2016

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A synthetic porcine reproductive and respiratory syndrome virus strain confers unprecedented levels of heterologous protection

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Running title: A synthetic PRRSV strain eliciting broad protection

Word count: Abstract – 172; Main text- 5,894

Key words: PRRSV, synthetic virus, broadly protective vaccine, centralized immunogen, reverse genetics

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Abstract

Current vaccines do not provide sufficient levels of protection against divergent porcine reproductive and respiratory syndrome virus (PRRSV) strains circulating in the field, mainly due to the substantial variation of the viral genome. We describe here a novel approach to generate a PRRSV vaccine candidate that could confer unprecedented levels of heterologous protection against divergent PRRSV isolates. Using a set of 59 non-redundant, full genome sequences of type-2 PRRSV, a consensus genome (designated as PRRSV-CON) was generated by aligning these 59 PRRSV full genome sequences, followed by selecting the most common nucleotide found at each position of the alignment. Next, the synthetic PRRSV-CON virus was generated through the use of reverse genetics. The PRRSV-CON virus replicates as efficiently as our prototype PRRSV strain FL12, both *in vitro* and *in vivo*. Importantly, when inoculated in pigs, the PRRSV-CON virus confers significantly broader levels of heterologous protection than the wild-type PRRSV. Collectively, our data demonstrates that the PRRSV-CON virus can serve as an excellent candidate for the development of a broadly protective PRRS vaccine.

Importance

The extraordinary genetic variation of RNA viruses poses a monumental challenge for the development of broadly protective vaccines against these viruses. To minimize the genetic dissimilarity between vaccine immunogens and the contemporary circulating viruses, computational strategies have been developed for generation of artificial immunogen sequences (so-called "centralized" sequences) that have equal genetic distances to the circulating viruses. Thus far, "centralized" vaccine immunogens have been carried out at the level of individual viral proteins. We expand this concept to for PRRSV, a highly variable RNA virus, by creating a synthetic PRRSV strain based on a "centralized" PRRSV genome sequence. This study provides the first example of "centralizing" the whole genome of an RNA virus to improve vaccine coverage. This concept may be significant for the development of vaccines against genetically variable viruses that require active viral replication in order to achieve complete immune protection.

Journal of Virology

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is widespread in most of swine-producing countries worldwide, causing significant economic losses to swine producers. In the U.S alone, the disease causes approximately $664 million losses to the American swine producers annually (1). Clinical signs of PRRS include reproductive failure in pregnant sows and respiratory diseases in young pigs. The causative agent of PRRS is a positive-sense, single-stranded RNA virus that belongs to the family Arteriviridae of the order Nidovirales and is referred to as porcine reproductive and respiratory syndrome (PRRSV) (2-4). The PRRSV genome is approximately 15 Kb in length and encodes at least 22 different viral proteins (5). Several viral proteins have been shown to elicit humoral and/or cell mediated immune responses in infected pigs but none of those proteins have been conclusively shown to elicit complete immune protection (6-9).

PRRS vaccines have been licensed for clinical application since 1994. Two types of PRRS vaccines are currently available including killed virus (KV) vaccines and modified live virus (MLV) vaccines. Sub-unit vaccines are not available, mainly due to the lack of information on which viral proteins should be incorporated into the vaccine in order to achieve optimal protection. The efficacy of MLV vaccines is far superior to that of KV vaccines (10-13). Current PRRS MLVs confer excellent protection against a PRRSV strain that is genetically similar to the vaccine strain (14, 15). However, the levels of protection against heterologous PRRSV strains are highly variable and overall are considered sub-optimal in all cases (10, 14-19).

The prominent genetic variation of PRRSV genome is the biggest hinder for the development of a broadly protective PRRS vaccine. PRRSV is classified into 2 major genotypes: type-1 (European) and type-2 (North American) that share approximately 65% genomic

sequence identity (20, 21). In addition, there exists a highly pathogenic variant of type-2 PRRSV (so called HP-PRRS) that is endemic in Asia, causing death in pigs of all ages with the mortality up to 100% (22). The genetic variation among PRRSV strains within each genotype is substantial. Based on phylogenetic analysis of the viral glycoprotein 5 (GP5, the most hypervariable surface envelope), type-2 PRRSV can be classified into 9 different lineages, with the pairwise interlineage genetic distance ranging from 10% to 18% (23). The average substitution rate of type-2 PRRSV ORF5 is estimated to be 9.6×10^{-3} substitution/site/year (23). Genetic divergence has been shown to occur when a PRRSV strain is serially passed from pig-to-pig (24). Further, co-circulation of multiple PRRSV variants within one herd or even within one animal has been demonstrated in the field (25).

Multiple strategies have been employed to overcome the formidable challenge posed by such substantial genetic diversity of PRRSV. Many swine producers choose to immunize their herds by means of exposing the animals to wild-type, highly virulent PRRSV that is autochthonous to their farm (for instance, though direct inoculation of viremic serum) so that their herds will acquire protective immunity specific to the residential PRRSV isolates (26). A polyvalent vaccine comprising 5 different live-attenuated PRRSV strains had been tested in pigs (27). However, this polyvalent vaccine did not seem to provide any significant improvement in the levels of heterologous protection as compared with the monovalent PRRS vaccine (27). Recently, several chimeric viruses have been generated by molecular breeding of different structural proteins from genetically divergent strains (28, 29). Although these chimeric viruses have been shown to elicit better cross-neutralizing antibody responses than did the parental PRRSV strains, the levels of heterologous protection conferred by these chimeric viruses remain to be tested (28, 29).

Genomic variation is a common characteristic of RNA viruses (30). One effective vaccinology approach to overcome the extraordinary genetic diversity of RNA viruses is to computationally design vaccine immunogen sequence, so-called "centralized sequences", that should be located at the center of a phylogenetic tree, thereby having equal genetic distances to all wild-type viruses (31, 32). As demonstrated in the case of human immune deficiency virus type 1 (HIV-1), the use of centralized sequences could effectively reduce the genetic distances between vaccine immunogens and the wild-type viruses by half of those between any wild-type viruses to each other (31-33). Three different computational methods have been developed to generate a centralized immunogen sequence including: Consensus, common ancestor and center of the tree (31, 32). A consensus sequence that caries the most common amino acid found at each position of the alignment is the simplest method for construction of a centralized immunogen (31). Studies on HIV-1 and influenza virus have clearly demonstrated that the vaccines based on the consensus sequences elicit broader immune responses than the vaccines based on naturally occurring sequences (34-38).

We describe here the generation and characterization of a synthetic PRRSV strain that was constructed based on a consensus, full genome sequence of type-2 PRRSV. We show that the PRRSV consensus genome (designated as PRRSV-CON) is fully infectious, and the synthetic PRRSV-CON virus displays typical characteristics of a naturally occurring PRRSV strain. Importantly, when inoculated to pigs, the PRRSV-CON virus confers exceptional levels of heterologous protection against divergent PRRSV strains as compared with a reference wild-type PRRSV strain.

Materials and Methods

Ethics Statement

All animal experiments in this study were conducted in compliance with the *Animal Welfare Act of 1966 and its amendments*, and the *Guide for the Care and Use of Agricultural Animals in Research and Teaching* (3rd edition). The animal care and use protocol was approved by the University of Nebraska-Lincoln (UNL) Institutional Animal Care and Use Committee (protocol # 930).

Cells, antibodies and PRRSV strains

Monkey-kidney cell line MARC-145 (39), porcine kidney 15 (PK-15, baby hamster kidney 21 (BHK-21) and Hela cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Immortalized porcine alveolar macrophages, clone 3D4/31 (PAM 3D4/31, ATCC CRL-2844) were cultured in RPMI-1640 supplemented with 10% FBS (40). All cell lines were cultured at 37^{o}C and 5% CO_2. PRRSV-specific hyper-immune antibody used for virus-neutralization assay was generated previously (41). This hyper-immune antibody can cross-neutralize different type-II PRRSV strains with high end-point neutralization titers (41). PRRSV-specific monoclonal antibodies (MAbs) used for indirect immunofluorescent assay include anti-GP5 (clone ISU25-C1 (42)), anti-M protein (clone 201 (43)) and anti-N protein (clone SDOW17 (44)). Alexa fluor® 488-conjugated goat anti-mouse antibody was purchased from Invitrogen (Eugene, OR). PRRSV strains used for immunization or challenge infection include: FL12, 16244B and MN184C. PRRSV strains FL12 was recovered from the full length infectious cDNA clone (45) derived from PRRSV strain NVSL 97-7895 (GenBank accession no. AY545985). PRRSV strain 16244B (GenBank accession no. AF046869) was isolated in 1997 from a piglet originated in a farm where sows

148 experienced severe reproductive failure (20). PRRSV strain MN184C (GenBank accession no.
149 EF488739 (46)) was kindly provided by Dr. Faaberg, National Animal Disease Center, USA.

150

151 **Collection of type-2 PRRSV full genome sequences and design of the consensus PRRSV**
152 **genome**

153 Through our studies on the "Immunologic Consequences of PRRSV Diversity" (Laegreid
154 et al., un-published data), we sequenced the full genome of 64 type-2 PRRSV strains/isolates
155 originating in Midwestern states (Iowa, Nebraska and Illinois) of the USA. In addition, we were
156 able to collect 20 genome sequences of type-2 PRRSV isolates from GenBank that also
157 originated in the USA. After removing redundant sequences, we attained a final set of 59
158 genome sequences of type-2 PRRSV: 39 genome sequences were sequenced by our laboratories
159 and 20 genome sequences were collected from GenBank. List of PRRSV genome sequences with
160 the GenBank accession number is presented in Table S1. The PRRSV genome sequences were
161 aligned using the MUSCLE 3.8 program (47). A consensus genome (PRRSV-CON) was
162 constructed using the Jalview program (48). The PRRSV-CON genome was aligned with the
163 reference PRRSV strain FL12 genome and frameshift mutations (insertion and deletion
164 mutations) were manually corrected to ascertain that the viral proteins would be properly
165 expressed. Finally, the 5' and 3' un-translated regions (UTR) of the PRRSV-CON genome were
166 replaced by the counterparts of FL12 genome. Phylogenetic tree of the 59 naturally occurring
167 PRRSV genomes, together with the PRRSV-CON, was constructed using PHYML 3.0, an
168 implementation of maximum likelihood method (49).

169

Generation of the synthetic PRRSV-CON virus

To generate an infectious virus based on the PRRSV-CON genome, a full genome cDNA clone of the PRRSV-CON was constructed following the strategy described previously (45). Four DNA fragments (A-D) encompassing the whole PRRSV-CON genome was chemically synthesized by Genscript (Piscataway, NJ). Each DNA fragment was flanked by a pair of restriction enzyme sites to facilitate the cloning purposes. The restriction enzymes sites used for assembling the full genome cDNA clone include: NotI, SphI, PmeI, SacI and PacI. NotI and PacI are restriction enzyme sites that are added to 5' and 3' ends of the PRRSV-CON cDNA genome, respectively. SphI, PmeI and SacI are naturally occurring restriction enzyme sites that reside inside the PRRSV-CON cDNA genome. 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 a pBR322 plasmid that was modified to carry the corresponding restriction enzyme sites. Once the full genome PRRSV-CON cDNA clone was assembled, standard reverse genetics techniques were employed to recover an infectious PRRSV-CON virus (43, 45, 50). Briefly, the plasmid containing 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% CO2 for up to 6 days. When clear cytopathic effect (CPE) was observed, culture supernatant containing the

192 rescued virus was collected and passed into naïve MARC-145 cells one more time to obtain
193 enough virus stock for future studies.
194

195 **Indirect immuno-fluorescent assay**

196 To study the reactivity of the viruses to different PRRSV specific monoclonal antibodies,
197 MARC-145 cells were mock-infected or infected with the PRRSV-CON virus and wild-type
198 FL12. At 48h post-infection, cells were washed twice with phosphate buffer saline (PBS, pH 7.4)
199 and then fixed with 4% paraformaldehyde for 20 minutes at room temperature. After two washes
200 in PBS, the cells were permeabilized with PBS containing 0.1% Triton X-100 for 15 minutes at
201 room temperature. Next, the cells were incubated with PRRSV-specific MAbs for 1h at room
202 temperature, followed by 3 washes in PBS. Finally, the cells were incubated with anti-mouse,
203 Alexa fluor® 488-conjugated antibody for 1 hour at room temperature. After 3 washes in PBS,
204 cells were observed under an inverted fluorescent microscope.

205 **Virus-neutralization assay**

206 Virus neutralization assay was done in MARC-145 cells, using a fluorescent focus
207 neutralization assay described previously (51). Neutralization titers were expressed as the
208 reciprocal of the highest dilution that showed 90% or greater reduction in the number of
209 fluorescent foci presenting in the control wells.

210 ***In vitro* Infectivity assay**

211 Immortalized PAM 3D4/31 (40), PK-15, BHK-21 and Hela cells were separately plated
212 in 24-well plates. At approximately 24h later, cells in each well were infected with 2 X $10^{4.0}$
213 $TCID_{50}$ of PRRSV-CON or PRRSV strain FL12. Forty eight hours after infection, the expression

of viral nucleocapsid protein was examined by using an indirect immuno-fluorescent assay described above.

Multiple step growth curve and plaque assay

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, culture supernatant was collected and virus titers were determined by titration in MARC-145 cells. Plaque morphology was examined in MARC-145 cells as previously described (52).

Assessment of the viral virulence in pigs

A total of 18 PRRSV-seronegative, 3 week-old pigs were purchased from the UNL's research farm. The pigs were randomly assigned into 3 treatment groups, 6 pigs per group. Each treatment group was housed in a separate room in the biosecurity level 2 (BL-2) animal research facilities at UNL. After 1 week of acclimation, pigs in group 1 were injected with PBS to serve as normal control. Pigs in group 2 and 3 were inoculated intramuscularly with $10^{5.0}TCID_{50}$ of PRRSV-CON and PRRSV strain FL12, respectively. Rectal temperature was measure daily from -1 to 13 days p.i.. Pigs were weighed right before challenge infection and on 15 days p.i.. Body weight was recorded. Average daily weight gain (ADWG) was calculated for the period of 15 days p.i.. Blood samples were collected periodically and serum samples were extracted and stored at -80^{o}C for evaluation of viremia levels and seroconversion. Viremia levels were quantitated by the Animal Disease Research and Diagnostic Laboratory, South Dakota State University, by using a commercial RT-PCR kit (Tetracore Inc., Rockville, MD). Results were reported as log10 copy/mL. For statistical purposes, samples that had undetected level of viral

RNA were assigned a value of 0 log10 copy/mL. Seroconversion was evaluated using the IDEXX PRRS X3 Ab test (IDEXX Laboratories, Inc. Westbrook, ME). At 14 day p.i., pigs were humanely sacrificed and necropsied. Gross and microscopic lung lesions were blindly evaluated by a pathologist, following a method described previously (53).

Assessment of heterologous protection in pigs

Two sets of immunization/challenge experiment were conducted. Three-week old, PRRSV seronegative pigs were obtained from UNL's research farm and were accommodated in BL-2 animal facilities at UNL. 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 the dose of $10^{4.0}$ TCID50 per pig, intramuscularly. At day 52 post-immunization, all control and immunized animals were challenged with a selected heterologous PRRSV field isolates at the challenge dose of $10^{5.0}$ $TCID_{50}$ per pig, intramuscularly. Parameters of protection include: growth performance; viremia and viral load in tissues. To measure growth performance, each pig was weighed right before challenge infection and at 15 days post-challenge (days p.c.) and average daily weigh gain (ADWG) was calculated for the period of 15 days p.c.. To quantitate levels of viremia after challenge infection, blood samples were taken periodically and serum samples were extracted and stored at -80^{o}C. Viremia levels were quantitated by the Animal Disease Research and Diagnostic Laboratory, South Dakota State University, by using a commercial RT-PCR kit (Tetracore Inc., Rockville, MD). Results were reported as log10 copy/mL. For statistical purposes, samples that had undetected level of viral RNA were assigned a value of 0 log10 copy/mL. To quantitate levels of viral load in tissues, pigs were humanely sacrificed and

necropsied at 15 day p.c.. Samples of tonsil, lung, mediastinal lymph node and inguinal lymph node were were snap-frozen in liquid nitrogen right after collected and stored in a -80^{o}C freezer. Tissue samples were homogenized in Trizol reagent (Life technologies, Carlsbad, CA) with the ratio of 300 mg tissue in 3mL Trizol reagent. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instruction. RNA concentration was quantified by the NanoDrop®ND-1000 (NanoDrop Technologies, Wilmington, DE) and adjusted to the final concentration of 200 ng/µL. Two different types of RT-PCR kits were used for quantitation of the viral load in tissues: (i) the commercial RT-qPCR kit (Tetracore, Rockville, MD) that detects total viral RNA resulting from primary infection and from challenge infection, and (ii) the differential RT-PCR kits developed in-house that selectively detects only the viral RNA from challenge infection. Design and validation of the differential RT-PCR kit is presented in the Appendix. Five µL of each RNA sample (equivalent to 1 µg RNA) was used for each RT-PCR reaction. Results were reported as log10 copy/µg of total RNA. For statistical purposes, samples that had undetected viral RNA level were assigned a value of 0 log RNA copy/1 µg of total RNA.

Statistical analysis

Each pig was considered an experimental unit and a random effect. Data was analyzed as a completely randomized design using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC). All means are presented as least-squares means and standard error of means (S.E.M.). Data was considered significant when $P \leq 0.05$. Viremia data was analyzed with repeated measures using the statistical model included treatment, time, and their interaction as fixed effects.

Results

Design of a consensus genome of type-2 PRRSV

We were able to obtain a set of 59 non-redundant, full genome sequences of type-2 PRRSV. Pairwise genetic distances among these 59 PRRSV genome sequences range from 0.1% to 17.8%. Phylogenetic analysis reveals that these 59 PRRSV full genome sequences can be divided into 4 subgroups (Fig. 1A), with the mean nucleotide distances between any 2 subgroups ranging from 8.0% to 15.7%. From this set of 59 full genome sequences, we created a consensus PRRSV genome (PRRSV-CON) by aligning these 59 PRRSV full genome sequences and selecting the most common nucleotide found at each position of the alignment. The PRRSV-CON genome is located precisely at the center of the phylogenetic tree (Fig 1A). Consequently, the PRRSV-CON genome has a balanced genetic distance to the wild-type PRRSV strains. As shown in Fig. 1B, the pairwise genetic distances between the PRRSV-CON and wild-type PRRSV strains are significantly shorter than the distances between each pair of wild-type PRRSV strains. Importantly, the distances between the PRRSV-CON and wild-type PRRSV are also significantly shorter than the distances between the type-2 PRRS vaccine strains and the wild-type PRRSV (Fig. 1B). Based on this data, we hypothesized that a vaccine formulated based on the PRRSV-CON virus would confer broader levels of heterologous protection than a conventional vaccine formulated based on a naturally occurring PRRSV strain.

The synthetic PRRSV-CON genome is fully infectious

The PRRSV-CON genome was chemically synthesized and assembled into a bacterial plasmid to produce a full genome cDNA clone (Fig. 2A). Standard reverse genetics techniques were employed to recover an infectious PRRSV-CON virus (43, 45, 50). Visible cytopathic effect

(CPE) was readily observed at approximately 4 days after MARC-145 cells were transfected with the RNA transcripts generated from the PRRSV-CON cDNA clone. The resultant PRRSV-CON virus reacted with different PRRSV-specific monoclonal antibodies including antibodies against GP5, M and N proteins (Fig. 2B). Importantly, the PRRSV-CON virus was neutralized by a PRRSV-specific hyper-immune antibody with an end-point titer equivalent to the PRRSV strain FL12 (Fig. 2C). The PRRSV-CON virus replicated efficiently in cell culture when compared with the reference wild-type PRRSV strain FL12 (45). As shown in Fig. 2D, no significant difference in growth kinetics was observed between the PRRSV-CON virus and the FL12. Further, the PRRSV-CON virus produced larger plaques than did the FL12 (Fig. 2E). Naturally occurring PRRSV has a very restricted cell tropism. Inside its natural host the virus mainly replicates in macrophages residing in lung and lymphoid organs (54). *In vitro*, the virus is mainly propagated in primary PAMs and (40) the monkey kidney cell MA-104 and its derivatives MARC-145 and CL-2621 (39). Interestingly, the virus does not infect immortalized cell lines derived from pigs such as PK-15 and the immortalized PAM 3D4/31, presumably due to the absence of the CD163 receptor (40, 55, 56). We asked if the synthetic PRRSV-CON virus shows any alterations on cell tropism. To address this question, we investigate the virus infectivity in different cell lines including immortalized PAM 3D4/31 (40), PK-15, BHK-21 and Hela cells. Similar to PRRSV strain FL12, the PRRSV-CON virus does not infect any of the cell lines tested (data not shown), indicating that the synthetic virus maintains the same cell tropism as the naturally occurring PRRSV.

The synthetic PRRSV-CON virus is highly virulent

To characterize the pathogenicity of the PRRSV-CON virus in pigs, an animal experimental comprising 3 groups of weaned (3 week-old) pigs. Pigs in group 1 were injected

with PBS to serve as normal control. Pigs in group 2 and 3 were inoculated intramuscularly with $10^{5.0}$ $TCID_{50}$ of PRRSV-CON and FL12, respectively. The PRRSV strain FL12 was included in this study for comparative purposes because its pathogenicity in pigs has been extensively characterized in our laboratories (45). After infection, the PRRSV-CON and FL12-groups displayed significantly higher rectal temperature than the PBS-group (Fig. 3A). There was no difference in rectal temperature between the PRRSV-CON group and the FL12-group. Pigs infected with the PRRSV-CON virus had the same kinetics and magnitude of viremia as those infected with the PRRSV strain FL12 (Fig. 3B). All pigs in PRRSV-CON and FL12 groups seroconverted by 10 days post infection (days p.i.). The level of antibody response in the PRRSV-CON group was slightly lower than in the FL12-group (Fig. 3C). At necropsy (14 days p.i.), pigs in the PRRSV-CON group displayed a similar level of lung lesions to those in the FL12-group (Fig. 3D and 3E). Collectively, the results of this experiment indicate that the synthetic PRRSV-CON virus displays the same level of virulence as the PRRSV strain FL12.

The PRRSV-CON virus confers exceptional levels of heterologous protection

Two sets of immunization (by infection)/challenge experiments were conducted to evaluate the cross-protective capacity of the PRRSV-CON virus. The experimental design to evaluate levels of cross-protection is presented in Fig. 4. In the first immunization/challenge experiment, we evaluated the level of cross-protection against the PRRSV strain MN184C which belongs to sub-group 1 in the phylogenetic tree (see Fig. 1A). During the period of 15 days post-challenge infection (days p.c.), pigs in the PRRSV-CON and FL12-groups had better average daily weight gain (ADWG) than those in the PBS-group (Fig. 5A). There was no statistical difference between the PRRSV-CON and the FL12 groups in regard to their growth

performance. The viremia levels after challenge infection are presented in Fig. 5B and Table 1. After challenge infection, all pigs in the PBS-group were viremic at all time-points tested. The PRRSV-CON group had only 3 viremic pigs, of which, 1 pig was viremic at 2 time-points (e.g. pig # 494 at 4 and 7 days p.c.) and 2 pigs were viremic at only one time-point (e.g. pigs # 394 and 495 at 15 days p.c.). The remaining 3 pigs in this group (pigs # 345, 410 and 459) were not viremic after challenge infection (Table 1). By contrast, 5 out of 6 pigs in the FL12-group were viremic at two time-points or more after challenge infection. There was only 1 pig in this group (pig # 440) that was not viremic at any time-point tested. Overall, the viremia level of the PRRSV-CON group was significantly lower than that of the FL12-group ($p<0.05$) and the PBS-group ($p<0.0001$) (Fig. 5B). To quantitate the levels of viral load in tissues, we first used a commercial RT-PCR kit (Tetracore, Rockville, MD) that detects total viral RNA resulting from primary infection (immunization) and from challenge infection. The results of total viral RNA are presented in Fig. 5C. The PRRSV-CON and FL12-groups contained significantly lower levels of total viral RNA than the PBS-group, regardless of the types of tissue tested. There was no difference between the PRRSV-CON and FL12 groups in terms of the total viral load in tissues (Fig. 5C). Next, we used a differential RT-PCR kit to specifically quantitate the levels of challenge virus-specific RNA (e.g. MN184C-specific RT-PCR kit). As shown in Fig. 5D, all pigs in the PBS-group carried the MN184C-specific RNA in their tissues. Four pigs in FL12-group had the MN184C-specific RNA in their tonsil and mediastinal lymph node whereas 5 pigs in this group had the MN184C-specific RNA in their inguinal lymph node (Fig. 5D). Remarkably, none of the pigs in PRRSV-CON group had detectable levels of the MN184C-specific RNA in any of the tissue samples tested (Fig. 5D). Collectively, the results of this immunization/challenge

experiment demonstrate that the PRRSV-CON conferred significantly better level of cross protection against challenge with the PRRSV strain MN184C than did the PRRSV strain FL12.

In the second immunization/challenge experiment, we evaluated the level of cross-protection against the PRRSV strain 16244B, which falls within the sub-group 2 in the phylogenetic tree (see Fig. 1A). During the period of 15 days p.c., the PRRSV-CON group had greater ADWG than the PBS- and FL12-groups (Fig 6A). In contrast, the FL12-group did not exhibit statistical difference in the growth performance compared with the PBS-group. The viremia levels after challenge infection are presented in Fig. 6B and Table 2. After challenge infection, all pigs in the PBS-group were viremic at all time-points tested. Two out of 5 pigs in the PRRSV-CON group (pigs # 442 and 445) did not resolve viremia at 50 day after primary infection (2 days before challenge infection), as low levels of viral RNA were still detected in their serum samples collected at this time-point (Table 2). After challenge infection, 3 pigs in the PRRSV-CON group were viremic at only 1 time-point. The remaining 2 pigs in this group (pigs # 436 and 438) were not viremic throughout the entire period of 15 days p.c. (Table 2). By contrast, all pigs in the FL12-group resolved viremia by 50 days post-primary infection. After challenge infection, all pigs in this group became viremic. Overall, the viremia level of the PRRSV-CON group was significantly lower than that of the FL12-group ($p<0.0001$) and the PBS-group ($p<0.0001$) (Fig. 6B). Similar to the first immunization/challenge experiment, we first used a commercial RT-PCR kit (Tetracore, Rockville, MD) to quantitate the total viral RNA in tissues of pigs. Both the PRRSV-CON and FL12-groups contained significantly lower levels of total viral RNA than the PBS-group in all of the tissues tested (Fig. 6C). However, there was no difference between the PRRSV-CON group and the FL12-group in regard to the levels of total viral RNA in tissues (Fig. 6C). Next, we used a differential PRT-PCR kit to specifically

quantify the levels of 16244B-specific RNA in tissues. Design and validation of the 16244B-specific RT-PCR kit is presented in supplementary section (S1 File). All pigs in the PBS- and FL12-groups carried the 16244B-specific RNA in their tissues (Fig. 6D). By contrast, only 1 pig in the PRRSV-CON group carried the 16244B -specific RNA in its inguinal lymph node while the remaining 4 pigs in this group did not carry the 16244B - specific RNA in any of the tissues tested. Collectively, the results of this immunization/challenge experiment demonstrate that the synthetic PRRSV-CON conferred better protection against challenge infection with the PRRSV strain 16244B than did the PRRSV strain FL12.

Genetic stability of the PRRSV-CON virus in pigs

To determine the stability of the PRRSV-CON genome, we isolated the virus from a serum sample collected at 21 days p.i. and sequenced its structural genes. Totally, there were 5 nucleotides changes in the structural genes of the virus: 1 in ORF3, 1 in the overlapping region between ORF3 and ORF4, 2 in ORF5 and 1 in ORF6 (Table 3). Two of these 5 nucleotides changes resulted in amino acid changes. The nucleotide change in the overlapping region between ORF3 and ORF4 led to amino acid change in ORF3 but not in ORF4.

Discussion

Advances in DNA synthesis have provided opportunities to manipulate viral genomes on a scale that otherwise cannot be done by the traditional molecular engineering approaches. This leads to the emergence of a new branch in the field of virus research termed synthetic virology (57). A number of synthetic viruses have been generated by *de novo* synthesis of the viral genomes in the absence of natural viral templates (58-64). These synthetic viruses provide powerful tools for studying viral biology and pathogenesis as well as for rational design novel

vaccines (58, 62, 65-67). In this study, we describe the generation of a synthetic PRRSV strain that can be used to develop a broadly protective vaccine.

Currently, all licensed PRRS vaccines are derived from naturally occurring PRRSV strains. The major limitation of the current PRRS vaccines is that they do not confer adequate levels of heterologous protection against divergent PRRSV strains circulating in the field, largely due to the substantially variable nature of the viral genome. Therefore, there is a need for a novel vaccine design to overcome the pronounced genetic variation of PRRSV. "Centralized" vaccine immunogen has been proven an effective method to reduce the genetic distances between the vaccine immunogen and the contemporary virus strains circulating in the field, thereby expanding the vaccine coverage (31, 32). Thus far, "centralized" vaccine immunogens are commonly generated based on amino acid sequence of selected viral proteins (34-37, 68). In the case of PRRSV, the viral proteins that are involved in eliciting protective immunity are not fully understood. None of the PRRSV encoded proteins are known to be able to elicit complete immune protection. The protective efficacy is best when the pigs are immunized by infection with a replicating PRRSV strain (10). Therefore, we aimed to generate a fully infectious PRRSV strain based on a "centralized" whole genome sequence. We demonstrated that the PRRSV-CON genome is biologically functional. Infectious virus is readily generated when the PRRSV-CON genome is transfected into a permissive cell line. Importantly, the PRRSV-CON virus confers significantly broader levels of heterologous protection against divergent PRRSV strains than does a wild-type PRRSV strain.

Globally, type-2 PRRSV can be classified into 9 different lineages, based on phylogenetic analysis of a large number of ORF5 nucleotide sequences collected from GenBank (23). The pairwise genetic distances among these 9 lineages vary from 10.1% to 18% (23). The

443 set of 59 PRRSV full genome sequences used for generation of the PRRSV-CON genome
444 originates exclusively in USA. At the full genome level, the pairwise genetic distances among
445 these 59 PRRSV genome sequences can be as large as 17.8%, which is the same order of
446 magnitude as the genetic distances among ORF5 nucleotide sequences of type-2 PRRSV
447 deposited on GenBank. We postulate that our set of 59 PRRSV full genome sequences would
448 represent the breadth of genetic diversity of type-2 PRRSV. We therefore expect that the
449 synthetic PRRSV-CON might be able to confer cross-protection against type-2 PRRSV strains
450 that are currently circulating worldwide.

451 As has been observed for HIV-1, genetic distances between 2 clades of the group M
452 envelope proteins can be up to 30%. A vaccine based on a single consensus envelope sequence
453 can elicit significantly broader cross-clade cellular immune responses than could a vaccine based
454 on a naturally occurring envelope sequence (34, 37). PRRSV is classified into 2 major types:
455 type-1 and type-2. There is very limited cross-protection between type-1 and type-2 PRRSV
456 strains (17, 18, 69). Genetically, type-1 and type-2 PRRSV share approximately 65% sequence
457 identity (20, 21). It is possible that a synthetic PRRSV strain whose genome is centralized
458 between type-1 and type-2 would be able to provide equal protection against both types of
459 PRRSV. The availability of such a PRRS vaccine would be extremely beneficial to the control
460 and eradication of the disease, especially in the areas where both types of PRRSV co-circulate.

461 Viral load in tissue samples collected after challenge infection is an important parameter
462 to evaluate the protective efficacy of a PRRS vaccine candidate. Currently, the tissue viral load is
463 usually quantified through the use of a commercial RT-PCR kit or through titration on a
464 permissive cell line such as MARC-145 cells. The use of these 2 methods will not allow
465 precisely quantifying the level of tissue viral load resulting from challenge infection in the case

466 that the pigs are immunized with a replicating vaccine (either with MLV vaccines or with
467 virulent PRRSV strains) (70). This is because PRRSV can persist in the infected animals for an
468 extended period of time (71, 72). At the time of tissue collection for evaluation of viral load, the
469 pigs that are immunized by infection with a live PRRSV may still carry in their lymphoid tissues
470 the PRRSV strain that is used from immunization. Consequently, the tissue samples will possibly
471 contain 2 populations of PRRSV: one from immunization and the other from challenge infection.
472 Neither the commercial RT-PCR kit nor titration on MARC-145 cells can differentiate the viral
473 strain used for primary infection from the PRRSV strain used for challenge infection. In the
474 present study, we used differential RT-PCR kits to specifically quantitate the viral RNA resulting
475 from challenge infection. Through the use of these differential RT-PCR kits, we demonstrate that
476 pigs previously infected with the PRRSV-CON virus contained undetectable levels of challenge
477 PRRSV strains while those infected with FL12 can only lower the level of challenge viral RNA
478 (Figs. 5D and 6D).

479 Of the 59 full genome sequences that were used in this study to design the PRRSV-CON
480 genome, only 3 sequences were of the live-attenuated PRRSV strains. The remaining 56
481 sequences were of the wild-type PRRSV strains/isolates. Therefore, it is expected that the
482 PRRSV-CON virus should display a virulent phenotype of wild-type PRRSV strains. Obviously,
483 the PRRSV-CON virus must be inactivated or attenuated before it can be used as a vaccine in
484 pigs. Both KV vaccines and MLV vaccines are being used in the field. MLV vaccines are
485 commonly developed by successively passaging virulent PRRSV strains in a non-natural host
486 cell lines. Recently, molecular approaches have been used to attenuate virulent PRRSV strains
487 (73, 74). Several studies have demonstrated that MLV vaccines are far more effective than KV
488 vaccines (10, 11). Even so, there are swine producers who prefer to use KV vaccines rather than

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MLV vaccines because of the concern that MLV vaccines might revert to virulence. It is highly possible that the killed PRRSV-CON virus vaccine may confer better levels of cross-protection than the KV made of naturally occurring PRRSV strains.

The mechanisms by which PRRS vaccines confer protection remain poorly understood (75). Passive immunization studies using both reproductive model and respiratory model have demonstrated that neutralizing antibodies (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 (41, 76). However, pigs infected with virulent PRRSV strains or vaccinated with MLV vaccines often develop weak and delayed NAb responses (10, 77, 78). Several vaccine studies have demonstrated that vaccinated pigs are protected from challenge infection in the absence of NAbs (10, 19, 79). Virus-specific IFN-γ producing cell has been suggested to be the correlate of vaccine-induced protection (10). However, the degrees of correlation between the frequencies of virus-specific IFN-γ producing cells and levels of protection are highly variable (80, 81). There exists a notion that the phenotype of IFN-γ producing cells as well as the magnitude of cytokine produced could affect the levels of protection (10). Since the PRRSV-CON virus confers outstanding levels of cross-protection, this virus may be a unique tool to elucidate the immune correlates of cross-protection. In addition, this synthetic virus will also provide us a tool to identify viral proteins involves in eliciting immune protection.

In summary, we describe here the generation and characterization of a synthetic PRRSV strain based on a synthetic genome that was computationally designed based on a large number of PRRSV full genome sequences. We demonstrate that this synthetic PRRSV strain confers outstanding levels of heterologous protection. This synthetic PRRSV strain could be an excellent candidate for the formulation of the next generation of PRRS vaccine with improved levels of

512 heterologous protection. In addition, this synthetic PRRSV strain will provide us a unique tool
513 and gold standard to investigate the mechanisms of cross-protection.

514

515 ## Acknowledgments

516 This study was funded primarily by the U.S. National Pork Board (grant # NPB 13-155 to
517 HLXV) and by the PRRS CAP, USDA NIFA (grant # 2008-55620-19132 to WWL). Partial
518 funding for development of the differential RT-PCR kits was from USDA NIFA (grant # 2013-
519 01035 to FAO). We thank Dr. Huyen Tran, Department of Animal Sciences, UNL, for her advice
520 on statistical analysis and Vicky Samek, Sara Fendric, Brittany Smola, Bayliegh Murphy, Molly
521 Johnson and Clinton Berg for their assistance in the animal experiments.

522

Appendix

Design and validation of the differential RT-PCR kits for quantification of the challenge virus- RNA in tissue samples

Two differential real-time RT-PCR kits for specific detection and quantification of the MN184C-specific and 16244B-speficic viral RNA in tissue samples were developed following the Taqman hydrolysis probe method. Specific primers and probes used in the differential RT-PCR are presented in Tables A1 and A2. All primers and probes were synthesized by Sigma-Aldrich (Woodland, TX). Real-time RT-PCR reactions were performed in 25µL reaction mixtures containing 4.475 µL distilled water, 12.5 µL One-step qRT-PCR master mix (Affymetrix), 1 µL of each primer (final concentration 400 nM), 0.625 µL probe (final concentration 250 nM) and 5 µL template. The thermal conditions were as followed: one cycle at 50^{o}C for 10 minutes, one cycle at 95^{o}C for 2 minutes and 40 cycles at 95^{o}C for 15 seconds and 60^{o}C for 60 seconds. Two sets of viral RNA templates with known copies number were used to establish the standard curves from which the RNA copy number in the test samples were calculated.

To evaluate the specificity of the differential RT-PCR kits, RNA samples were extracted from MN184C, FL12 and PRRSV-CON virus stocks using the QIAamp viral RNA mini kit (Qiagen, Valencia, CA). Viral genome copies in each of these RNA samples were quantified using a commercial RT-PCR kit (Tetracore), following the manufacturer's instruction. After that, these viral RNA samples were diluted to different concentration, ranging from 10^{1} copies per µL to 10^{5} copies per µL. Five µL of each dilution of these viral RNA samples were used in the differential RT-PCR reactions. Data demonstrating the specificity of the differential RT-PCR kits are presented in Tables A3 and A4.

To validate the compatibility of the differential RT-PCR kits, we compared the performance of the differential RT-PCR kits with that of the commercial RT-PCR kit, using the RNA samples extracted from tissue samples collected from the PBS-groups because these pigs should only carry viral RNA of the viral strains used for challenge infection. In general, the viral RNA copy numbers quantitated by the differential RT-PCR kits were approximately 0.2 – 0.3 log lower than the copy numbers quantitated by the commercial RT-PCR kits (Tables A5 and A6).

Table A1: Primers and probes used in the differential RT-PCR kit for quantitation of the PRRSV strain MN184C-specific RNA (GenBank accession no. EF488739)

	Sequence (5' -> 3')	Binding sites
Forward primer (sense)	AGCTGGCATTCTTGAGACAT	14871 - 14891
Reverse primer (antisense)	AGGTGACTTAGAGGCACAATATC	14935 - 14957
Probe (sense)	AGGATGTGTGGTGAATGGCACTGA	14908 - 14932

Table A2: Primers and probes used in the differential RT-PCR kit for quantitation of the PRRSV strain 16244B-specific RNA (GenBank accession no. AF046869)

	Sequence (5' -> 3')	Binding sites
Forward primer (sense)	GGCTGGCATTCTTGAGGCAT	15262 - 15282
Reverse primer (antisense)	CACGGTCGCCCTAATTGAATA	15348 - 15369
Probe (antisense)	CAGTGCCATTCACCACACATTCTTCC	15297 - 15323

560 **Table A3: Specificity of the MN184-specific RT-PCR kit**

561

RNA copies per reaction	Crossing point (CP)		
	MN184C	FL12	PRRSV-CON
$5x10^1$	38.93	nd	Nd
$5x10^2$	34.68	nd	Nd
$5x10^3$	31.54	nd	Nd
$5x10^4$	28.05	nd	Nd
$5x10^5$	24.67	nd	Nd

562 nd: not detected

563

564 **Table A4: Specificity of the 16244B-specific RT-PCR kit**

RNA copies per reaction	Crossing point (CP)		
	16244B	FL12	PRRSV-CON
$5x10^1$	40.00	nd	nd
$5x10^2$	36.27	nd	nd
$5x10^3$	33.92	nd	nd
$5x10^4$	29.99	nd	nd
$5x10^5$	26.55	nd	nd

565 nd: not detected

566

567 **Table A5: Comparison between the MN184C-specific RT-PCR kit and the commercial RT-**

568 **PCR kit**

Tissue types	Pig ID	Copies per µg total RNA (log10)	
		Commercial RT-PCR kit	MN184C-specific RT-PCR kit
Tonsil	365	6.08	6.00
	389	6.70	6.50
	407	6.94	6.80
	416	Not done	Not done
	417	4.44	4.60
	435	6.34	6.50
Inguinal LN	365	6.21	5.64
	389	6.20	5.90
	407	6.99	6.49
	416	5.71	5.38
	417	5.51	5.26
	435	5.97	5.73
Mediastinal LN	365	4.78	4.52
	389	5.04	4.87
	407	6.40	6.28
	416	4.71	4.53
	417	4.73	4.34
	435	5.34	5.19
	Means ± SD	5.77 ± 0.82	5.56 ± 0.80

569

570

571

572 **Table A6: Comparison between the 16244B-specific RT-PCR kit and the commercial RT-**

573 **PCR kit**

Tissue types	Pig ID	Copies per µg total RNA (log10)	
		Commercial RT-PCR kit	16244B-specific RT-PCR kit
Tonsil	440	4.92	4.76
	441	4.91	4.79
	544	5.92	5.76
	545	6.72	6.39
	546	6.33	5.33
	547	5.63	6.14
Mediastinal LN	440	4.41	3.83
	441	4.53	4.08
	544	5.37	5.05
	545	5.20	4.93
	546	4.85	4.54
	547	5.09	4.78
Inguinal LN	440	4.28	3.93
	441	5.21	4.96
	544	5.55	5.16
	545	5.33	4.82
	546	5.04	4.64
	547	5.15	4.72
	Mean ± SD	5.25 ± 0.63	4.92 ± 0.68

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Tables

Table 1: Levels of viremia after challenge infection with MN184C (log10 copy/mL of serum)

Treatment groups	Pig ID	Day post-challenge infection					
		0	1	4	7	10	15
Group 1 (Injected with PBS)	365	0.00	4.94	5.43	5.45	6.79	6.32
	389	0.00	6.26	6.08	5.40	7.60	6.93
	407	0.00	4.91	6.00	5.86	7.56	6.75
	416	0.00	6.20	6.04	5.20	7.18	6.78
	417	0.00	5.18	5.59	4.86	5.90	6.45
	435	0.00	5.83	5.08	5.94	5.57	5.36
	Mean	**0.00**	**5.55**	**5.70**	**5.45**	**6.77**	**6.43**
	SD	**0.00**	**0.62**	**0.40**	**0.40**	**0.86**	**0.57**
Group 2 (Immunized by infection with PRRSV-CON)	345	0.00	0.00	0.00	0.00	0.00	0.00
	394	0.00	0.00	0.00	0.00	0.00	2.58
	410	0.00	0.00	0.00	0.00	0.00	0.00
	459	0.00	0.00	0.00	0.00	0.00	0.00
	494	0.00	0.00	3.58	5.98	0.00	0.00
	495	0.00	0.00	0.00	0.00	0.00	2.98
	Mean	**0.00**	**0.00**	**0.60**	**1.00**	**0.00**	**0.93**
	SD	**0.00**	**0.00**	**1.46**	**2.44**	**0.00**	**1.44**
Group 3 (Immunized by infection with FL12)	349	0.00	0.00	2.81	2.92	0.00	0.00
	381	0.00	0.00	0.00	3.04	2.86	0.00
	440	0.00	0.00	0.00	0.00	0.00	0.00
	455	0.00	0.00	4.18	4.34	0.00	0.00
	487	0.00	3.59	5.28	2.40	5.60	2.68
	507	0.00	2.32	5.56	3.70	0.00	0.00
	Mean	**0.00**	**0.99**	**2.97**	**2.73**	**1.41**	**0.45**
	SD	**0.00**	**1.58**	**2.50**	**1.50**	**2.35**	**1.09**

Samples that contained undetected levels of viral RNA are assigned a value of 0 log10 copies/ mL of serum.

842

843 **Table 2: Levels of viremia after challenge infection with 16244B (log10 copy/mL)**

Treatment groups	Pig ID	Day post-challenge infection					
		0	1	4	7	11	14
Group 1 (Injected with PBS)	440	0.00	6.62	6.99	6.79	6.15	4.67
	441	0.00	6.61	6.93	7.11	5.79	4.81
	544	0.00	6.85	6.82	6.96	3.91	5.68
	545	0.00	7.11	7.41	7.11	6.81	5.93
	546	0.00	6.74	7.45	7.30	5.67	5.40
	547	0.00	6.77	7.51	7.36	6.73	5.52
	Mean	**0.00**	**6.78**	**7.18**	**7.11**	**5.84**	**5.34**
	SD	**0.00**	**0.18**	**0.30**	**0.21**	**1.06**	**0.50**
Group 2 (immunized by infection with PRRSV-CON)	435	Removed from experiment on day 23rd after primary infection					
	436	0.00	0.00	0.00	0.00	0.00	0.00
	437	0.00	2.48	0.00	0.00	0.00	0.00
	438	0.00	0.00	0.00	0.00	0.00	0.00
	442	2.81	0.00	0.00	0.00	0.00	2.93
	445	3.00	3.32	0.00	0.00	0.00	0.00
	Mean	**1.16**	**1.16**	**0.00**	**0.00**	**0.00**	**0.59**
	SD	**1.59**	**1.62**	**0.00**	**0.00**	**0.00**	**1.31**
Group 3 (immunized by infection with FL12)	439	0.00	4.34	6.78	3.54	2.48	0.00
	444	0.00	3.04	6.58	0.00	0.00	0.00
	446	0.00	5.26	4.84	0.00	0.00	0.00
	526	0.00	2.98	4.40	4.15	0.00	0.00
	540	0.00	3.90	4.18	5.08	3.95	0.00
	543	Removed from experiment on day 14th after primary infection					
	Mean	**0.00**	**3.90**	**5.35**	**2.55**	**1.29**	**0.00**
	SD	**0.00**	**0.95**	**1.23**	**2.39**	**1.84**	**0.00**

844 Samples that contained undetected levels of viral RNA are assigned a value of 0 log10 copies/

845 mL of serum.

846 Pigs # 435 (group 2) and 543 (group 3) were removed from the experiment due to lameness in

847 their limbs.

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850 **Table 3: Genetic stability of the PRRSV-CON virus at 21 days p.i.**

Nucleotide position	Open reading frame (ORF)	Nucleotide change	Amino acid change
12883	3	A->G	Synonymous
13440	3 & 4	C->T	ORF3: Ala -> Val
			ORF4: Synonymous
14280	5	G->A	Arg - > Lys
14311	5	C->T	Synonymous
14703	6	T->C	Synonymous

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Figure Legends

Fig. 1: Phylogenetic analysis of full genome sequences of type-2 PRRSV.

(A) Phylogenetic tree constructed from a set of 59 type-2 PRRSV full genome sequences, together with a consensus sequence (PRRSV-CON) derived from these 59 PRRSV genomes. Scale bare represents the nucleotide substitution per site. Locations of the PRRSV strains involved in the cross-protection experiments are indicated by the arrows. The phylogenetic tree with tip labels is presented in Fig. S1. (B) Pairwise nucleotide distances between wild-type PRRSV; between wild-type and the PRRSV-CON; and between wild-type and different PRRS vaccine strains. The lower and upper boundaries of the box indicate the 25th and 75th percentile, respectively. The solid line within the box represents the median. Whiskers above and below the box indicate the minimum and maximum of the data. Letters on top of the whiskers indicate the statistical difference.

Fig. 2: Generation and *in vitro* characterization of the synthetic PRRSV-CON virus.

(A) Strategy to construct the PRRSV-CON full genome cDNA clone. The upper part of the figure depicts the schematic representation of the PRRSV genome, together with the restriction enzyme sites used for cloning purposes. The horizontal black lines, with the letters A-D on top, represent the DNA fragments that were synthesized. The numbers inside the parenthesis below the lines indicate the length (in nucleotides) of each corresponding fragments. ΦT7 represents the T7 RNA polymerase promoter. Individual DNA fragments of the genome were sequentially inserted into the shuttle vector (shown in the bottom) in the order from fragment A to fragment D. (B) Reactivity of the indicated PRRSV strains with different PRRSV-specific monoclonal antibodies. ISU-25: anti-GP5; MAb-201: Anti-M protein and SDOW-17:

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Anti-N protein. (C) Susceptibility to neutralization by a hyper-immune antibody. (D) Multiple step growth curves of the indicated PRRSV strains in MARC-145 cells. (E) Plaque morphology of the indicated PRRSV strains in MARC-145 cells.

Fig. 3: The PRRSV-CON virus is highly virulent.

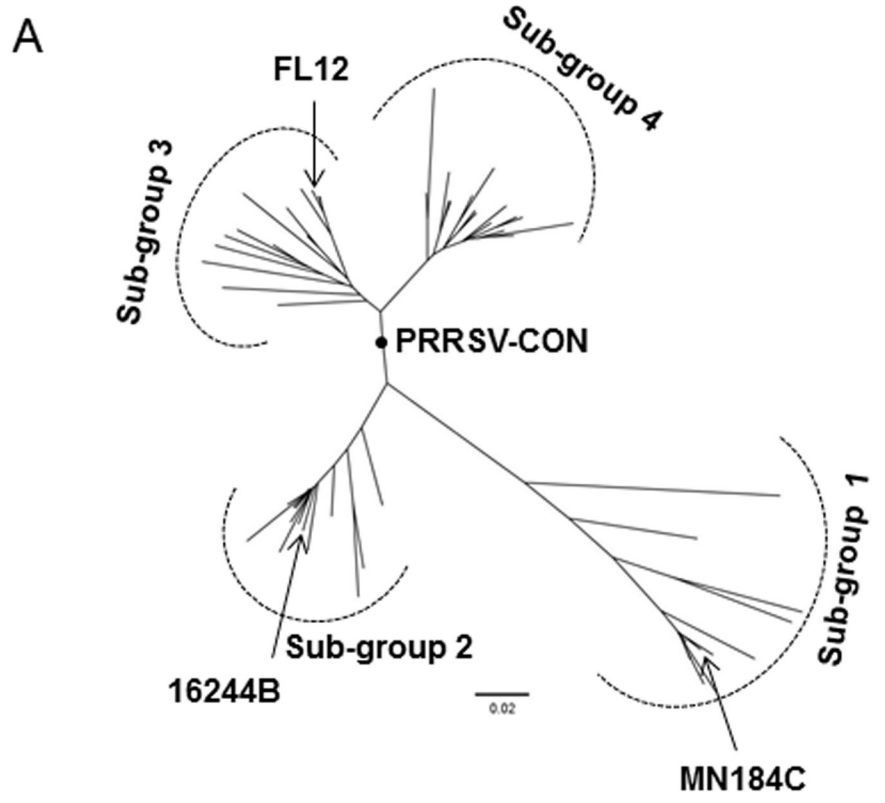
(A) Rectal temperature measured daily from -1 to 13 days p.i.. (B) Viremia levels determined by a commercial, universal RT-qPCR (Tetracore Inc., Rockville, MD). (C) Levels of antibody response after inoculation, determined by IDEXX ELISA. The horizontal dotted line indicates the cut-off of the assay. (D) Gross lung lesion evaluated at necrosy. (E) Micro-scopic lung lesion.

Fig. 4: Experimental design to evaluate levels of cross-protection. (A) Treatment groups, together with the corresponding PRRSV strains used for primary infection and challenge infection. (B) Chronology of cross-protection experiments. Triangles indicate blood sampling dates.

Fig. 5: Cross-protection against PRRSV strain MN184C. (A) Average daily weight gain (ADWG) within 15 days p.c.. (B) Viremia levels after challenge infection determined by a commercial RT-PCR (Tetracore, Rockville, MD). (C) Total viral RNA levels in different tissues collected at 15 days p.c. as determined by a commercial RT-PCR kit (Tetracore, Rockville, MD). (D) MN184C-specific RNA levels as determined by a differential RT-PCR developed in-house.

Fig. 6: Cross-protection against PRRSV strain 16244B. (A) Average daily weight gain (ADWG) within 15 days p.c.. (B) Viremia levels after challenge infection determined by a

commercial RT-PCR (Tetracore, Rockville, MD). (C) Total viral RNA levels in different tissues collected at 15 days p.c. as determined by a commercial RT-PCR kit (Tetracore, Rockville, MD). (D) 16244B-specific RNA levels as determined by a differential RT-PCR developed in-house.



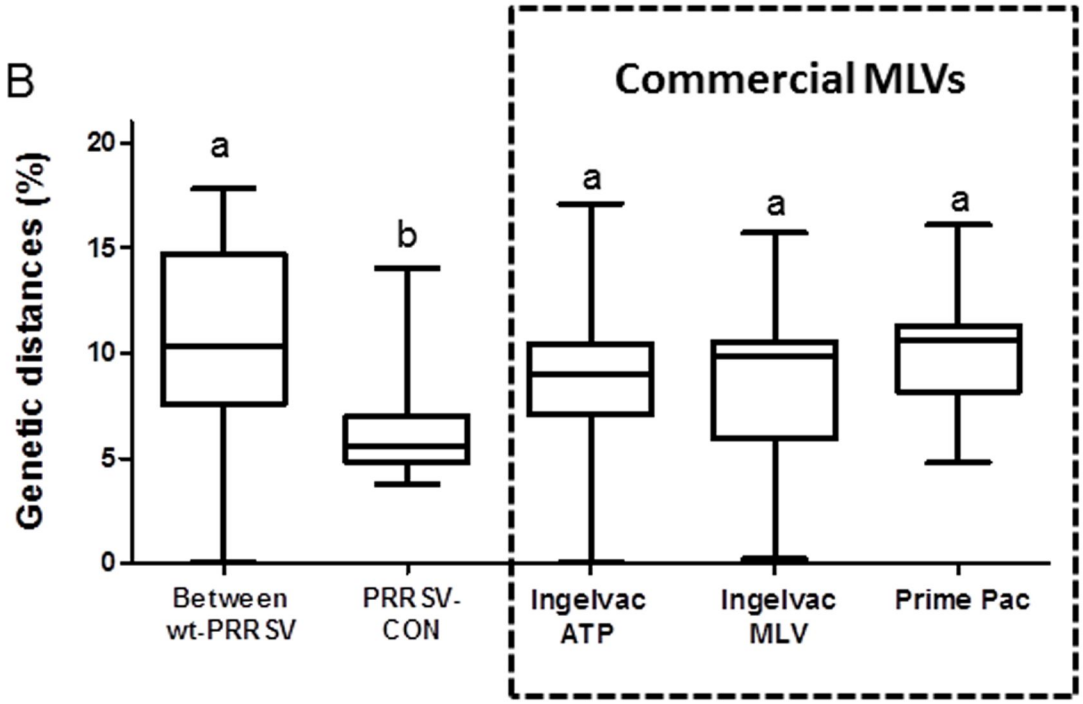
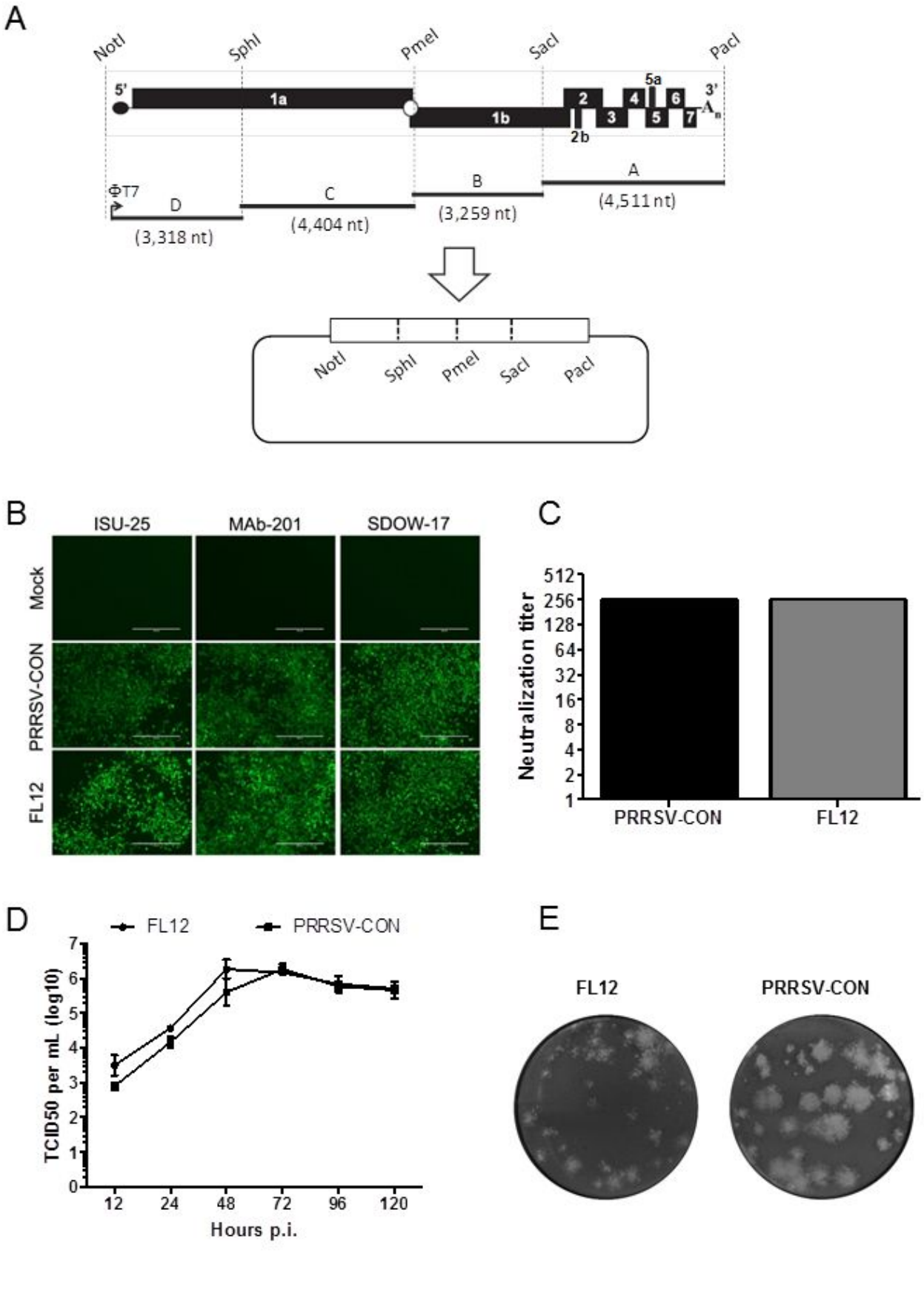
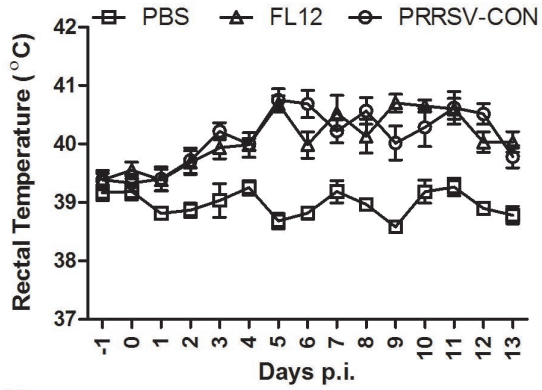


Fig. 2



A.



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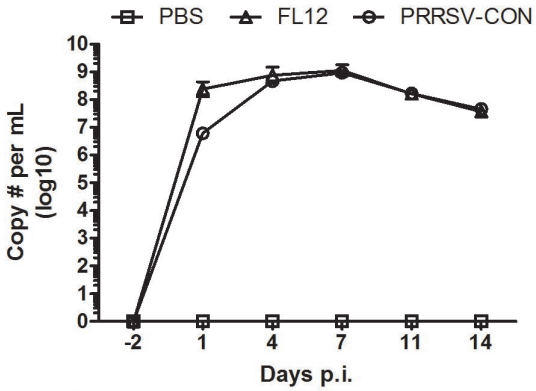

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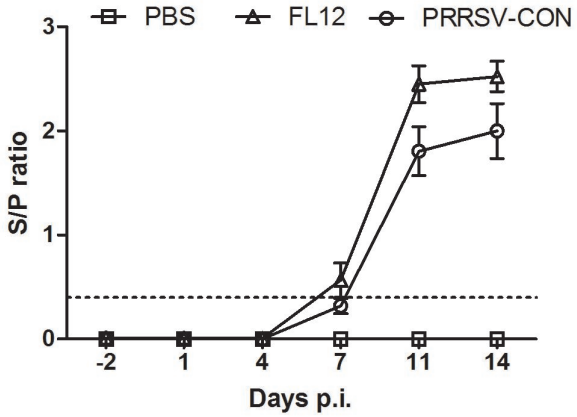

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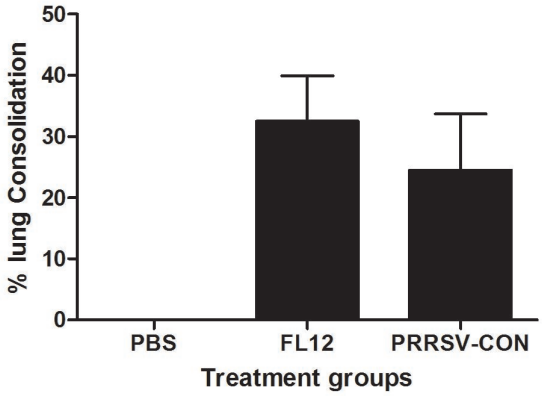

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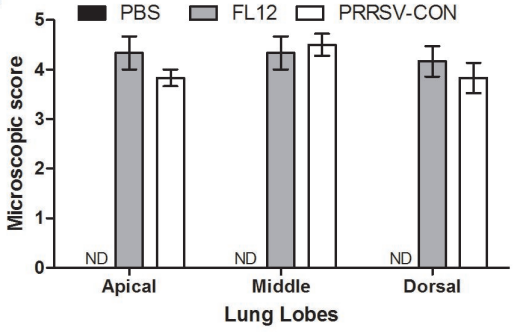
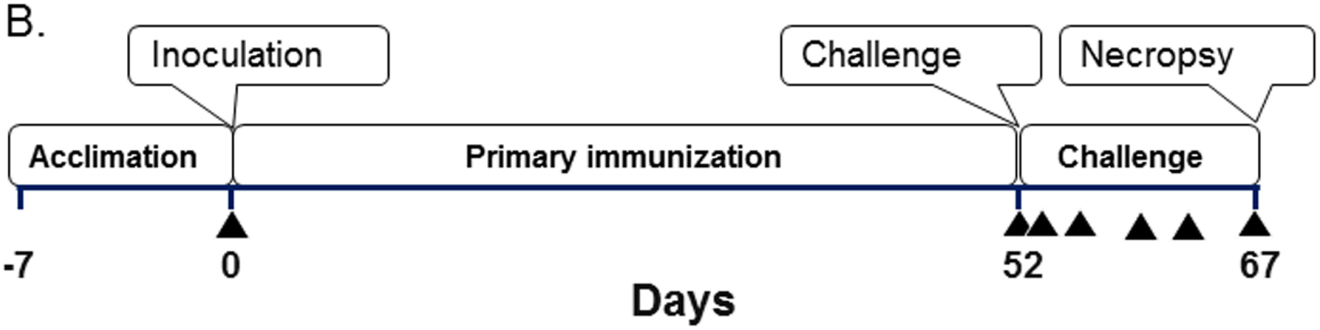
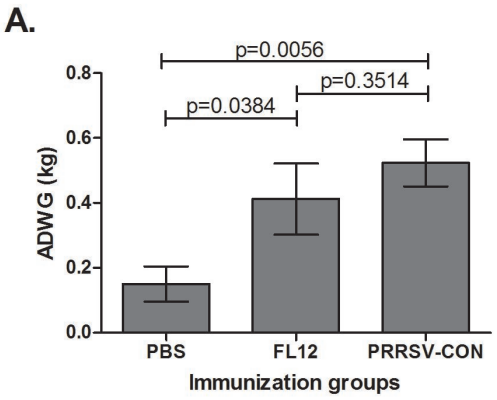


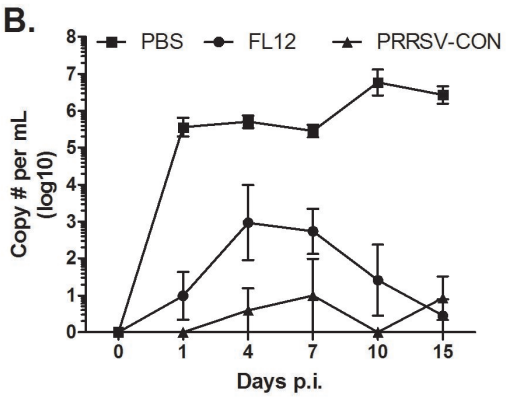
A.

Groups	Immunized with	Challenged with	
		Exp. # 1	Exp. # 2
1 (n=6)	PBS	MN184C (sub-group 1)	16244B (sub-group 2)
2 (n=6)	PRRSV-CON		
3 (n=6)	FL12		

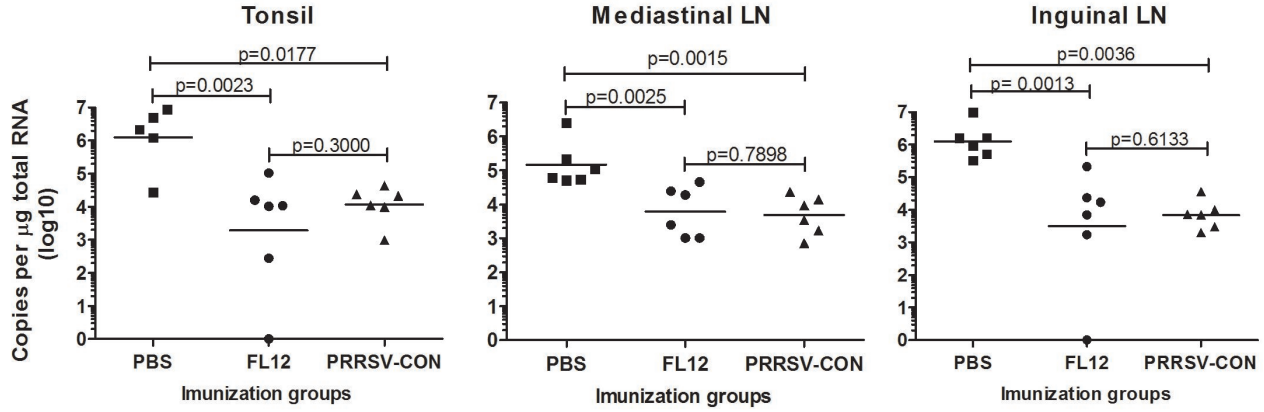
B.



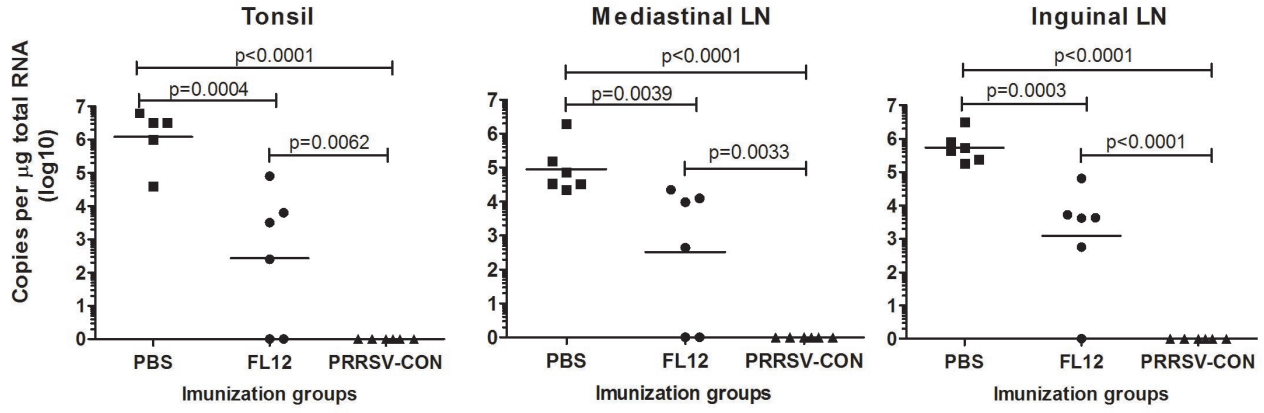


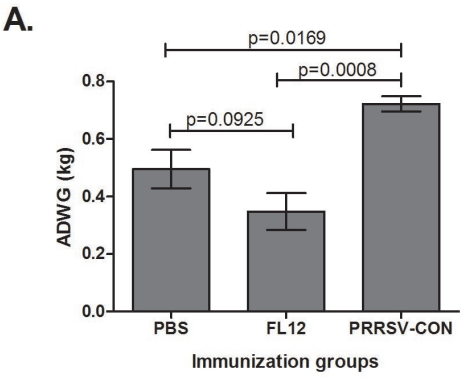


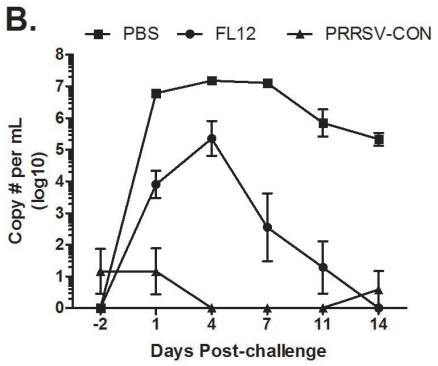
C. Total viral RNA

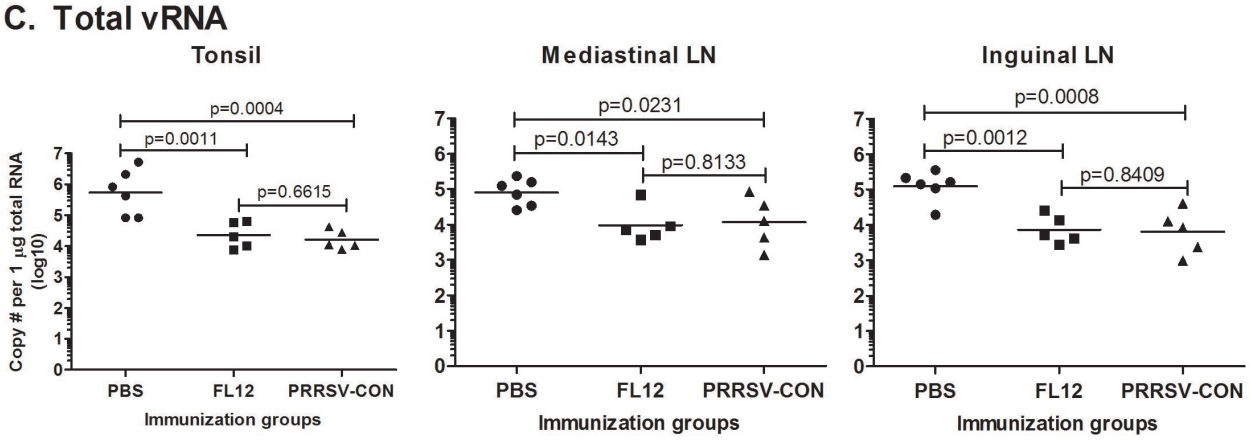


D. MN-184 specific vRNA









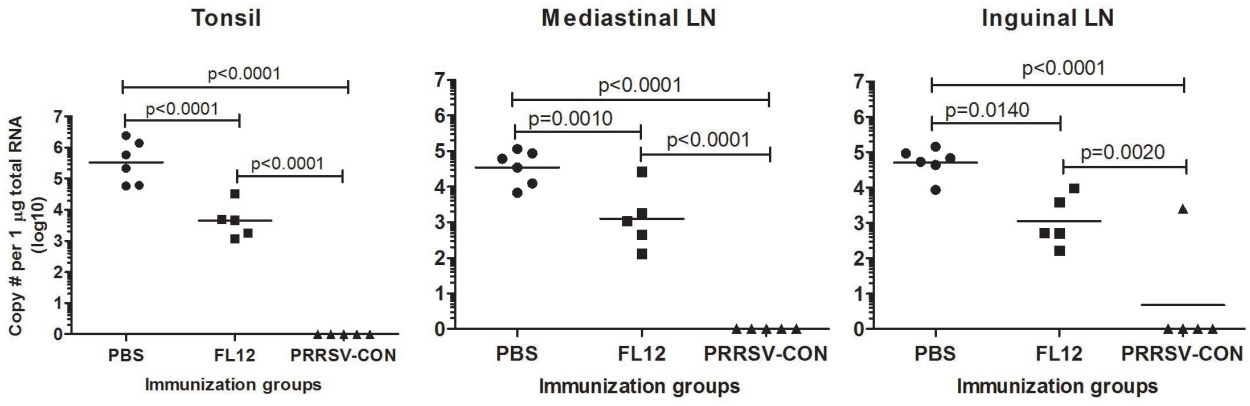


Table S1: List of type-2 PRRSV full genomes used in this study

No	Identity	GenBank assession number
1	NADC30	JN654459
2	18565-01	Pending
3	NADC31	JN660150
4	Hawkeye-2	EF532811
5	Hawkeye-7	EF532815
6	Biss	EF532803
7	Lewis	EF532818
8	MN184C	EF488739
9	18066-04	Pending
10	MFF	EF532819
11	MN184B	DQ176020
12	MN184A	Pending
13	VR-2385	JX044140
14	3805-00	Pending
15	PrimPac	DQ779791
16	ISU-P	EF532816
17	43807-00	Pending
18	9974-97	Pending
19	16244B	AF046869
20	12711-01	Pending
21	67516A-01	Pending
22	10277-97	Pending
23	3283-98	Pending
24	4190-01	Pending
25	16480-97	Pending
26	16138-96	Pending
27	Ingelvac MLV	AF066183
28	VR-2332	AY150564
29	P129	AF494042
30	4684-98	Pending
31	1648-01	Pending
32	12697-01	Pending
33	13867-00	Pending
34	MN30100	EF536000
35	1692-98	Pending
36	19248-01	Pending
37	SDSU-73	JN654458
38	21599-00	Pending
39	6527-00	Pending

40	58219C-00	Pending
41	FL12 (97-7895)	AF325691
42	Ingelvac-ATP	DQ988090
43	11604-05	Pending
44	2330-03	Pending
45	FF3	EF532808
46	13392-01	Pending
47	5564-04	Pending
48	5424-00	Pending
49	15571-00	Pending
50	46517-00	Pending
51	12120-01	Pending
52	51220-00	Pending
53	26078-00	Pending
54	15286-99	Pending
55	55406A-00	Pending
56	12817-01	Pending
57	25617-00	Pending
58	3232B-02	Pending
59	6258B-01	Pending

Fig. S1: Phylogenetic tree constructed from a set of 59 type-2 PRRSV full genome sequences, together with a consensus sequence (PRRSV-CON) derived from these 59 PRRSV genomes