells, as an indicator virus to show interferon inhibition by PRRSV. Thus, MARC-145 cells were transfected with PRRSV, followed by a further stimulation with poly(IC), and removal of the cell supernatant to use in the NDV-GFP bioassay. However, we found that this bioassay system was extremely difficult to optimize for reliable and consistent results. In the end, the second step of the bioassay, using NDV-GFP, was omitted since the chosen ELISA system reliably measured IFN-β protein down to 1 picogram per milliliter of cell supernatant. A commercially prepared qRT-PCR test was implemented to assess the level of cellular mRNA for IFN-β. The NS1 gene of pandemic influenza was to be used as our positive control, but the inhibition by this gene product was not seen, most likely because the pandemic flu NS1 gene was not found to globally inhibit type 1 interferon, contrary to our understanding at the time. We showed that the transfection of poly(I:C) could stimulate IFN-β mRNA (Trial 4), so we were assured that the MARC-145 cells used were capable of inducing type 1 interferon. In the end, however, this assay system did not detect MARC-145 cell IFN-β inhibition by MN184 proteins. Rather, we saw that some strain MN184 nonstructural proteins actually induced the transport of IFN-β protein into the MARC-145 cell supernatant (Trials 1-3). Because these results were obtained recently, we have not had time to explore the MN184 protein expression product size levels in the supernatants in order to confirm our findings to date. The reasons why we did not detect type 1 IFN inhibition may lie in the strain of PRRSV tested, the type of cells utilized (MARC-145 vs others), the methods used to detect interferon, and the IFN chosen for ELISA or mRNA detection (numerous α genes vs. one β gene). However, the MN184 protein expression system developed by this funding will be used to further define the host response to PRRSV infection.

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