RESEARCH REPORT
SWINE HEALTH

Title: Rational design of a broadly protective vaccine against porcine reproductive and respiratory syndrome virus - **NPB #13-155** revised

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Industry summary:
Current PRRS vaccines are not adequately effective for control and eradication of PRRS. The main limitation of the current PRRS vaccines is their sub-optimal coverage against divergent PRRSV strains circulating in the field. The substantial genetic variation among the PRRSV strains is the biggest obstacle for the development of a broadly protective PRRS vaccine. Thus far, all commercial PRRS vaccines are formulated using natural PRRSV strains. In this current project, we proposed a novel approach to the development of a PRRSV vaccine strain that could confer broader cross-protection. Specifically, we applied a validated bioinformatics algorithm to design an artificial PRRSV genome based on a large set of full-genome sequences of PRRSV isolates which represent the widest genetic diversity of PRRSV strains circulating in the U.S. swine herds. This artificial PRRSV genome was designed in such a way that it should have the highest degree of genetic similarity to all the PRRSV field-isolates when compared to any natural PRRSV strains. After that, we chemically synthesized this computer-designed PRRSV genome (herein designated as PRRSV-CON) and used reverse genetics techniques to generate a viable PRRSV-CON virus. Our data showed that the PRRSV-CON virus replicates as efficiently as our prototype PRRSV strain FL12, both in cell culture and in pigs. To this end, we conducted two sets of standard cross-protection experiments in pigs to evaluate the cross-protective capacity of the PRRSV-CON virus. Each set of experiments consisted of 3 groups of weaning pigs, 6 pigs per group. Pigs in group 1 served as non-immunization control whereas those in groups 2 and 3 were infected with either with the PRRSV-CON virus or the PRRSV strain FL12. The PRRSV strain FL12 that is closely similar to the parental strain of the Ingelvac PRRS® ATP was used for the comparison purposes. At 52 days post-primary infection, all control and immunized pigs were challenged with a heterologous PRRSV isolate. The results of these experiments showed that the PRRSV-CON confers significantly broader protection than the prototype PRRSV strain FL12. Collectively, our data demonstrate that the PRRSV-CON can serve as a potential vaccine candidate for the development of a novel PRRS vaccine with broader cross-protection.

Keywords: PRRSV, Cross-protection, Synthetic vaccine

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Scientific abstract

Substantial genetic variation among PRRSV strains represents a major obstacle for the development of a broadly protective vaccine. We describe here a novel approach to generate a PRRSV vaccine strain that could confer broad cross-protection against divergent PRRSV isolates. We initially obtained a set of 60 non-redundant, full-genome sequences of type-II PRRSV. After that, we generated the consensus genome (designated as PRRSV-CON) by aligning the 60 PRRSV full-genome sequences, followed by selecting the most common nucleotide found at each position of the alignment. Our analysis demonstrates that the PRRSV-CON has the highest degree of sequence identity to the PRRSV field-isolates when compared to any current PRRS vaccine strains, both at the full-genome level and the individual gene level. Next, we chemically synthesized the PRRSV-CON genome and assembled it into a bacterial plasmid under the control of the T7 promoter. The resulting PRRSV-CON cDNA clone is fully infectious. Viable virus is consistently produced after MARC-145 cells are transfected with the RNA transcript produced from the PRRSV-CON cDNA clone. Moreover, the PRRSV-CON virus replicates as efficiently as our prototype PRRSV strain FL12, both *in vitro* and *in vivo*. Importantly, primary infection of pigs with PRRSV-CON virus confers significantly broader protection than the prototype PRRSV strain FL12 when tested upon subsequent challenge with a third unrelated heterologous PRRSV strain. Collectively, our data demonstrate that the PRRSV-CON virus can serve as a potential vaccine candidate for the development of a novel PRRS vaccine with broader cross-protection.

Introduction

PRRS remains one of the most economically important diseases affecting the U.S. swine herds, in spite of the fact that PRRS vaccines have been in use for 20 years (since 1994). Current PRRS vaccines are not adequately effective for control and eradication of PRRS. There are 2 types of PRRS vaccines currently available in the market, including modified-live and inactivated virus adjuvanted vaccines. In addition, several subunit vaccines for PRRS are being tested in different laboratories worldwide but none of them are licensed for clinical application. The protective capacity of inactivated PRRS vaccines is very limited. Multiple evidences indicate that the best protection that can be obtained when vaccinating pigs is through the use of modified-live vaccines (MLVs). The homologous immunity conferred by MLV is considered to be fully protective, reaching in many cases sterilizing conditions. On the other hand, the extent and duration of protection against heterologous strains is highly variable. Extensive genetic diversity among PRRSV isolates is the main factor that accounts for the sub-optimal heterologous protection of the current PRRS vaccines. PRRSV is classified into two major genotypes: European (Type 1) and North American (Type 2). There is only limited cross-protection between viral isolates of these two genotypes. Considerable genetic variation exists among PRRSV isolates of each of these genotypes. Importantly, genetic divergence has been shown to occur when a PRRSV strain is serially passed from pig to pig. This leads to the co-circulation of multiple PRRSV variants within one herd or even within one animal that is persistently infected with PRRSV. Therefore, in order to produce an effective PRRS vaccine with a broad spectrum of coverage, it is important to have an applicable method to overcome the pronounced genetic variation of the PRRSV genome. In this project, we demonstrate a novel vaccinology approach to the development of a PRRSV vaccine strain that confers broad cross-protection against divergent PRRSV isolates.

Objective

Objective # 1: Generation of the synthetic PRRSV-CON virus

Objective # 2: Evaluation of the cross-protective capacity of the PRRSV-CON virus against divergent PRRSV strains

Materials and methods

Computational design of the artificial PRRSV-CON genome

Through CAP2 project, we had achieved 64 full-genome sequences of PRRSV isolates originating from the Midwestern states (Iowa, Nebraska and Illinois) of the U.S. In addition, we were able to collect from GenBank more than 20 full-genome sequences of PRRSV isolates that originating from the U.S. After removing redundant sequences (sharing > 99% sequence identity), we attained a final set of 60 full-genome sequences of PRRSV, which represents the breadth of genetic/antigenic diversity of PRRSV. The 60 PRRSV full-genome sequences were aligned using the MUSCLE program. After that, the PRRSV-CON genome was constructed using the Jalview program.

Generation of the synthetic PRRSV-CON virus

The full-length cDNA clone of the PRRSV-CON was constructed following the strategy described previously. Four DNA fragments (A-D) encompassing the entire genome of the PRRSV-CON was chemically synthesized by Genscript (Piscataway, NJ). Each DNA fragment was flanked by a pair of unique restriction enzyme sites to facilitate the cloning purposes (Figure 2). The T7 RNA polymerase promoter was incorporated into fragment D, preceding the viral 5'end, to facilitate the *in vitro* transcription of the viral genome. Individual DNA fragments were sequentially cloned into the shuttle vector that carries the corresponding restriction enzyme sites. Once the full-length PRRSV-CON cDNA clone was achieved, we applied our laboratory's standard reverse genetics techniques to recover the viable PRRSV-CON virus. Briefly, the plasmid containing full-length cDNA genome was digested with AclI for linearization. The purified, linear DNA fragment was used as the template for an *in vitro* transcription reaction using the mMESSAGE mMACHINE Ultra T7 kit (Ambion, Austin, TX) to generate the 5' capped viral RNA transcript. After that, approximately 5 μg of the full-genome RNA transcripts was transfected into MARC-145 cells cultured in a 6-well plate, using the TransIT®-mRNA Transfection Kit (Mirus Bio, Madison, WI). Transfected cells were cultured in DMEM containing 10% FBS at 37^{o}C, 5% CO_2 for up to 6 days. When clear cytopathic effect (CPE) was observed, culture supernatant containing the rescued virus was collected and passed into naïve MARC-145 cells once to obtain enough virus stock for future studies.

In vitro characterization of the PRRSV-CON virus

To study the reactivity of the viruses to different PRRSV specific monoclonal antibodies, MARC-145 cells were mock infected or infected with the PRRSV-CON virus (labeled CON) and FL12 wild type virus. At 48h post-infection, the cells were immuno-stained with antibodies specific to the viral nucleocapsid protein (N protein) and to the viral nonstructural protein 1 beta (nsp1b). To study the growth kinetics of the viruses in cell culture, MARC-145 cells were infected with the PRRSV-CON or FL12 at multiplicity of infection (MOI) 0.01. At different time-points post infection (p.i.), culture supernatant was collected and viral titers were determined by titration in MARC-145 cells.

Evaluation of the cross-protection conferred by the PRRSV-CON virus

To thoroughly evaluate the cross-protective capacity of the PRRSV-CON virus, we performed 2 sets of cross-protection experiments, following the young pig model. The experimental design is presented in table 1. PRRSV seronegative pigs were obtained from UNL research farm and were accommodated in BL-2 animal facilities at UNL, following the regulations established by Institutional Animal Care and Use Committee. Each set of experiments consisted of 3 groups of 6 weaning pigs. Pigs in group 1 served as non-immunization control whereas those in groups 2 and 3 were immunized by infection either with the PRRSV-CON virus or with the PRRSV strain FL12. At day 52 post-immunization, all control and immunized animals were challenged with a selected heterologous PRRSV field isolates. Blood samples were taken at days 1, 4, 7, 10, and 15 post-challenge

for quantification of viremia. On day 15 post-challenge, pigs were humanely sacrificed and necropsied. Samples of tonsil, lung, mediastinal lymph node and inguinal lymph node were obtained for determination of tissue viral load. Viremia and tissue viral load were determined by quantitative RT-PCR.

Results

The synthetic PRRSV-CON genome is fully infectious

The design and construction of the PRRSV-CON cDNA clone are described in the Material and Method section. Clear cytopathic effect was observed at about four days after MARC-145 cells were transfected with the PRRSV-CON RNA transcript. The PRRSV-CON virus displays typical characterizations of a natural PRRSV strain. It reacts with different PRRSV-specific monoclonal antibodies including antibodies against nsp1-betta and N protein (Figure 2B). It is able to form clear and distinct plaque morphology (Figure 2C). In addition, it replicates as efficiently as the natural PRRSV strain FL12 in cell culture (Figure 2D).

The PRRSV-CON virus can infect pigs as efficiently as the natural PRRSV strain

After successful generation of the infectious PRRSV-CON virus, we wanted to know how well this virus infects pigs. To address this question, we infected 4 pigs with the PRRSV-CON virus. Serum samples were collected at different time-points post-infection to determine viremia levels and seroconversion. As shown in Figure 3A, all pigs infected with the PRRSV-CON were viremic. The PRRSV-CON - infected pigs had the same magnitudes of viremia as compared to those infected with the natural PRRSV strain FL12 (Figure 3A). Likewise, all pigs infected with the PRRSV-CON were seroconverted. There was no significant difference in the kinetics and magnitude of seroconversion between pigs infected with the PRRSV-CON and those infected with PRRSV strain FL12 (Figure 3B).

The PRRSV-CON confers broad protection against divergent RRSV strains

We conducted 2 sets of immunization/challenge experiments to evaluate the cross-protective capacity of the PRRSV-CON. The experimental design is presented in table 1. The PRRSV strain FL12, which is closely similar to the parental strain of the MLV ATP, was used for the comparison purposes. In the first cross-protection experiment, we evaluated the cross-protective capacity of the PRRSV-CON against the PRRSV strain MN-184 which belongs to sub-group 2 in the phylogenetic tree. The results show that the PRRSV-CON conferred superior cross-protection against MN-184, as compared to the PRRSV strain FL12. Within the period of 15 days post challenge, pigs in the PRRSV-CON group had significantly greater daily weight gain than those in the control-group (Figure 4A). More importantly, pigs in the PRRSV-CON group had minimal levels of viremia after challenge infection. The viremia levels of the PRRSV-CON group were significantly lower than that of the FL12- and the control-group (Figure 4B). It has been well characterized that PRRSV can colonize and persist in lymphoid tissues of infected pigs up to 150 days post-infection. In our experiment, we evaluate the tissue viral load at 15 days post-challenge, which corresponds to 67 days after the primary infection. At that time, it is likely that the pigs in the PRRSV-CON and FL12 groups still contained residual virus of the primary infection. Consequently, pigs in these two groups may eventually carry 2 populations of viruses: one from the primary (immunizing) infection and the other from the challenge infection. Therefore, we used 2 different RT-qPCR assays to determine the levels of viral RNA in tissues collected at 15 days after challenge: the Tetracore RT-qPCR to detect total PRRSV RNA and the MN-184 specific RT-qPCR to detect the RNA of the challenge virus. The results show that, pigs in the PRRSV-CON and FL12-groups contained significantly lower levels of total viral RNA than those in the control-group (Figure 4C), regardless of the tissue types tested. There was no

significant difference between PRRSV-CON-group and FL12-group in term of total viral RNA. However, pigs in the PRRSV-CON group had significant lower levels of the challenge viral RNA than those in the FL12- and Control-groups (Figure 4D).

In the second cross-protection experiment, we evaluated the cross-protective capacity of the PRRSV-CON against PRRSV strain 16248B which belongs to sub-group 3 in the phylogenetic tree. Similar to the first cross-protection experiment, we observed that during the 15 days after challenge, pigs in the PRRSV-CON-group gained weight significantly better than those in the FL12 and Control groups (Figure 5A). Moreover, the PRRSV-CON groups had significantly lower levels of viremia than the FL12 and control groups. We are in the process of determining the levels viral RNA in tissue samples collected at 15 days after challenge infection.

Discussion

A novel PRRS vaccine capable of conferring cross-protection against all PRRSV strains circulating in the field is badly needed for control and eradication of PRRSV. Current PRRS vaccines can provide solid protection against PRRSV strains that are closely similar to the vaccine strain. Unfortunately, the current vaccines do not confer adequate protection against heterologous PRRSV strains. The pronounced genetic diversity among PRRSV strain is the main hurdle to the development of a broadly protective PRRS vaccine. Up to the present, PRRS vaccines are produced following the traditional method which uses natural PRRSV strains as the vaccine immunogene. Given that the PRRSV genome is highly variable, it is highly unlikely that the traditionally prepared vaccines could be able to provide adequate cross-protection. In this project, we applied a novel vaccinology approach to develop a universal, broadly protective PRRS vaccine in which we uses the synthetic PRRSV strain, instead of the natural PRRSV strain, as the vaccine immunogene to immunize pigs. We demonstrate that the synthetic PRRSV-CON confers superior cross-protective against different heterologous PRRSV strains, as compared to the natural PRRSV strain FL12. Thus, this PRRSV-CON can serve as potential seed strain for the formulation of a universal PRRS vaccine. In addition, it provides an important tool to study the mechanism of heterologous protection against divergent PRRSV strains.

Table 1: Design of the cross-protection experiments

Groups	Immunized with	Challenged with	
		Experiment 1	Experiment 2
1	PBS	MN-184 (Sub-group 2)	16244B (Sub-group 3)
2	PRRSV-CON		
3	PRRSV strain FL12		

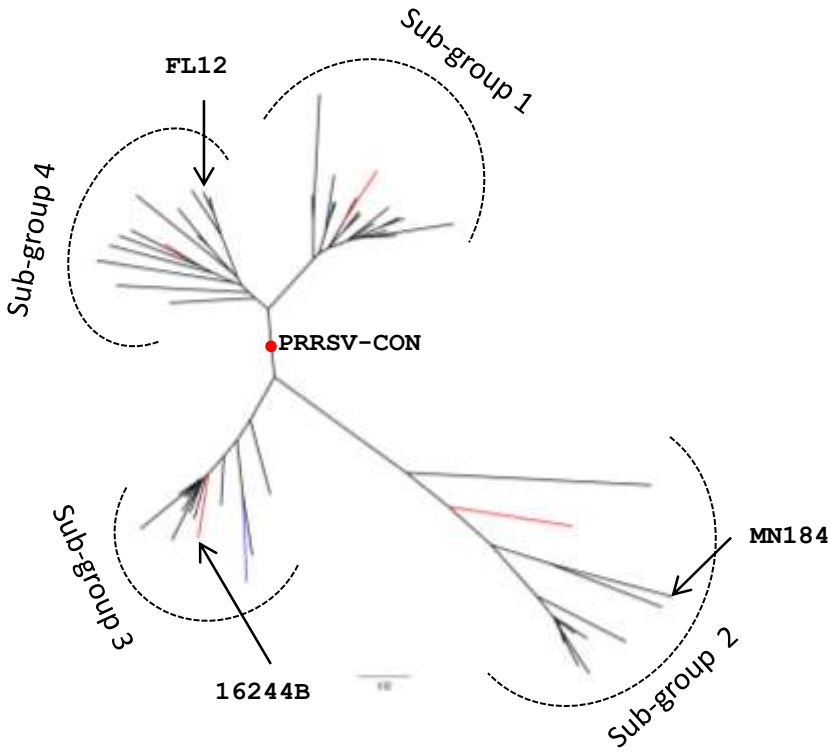

Figure 1: Phylogenetic tree constructed from a set of 60 PRRSV full-genome sequences. These 60 PRRSV genomes are classified into 4 sub-groups. The locations of the viruses involved in the cross-protection experiments are indicated by the arrows.

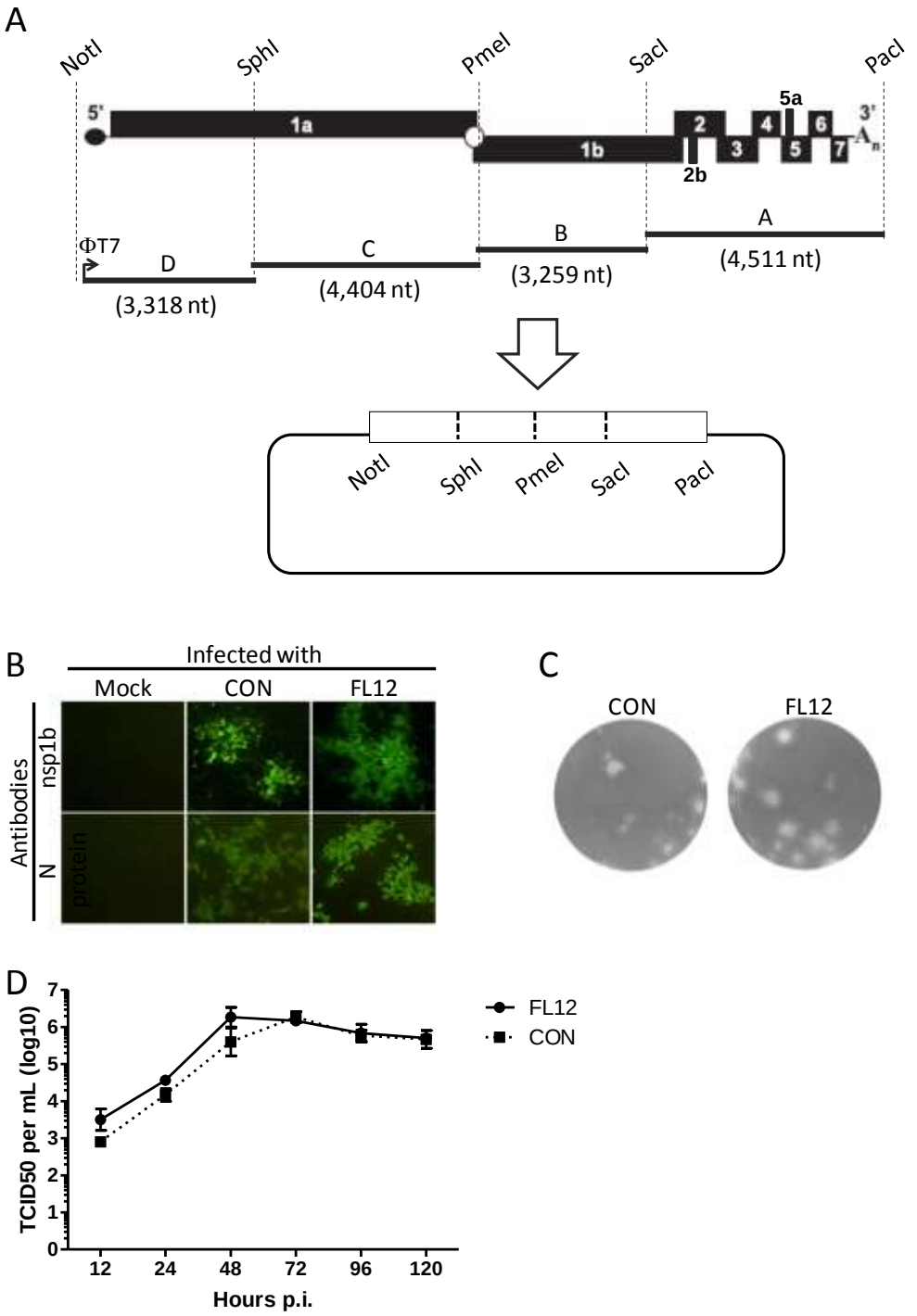

Figure 2: Generation of the PRRSV-CON virus. (A) Strategy to construct the PRRSV-CON full-genome cDNA clone. The unique restriction enzyme sites that were utilized for cloning purpose were indicated. (B) Reactivity of the viruses with different PRRSV-specific monoclonal antibodies. MARC-145 cells were mock infected or infected with PRRSV-CON and PRRSV strain FL12. At 48h post-infection, the cells were stained with antibodies specific to the viral nucleocapsid protein (N protein) and to the viral nonstructural protein 1 beta (nsp1b). (C) Plaque morphology of the viruses in MARC-145 cells. (D) Multiple step growth curve. MARC-145 cells were infected with the indicated viruses at multiplicity of infection (MOI) 0.01. At different time-points post- infection (p.i.), culture supernatant was collected and viral titer was determined by titration on MARC-145 cells.

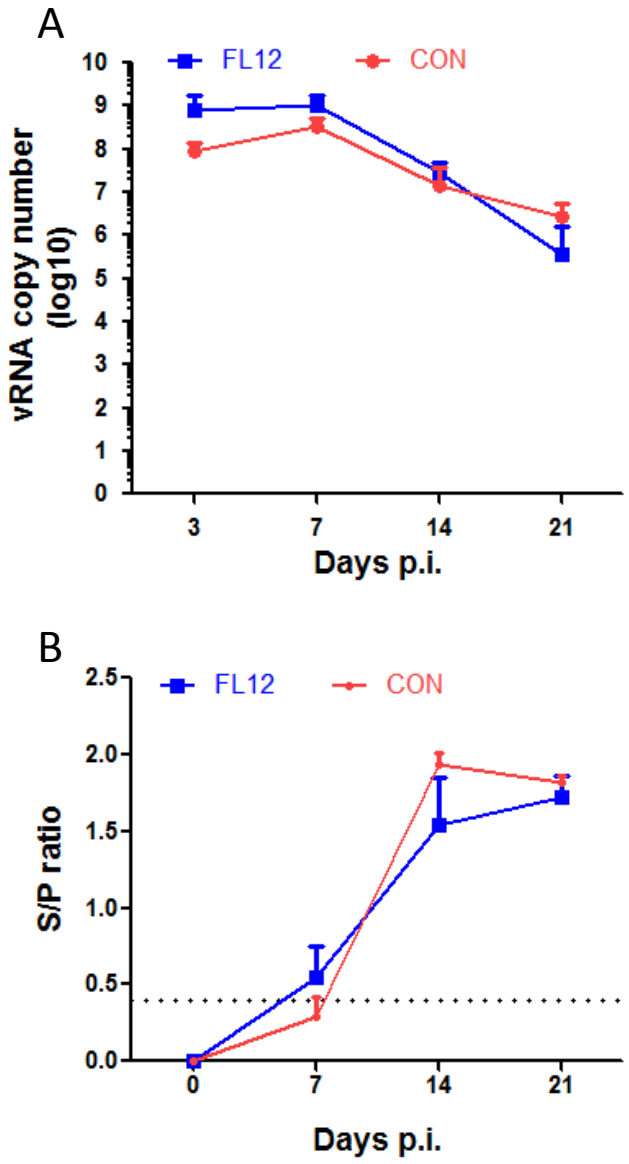

Figure 3: Replication of the PRRSV-CON virus in pigs. (A.) Viremia was determined by quantitative RT-PCR. (B.) Seroconverson was determined by IDEXX ELISA. The horizontal dotted line indicates the cut-off of the assay.

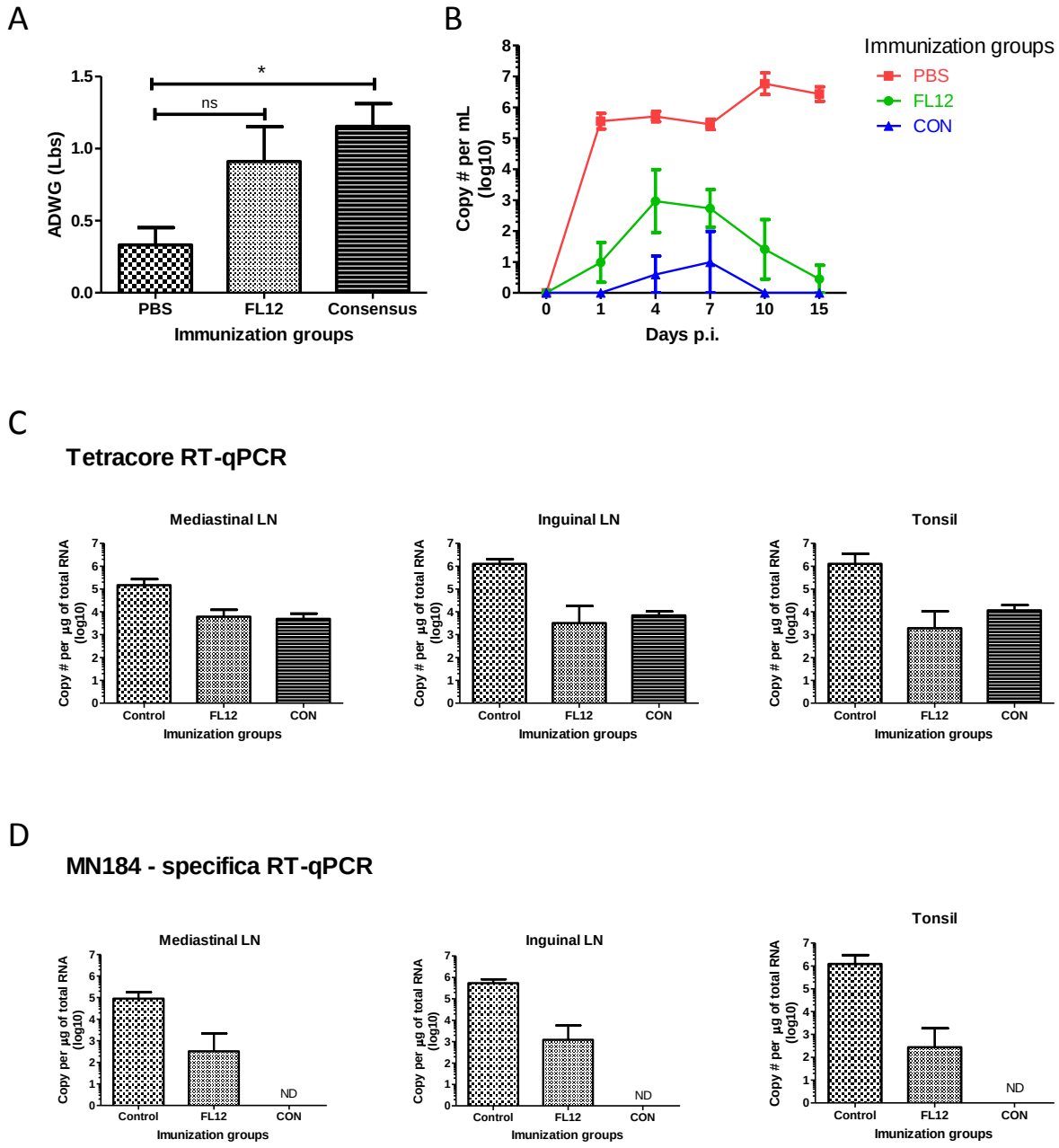

Figure 4: Cross-protection against PRRSV-strain MN-184. (A) Average daily weight gain (ADWG) within the period of 15 days after challenge-infection. (B) Viremia levels after challenge infection determined by quantitative RT-PCR. (C) Total viral RNA levels in different tissues collected at 15 days post challenge determined by Tetracore RT-qPCR. (D) MN-184 specific RNA levels. ND: non-detected

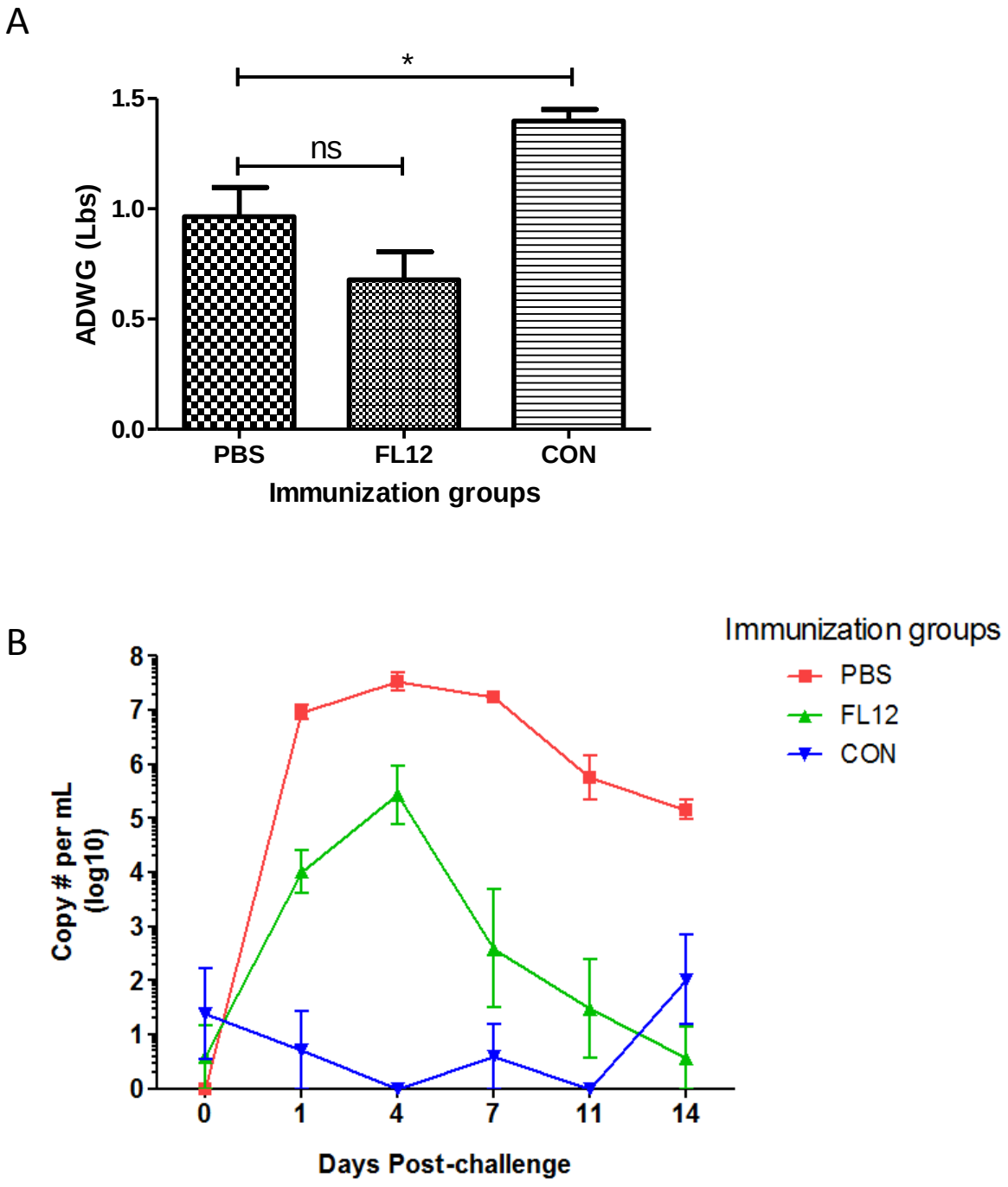

Figure 5: Cross-protection against PRRSV strain 16244B. (A) Average daily weight gain (ADWG) within the period of 15 days after challenge-infection. (B) Viremia levels after challenge infection determined by quantitative RT-PCR.