RESEARCH REPORT
SWINE HEALTH

Title: Determine the mechanisms of cross-protection against infection with a divergent porcine reproductive and respiratory virus strain - **NPB # 14-200**

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Industry Summary:

PRRS modified live virus (MLV) vaccines have been licensed for clinical administration for over 20 years. The vaccines confer solid protection against homologous PRRSV strains. However, the extent and duration of protection against heterologous strains is highly variable and mostly sub-optimal. We recently generated a synthetic PRRSV strain (designated as PRRSV-CON) that is able to confer unprecedented levels of protection. In this project we characterized and compared the immune responses between pigs infected with a wild-type PRRSV strain and those infected with the PRRSV-CON. The primary goal is to determine the mechanisms by which the PRRSV-CON confers cross-protection, as this knowledge is useful for rational design of PRRS MLV vaccines in order to achieve optimal levels of heterologous protection. We found that the synthetic PRRSV-CON virus provides better levels of innate and adaptive immunity than the reference, wild-type PRRSV strain FL12. We are currently conducting further studies, with different experimental conditions, to determine how the difference in innate and adaptive immunity affects the protection outcomes.

Keywords: PRRSV, heterologous protection, immune response, type-I interferons, neutralizing antibodies, interferon gamma producing cells

Scientific Abstract:

We have recently generated a synthetic PRRSV strain (so-called PRRSV-CON) that confers outstanding levels of heterologous protection. We report herein the use of the PRRSV-CON as gold standard to elucidate the mechanisms of cross-protection against divergent PRRSV strains. When inoculated into pigs, the PRRSV-CON seems to elicit better levels of cross-neutralizing antibodies than the PRRSV strain FL12. Cross-neutralizing

antibodies were detected in 2 out of 6 pigs infected with the PRRSV-CON whereas cross-neutralizing antibodies were not detected in any of the pigs infected with the PRRSV strain FL12. We did not observe any significant difference in the levels of interferon-gamma secreting cells between pigs infected with the PRRSV-CON and those infected with FL12. Interestingly, we found that the synthetic PRRSV-CON virus can induce high levels of type-I interferons in cell culture. This observation is surprising because it has been well demonstrated that naturally occurring PRRSV actively suppress type-I interferons. We further identified that the 3.3 kb fragment at the 5'end of the PRRSV-CON, containing the non-structural protein (nsp) 1 and a part of nsp2, is responsible for the induction of type-I interferons. We are conducting experiments to determine the relationship between the viral capability of inducing type-I IFNs and the viral ability to confer protection against heterologous PRRSV strains.

Introduction:

PRRS continues causing substantial losses to the swine producers. Current PRRS vaccines are not adequately effective for control and eradication of the disease. There are 2 types of PRRS vaccines currently available in the market, including modified-live and inactivated virus vaccines. In addition, several subunit vaccines for PRRS are being tested in different laboratories worldwide but none of them are licensed for clinical applications. The protective capacity of inactivated PRRS vaccines is very limited. Multiple evidences indicate that the best protection that can be obtained through vaccination is by the use of modified-live vaccines (MLVs) (1-3). The homologous protective immunity conferred by PRRS MLVs is considered to be fully protective, reaching sterilizing conditions in many cases (1, 3). On the other hand, the extent and duration of protection against heterologous strains is highly variable and sub-optimal (4, 5). The levels of heterologous protection conferred by PRRS MLVs in pregnant sows vary across a wide range between 52 % and 85.9 % (1, 2). The mechanisms of cross-protective immunity to PRRSV infection remain elusive. Both cell-mediated immunity and NAbs have been demonstrated to be important for protection (3, 6). However, current PRRS vaccines does not elicit robust NAb responses (7). Several vaccine studies have demonstrated that vaccinated pigs are protected from challenge infection in the absence of NAbs (3, 8, 9). These observations lead to the notion that cell-mediated immunity plays an important role in protection against PRRSV infection.

Thanks to the initial support from the Pock-checkoff, we have recently generated a *de novo* synthesized PRRSV strain based on a consensus, full-genome sequence (herein designated as PRRSV-CON) derived from a large set (n=59) of PRRSV strains. The synthetic PRRSV-CON virus replicates efficiently both in cell culture and in pigs. Unlike most of naturally occurring PRRSV strains, the PRRSV-CON induces significant levels of type-I interferon response, rather than suppressing. Through two different immunization/challenge experiments, we proved that the PRRSV-CON confers exceptional levels of cross-protection against heterologous PRRSV strains. In the current project, we used the synthetic PRRSV-CON as a gold standard to elucidate the mechanisms of cross-protection against divergent PRRSV strains.

Objective:

To elucidate the mechanisms of cross-protection against divergent PRRSV strains

Materials & Methods

Cross-protection experiment in pigs: Three groups of 6 weaning pigs were purchased from the UNL research farm which holds certified records of absence of PRRSV infection. The pigs were accommodated in

BSL-2 animal facilities at UNL, following the regulations established by Institutional Animal Care and Use Committee. Pigs in group 1 served as non-immunization control whereas those in groups 2 and 3 were immunized by infection with either FL12 or PRRSV-CON, at the dose of $10^{4.0}$ TCID50 per pig, intramuscularly. Blood samples were drawn periodically. Serum and peripheral blood monocytes (PPBMCs) were isolated and preserved, following the method described previously (10). Serum samples were used for virus-neutralization assay while PBMCs were used for IFN-γ ELISPOT assay to determine the cross-reactive T-cell responses after immunization. At day 52 post-infection, all control and immunized animals were challenged with the heterologous PRRSV strain MN184. Parameters of protection included: growth performance, viremia, and viral load in tissues. Results of this cross-protection experiment have been reported in our recent publication in Journal of Virology (11).

Virus-neutralization assay: Neutralizing antibody titers of serum samples against a specific PRRSV studied were determined using a fluorescent focus neutralization assay described previously. Heat-inactivated antisera were diluted 2-fold serially in 50 µl of DMEM supplemented with 5% FBS on a 96 well plate, then incubated with an equal volume (50 µl) containing 100 TCID50 of testing virus for 1 h at 37ºC. Total mixture of serum and virus (100 µl/well) was transferred to another 96-well plate containing confluent MARC-145 cells which had been seeded 48 h earlier. The plate was further incubated for 36 h at 37ºC in a humidified atmosphere containing 5% CO2. After incubation, the cell monolayer was washed twice with PBS, followed by fixing with a cold solution of 50% methanol and 50% acetone (v/v) for 10 minutes. The cells were incubated for 1 h with anti-N monoclonal antibody SDOW-17 diluted 1:500 in PBS, followed by three times wash with PBS. Next, cells were incubated for 1 h with Alexa fluor® 488 conjugated goat anti-mouse immunoglobulin G. Again, cells were washed three times with PBS. The presence of PRRSV is observed under the fluorescent microscope. Neutralization titers were expressed as the reciprocal of the highest dilution that showed 90% or greater reduction in the number of fluorescent foci presenting in the control wells.

Measure cross-reactive T-cell responses post-immunization: IFN-γ ELISPOT assay will be used to quantify the frequencies of antigen-specific T-cells presenting in PBMCs collected and preserved as described in the previous section. Details of this assay were described in our recent publication (10). Whole virus and peptide pools containing individual viral proteins were used as the recall antigens to stimulate PBMCs.

Measure of type-I interferons production: MARC-145 cells were infected with different PRRSV strains at multiplicity of infection (MOI) 0.01. At various time-points after infection, total viral RNA was collected for evaluation of the IFN-β mRNA levels, using the commercial real-time PCR kit.

Results

1. Cross-neutralizing antibodies

We collected serum samples at 52 days post-infection from 3 groups of pigs as described in the Material and Method section. We then measured neutralizing activity of these serum samples against 3 different PRRSV strains: FL12, PRRSV-CON and MN184C. As shown in table 1, homologous neutralizing antibody titers (e.g. the titers measured against the corresponding virus strains that were used for infection) were similar between pigs infected with the PRRSV-CON and those infected with FL12. However, pigs infected with the PRRSV-CON seemed to develop better levels of cross-neutralizing antibodies than those infected with FL12. Two out of 6 pigs

infected with PRRSV-CON develop cross-neutralizing antibodies against MN184C and FL12 whereas none of the pigs infected with FL12 developed cross-neutralizing antibodies against PRRSV-CON and MN184C.

2. Cross-reactive T cells responses

We first measured cross-reactive T cells in PBMCs collected at days 52 post-infection by using the interferon – gamma ELISPOT assay, with whole virus as the stimulating antigen. PBMCs collected from pigs infected with either PRRSV-CON or FL12 contained similar numbers of interferon-gamma secreting cells (IFN-γ SC), regardless of which viral strains were used for stimulating the cells (Figure 1). We next measured cross-reactive T cells in PBMCs by using peptide pools containing individual viral proteins as the stimulating antigens. Again, we observed no significant difference in the numbers of IFN-γ SC between PBMCs collected from the PRRSV-CON infected pigs and the cells collected from FL12 infected pigs.

3. **The PRRSV-CON induces type-I interferons in MARC-145 cells**

Most of naturally occurring PRRSV strains suppress type-I IFN induction, except only one PRRSV strain that was reported to induce type-I IFN (12). Intriguingly, we found that the PRRSV-CON virus can induce high levels of type-I IFNs in cell culture. As shown in figure 3A, large amounts of interferon-beta (IFN-β) mRNA were detected in MARC-145 cells infected with PRRSV-CON, but not in cells infected with FL12. Likewise, ISG-56, an interferon-stimulated gene that is expressed in response to interferons, was only detected from PRRSV-CON infected cells but not from FL12- infected cells (Fig. 3B). We used the Vesicular Stomatitis Virus (VSV) bio-assay to detect the presence of type-1 interferons in culture medium of PRRSV-infected cells (13). VSV is highly sensitive to the antiviral effects of type-I IFN. Therefore, in the presence of type-I IFNs, VSV replication will be inhibited. As shown in figure 3C, UV-treated culture medium collected from PRRSV-CON infected cells inhibited the replication of VSV while the UV-treated medium collected from FL12-infected cells did not. The results of this VSV-bioassay indicate that the culture supernatant collected from PRRSV-CON infected cells contains type-I IFNs.

4. Nsp1 and a part of nsp2 of the PRRSV-CON are responsible for the viral capacity to induce type-I IFNs

We sought to identify which genes of the PRRSV-CON are responsible for the induction of type-I IFNs. Based on the availability of unique restriction enzyme sites, we divided the PRRSV-CON genomes into 3 fragments (Fig.4A). Next, we separately exchanged each of the 3 genomic fragments of the PRRSV-CON into the FL12 infectious clone, a PRRSV strain that suppresses type-I IFN production. As shown in figure 4, significant levels of IFN-β mRNA and ISG-56 protein were detected from MARC-145 cells infected with the FL12/A chimeric virus, but not from the cells that were infected with the FL12/B or FL12/C. The results indicate that the genomic fragment A of the PRRSV-CON is solely responsible for the induction of type-I IFNs. The fragment A encodes the nsp1α, nsp1β and a part of nsp2. In total, the genomic fragment A of PRRSV-CON has 73 amino acid differences from the corresponding fragment of the PRRSV FL12. We are conducting animal experiments to compare the protection levels between wild-type FL12 (suppressing type-I IFNs) and FL12/A chimeric virus (inducing type-I IFNs) to determine how the effects of type-I interferon production on the levels of heterologous protection.

Discussion:

Although PRRS vaccines have been used for over 20 years, the mechanisms of protective immunity to PRRSV are not well-understood. Virus neutralizing antibody (NAb) appears to be a component of PRRSV protective immunity (6). Passive immunization studies using both reproductive model and respiratory model have demonstrated that NAbs can protect pigs against infection with a virulent PRRSV strain, providing that sufficient amounts of NAbs are present in the pigs prior to challenge infection (6, 14). However, pigs infected with a virulent PRRSV strain or vaccinated with a PRRS vaccine often develop weak and delayed NAb responses (3, 7, 15). Moreover, the neutralizing antibodies elicited by naturally occurring PRRSV strains often have very limited cross-neutralizing activities. Virus-specific IFN-γ secreting cells have been suggested to be the correlate of vaccine-induced protection (3). However, the degrees of correlation between virus-specific IFN-γ and vaccine-protection are highly variable (16, 17). PRRSV is well characterized for its ability to suppress type-I IFNs (IFN-α/β) (18-21). It is widely accepted that the viral ability to modulate the innate immune response may prime the host towards an enduring defect in the overall acquired protective immune response. Consequently, significant efforts have been made to discover the mechanisms by which PRRSV suppresses the production of type-I IFNs. Until now, the effects of type-I IFNs on the levels of vaccine-induced protection against PRRSV remains uncertain.

In this study, we did not observe any significant difference in the levels of interferon-gamma secreting cells between pigs infected with the PRRSV-CON and those infected with FL12. We observed that the PRRSV-CON seems to elicit better levels of cross-neutralizing antibodies than the PRRSV strain FL12. The most striking observation would be that the synthetic PRRSV-CON virus can induce significantly greater levels of type-I interferons than the PRRSV strain FL12. We further identified that the 3.3 kb fragment at the 5'end of the PRRSV-CON, containing the non-structural protein (nsp) 1 and a part of nsp2, is responsible for the induction of type-I interferons. We are conducting experiments to determine how the effects of type-I interferons on the levels of heterologous protection.

Table 1: Cross-neutralizing antibody titers of serum samples collected at 52 days post-infection (before challenge infection)

Immunization with	Animal ID	**Neutralization titers measured against**		
		FL12	**PRRSV-CON**	**MN184C**
PBS	365	0	0	0
	389	0	0	0
	407	0	0	0
	416	0	0	0
	417	0	0	0
	435	0	0	0
	Geometric Mean	0	0	0
PRRSV-CON	345	0	8	0
	394	0	8	0
	410	4	32	4
	459	8	32	8
	494	0	32	0
	495	0	8	0
	Geometric Mean		12.07	
FL12	349	8	0	0
	381	16	0	0
	440	32	0	0
	455	8	0	0
	487	16	0	0
	507	4	0	0
	Geometric Mean	11.31		

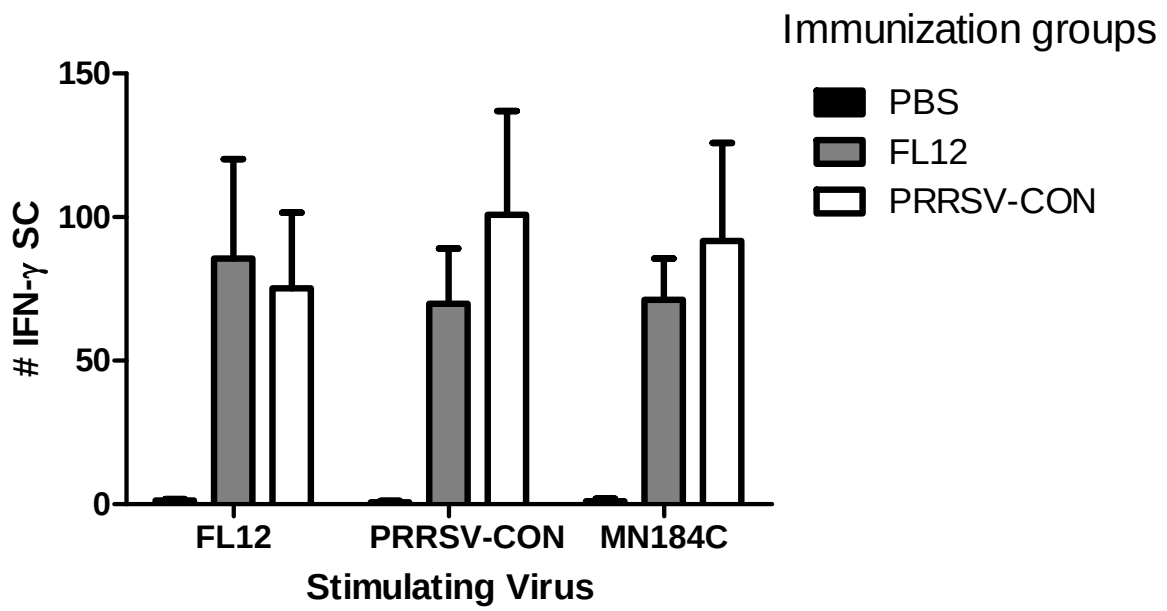

Figure 1: Frequency of interferon-gamma secreting cells (IFN-γ SC) in PBMCs collected right before challenge infection. The frequency of IFN-γ SC was measured by using the IFN-γ ELISPOT assay, with whole virus as the stimulating antigens.

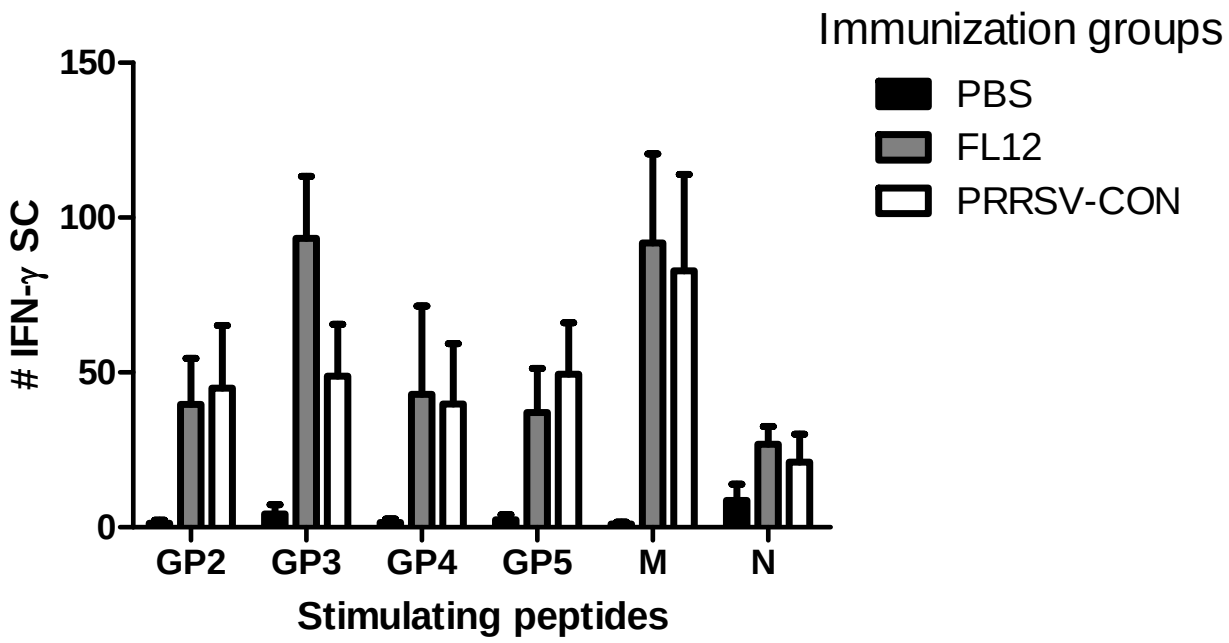

Figure 2: Frequency of interferon-gamma secreting cells (IFN-γ SC) in PBMCs measured by using peptide pools containing individual viral structural proteins.

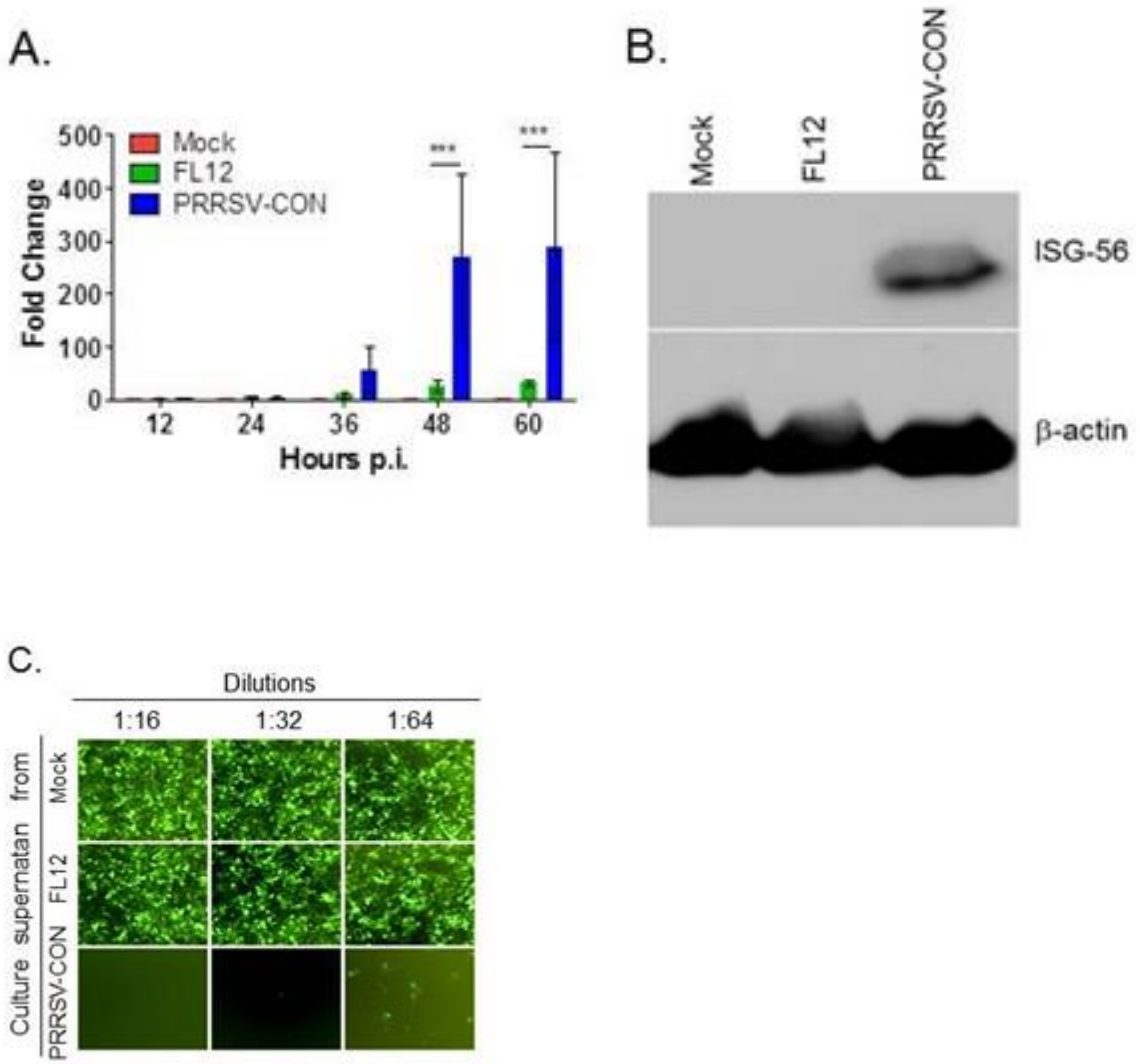

Figure 3: PRRSV-CON induces type I IFNs response in MARC-145 cells. (A) MARC-145 cells were infected with the indicated viruses at MOI of 0.01. The levels of IFN-β mRNA expression were determined by RT-PCR. (B) MARC-145 cells were infected the indicated-viruses. At 48 hours p.i., total protein lysate was collected and analyzed by western blotting using antibody to ISG-56. Beta-actin was used as loading control. (C) Naïve MARC-145 cells were incubated with different dilutions of UV-treated culture supernatants collected from mock or virus infected MARC-145 cells 24 hours. The UV-treated supernatants were then removed and the treated MARC-145 cells were subsequently infected with Vesicular Stomatitis Virus (VSV) expressing GFP. VSV is highly sensitive to interferons. Thus, in the presence of interferons, VSV replication is inhibited.

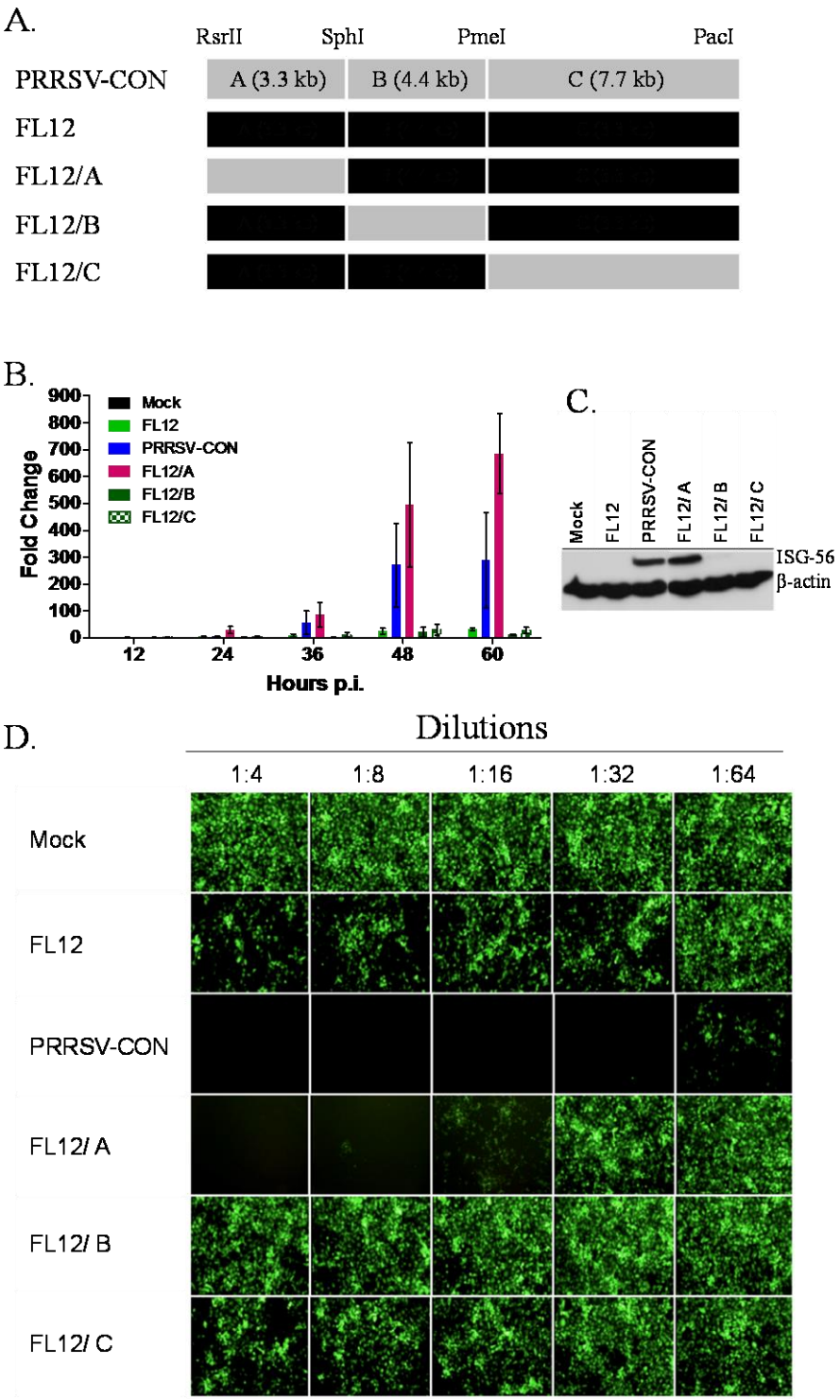

Figure 4: Identification of the genomic region of the PRRSV-CON that is responsible for inducing type-I IFNs. (A) Schematic representation of the chimeric PRRSV constructed by exchanging the genomic fragments the between PRRSV-CON and FL12. The restriction enzymes used for exchanging the genomic fragments are shown on top. The size of each fragment is shown. (B) Levels of IFN-β mRNA in MARC-145 cells infected with the indicated PRRSV strains. (C) Detection of ISG-56 by western blotting. (D) VSV-bioassay. Please see figure 3 for detailed descriptions.

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