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A Synthetic Porcine Reproductive and Respiratory Syndrome Virus Strain Confers Unprecedented Levels of Heterologous Protection

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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. By using a set of 59 nonredundant, full-genome sequences of type 2 PRRSVs, a consensus genome (designated 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 strain was generated through the use of reverse genetics. PRRSV-CON replicates as efficiently as our prototype PRRSV strain FL12, both *in vitro* and *in vivo*. Importantly, when inoculated into pigs, PRRSV-CON confers significantly broader levels of heterologous protection than does wild-type PRRSV. Collectively, our data demonstrate that PRRSV-CON can serve as an excellent candidate for the development of a broadly protective PRRSV 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 contemporary circulating viruses, computational strategies have been developed for the generation of artificial immunogen sequences (so-called "centralized" sequences) that have equal genetic distances to the circulating viruses. Thus far, the generation of centralized vaccine immunogens has been carried out at the level of individual viral proteins. We expand this concept to 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.

Porcine reproductive and respiratory syndrome (PRRS) is widespread in most swine-producing countries worldwide, causing significant economic losses to swine producers. In the United States alone, the disease causes approximately $664 million in losses to 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 virus (PRRSV) (2–4). The PRRSV genome is ~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. Subunit 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 MLV vaccines 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 are considered suboptimal in all cases (10, 14–19).

The prominent genetic variation of the PRRSV genome is the greatest hindrance to 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 ~65% genomic sequence identity (20, 21). In addition, there is a highly pathogenic variant of type 2 PRRSV (HP-PRRS) that is endemic in Asia, causing death in pigs of all ages, with a mortality rate of up to 100% (22). The genetic variation among PRRSV strains within each genotype is substantial. Based on phylogenetic analysis of

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viral glycoprotein 5 (GP5) (the most hypervariable surface envelope protein), type 2 PRRSV can be classified into 9 different lineages, with pairwise interlineage genetic distances ranging from 10% to 18% (23). The average substitution rate for type 2 PRRSV open reading frame 5 (ORF5) is estimated to be 9.6×10^{-3} substitutions/site/year (23). Genetic divergence has been shown to occur when a PRRSV strain is serially passed from pig to pig (24). Furthermore, cocirculation 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 the 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, through 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 was tested in pigs previously (27). However, this polyvalent vaccine did not seem to provide any significant improvement in the levels of heterologous protection compared with the monovalent PRRS vaccine (27). Recently, several chimeric viruses were 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 those elicited by 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 sequences, 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 immunodeficiency 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 virus and another (31–33). Three different computational methods have been developed to generate a centralized immunogen sequence: consensus, common ancestor, and center of the tree (31, 32). A consensus sequence that carries the most common amino acid found at each position of the alignment is the simplest method for the construction of a centralized immunogen (31). Studies on HIV-1 and influenza virus have clearly demonstrated that vaccines based on the consensus sequences elicit broader immune responses than do 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 PRRSV-CON) is fully infectious, and the synthetic PRRSV-CON strain displays characteristics typical of a naturally occurring PRRSV strain. Importantly, when inoculated into pigs, PRRSV-CON confers exceptional levels of heterologous protection against divergent PRRSV strains 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 (82) and the *Guide for the Care and Use of Agricultural Animals in Research and Teaching* (39). The animal care and use protocol was approved by the University of Nebraska—Lincoln (UNL) Institutional Animal Care and Use Committee (protocol no. 930).

Cells, antibodies, and PRRSV strains. Monkey kidney MARC-145 (40), porcine kidney 15 (PK-15), baby hamster kidney 21 (BHK-21), and HeLa cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Immortalized porcine alveolar macrophage (PAM) clone 3D4/31 (PAM 3D4/31) (ATCC CRL-2844) cells were cultured in RPMI 1640 supplemented with 10% FBS (41). All cell lines were cultured at 37°C with 5% CO_2. The PRRSV-specific hyperimmune antibody used for virus neutralization assays was generated previously (42). This hyperimmune antibody can cross-neutralize different type 2 PRRSV strains with high endpoint neutralization titers (42). PRRSV-specific monoclonal antibodies (MAbs) used for indirect-immunofluorescence assays include anti-GP5 (clone ISU25-C1 [43]), anti-M protein (clone 201 [44]), and anti-N protein (clone SDOW17 [45]). 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 strain FL12 was recovered from a full-length infectious cDNA clone (46) 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 originating from a farm where sows experienced severe reproductive failure (20). PRRSV strain MN184C (GenBank accession no. EF488739 [47]) was kindly provided by K. S. Faaberg, National Animal Disease Center.

Collection of type 2 PRRSV full-genome sequences and design of the consensus PRRSV genome. Through our studies on the immunologic consequences of PRRSV diversity (W. W. Laegreid, F. A. Osorio, T. Golberg, J. Hennings, and E. A. Nelson, unpublished data), we sequenced the full genomes of 64 type 2 PRRSV strains/isolates originating in Midwestern states (Iowa, Nebraska, and Illinois) of the United States. In addition, we were able to collect 20 genome sequences of type 2 PRRSV isolates from GenBank that also originated in the United States. After removing redundant sequences, we attained a final set of 59 genome sequences of type 2 PRRSV: 39 genome sequences were sequenced by our laboratories, and 20 genome sequences were collected from GenBank. The list of PRRSV genome sequences with GenBank accession numbers is presented in Table S1 in the supplemental material. The PRRSV genome sequences were aligned by using the MUSCLE 3.8 program (48). A consensus genome (PRRSV-CON) was constructed by using the Jalview program (49). The PRRSV-CON genome was aligned with the reference PRRSV strain FL12 genome, and frameshift mutations (insertion and deletion mutations) were manually corrected to ascertain that the viral proteins would be properly expressed. Finally, the 5′ and 3′ untranslated regions (UTRs) of the PRRSV-CON genome were replaced by the counterparts of the FL12 genome. A phylogenetic tree of the 59 naturally occurring PRRSV genomes, together with PRRSV-CON, was constructed by using PHYML 3.0, an implementation of the maximum likelihood method (50).

Generation of the synthetic PRRSV-CON strain. To generate an infectious virus based on the PRRSV-CON genome, a full-genome cDNA clone of PRRSV-CON was constructed according to a strategy described previously (46). Four DNA fragments (fragments A to D) encompassing the whole PRRSV-CON genome were chemically synthesized by Genscript (Piscataway, NJ). Each DNA fragment was flanked by a pair of restriction enzyme sites to facilitate cloning. The restriction enzyme sites used for assembling the full-genome cDNA clone include NotI, SphI, PmeI, SacI, and PacI. NotI and PacI are restriction enzyme sites that were added to the 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 strain (44, 46, 51). 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 this, ~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°C with 5% CO_2 for up to 6 days. When a clear cytopathic effect (CPE) was observed, the culture supernatant containing the rescued virus was collected and passed into naive MARC-145 cells one more time to obtain enough virus stock for future studies.

Indirect-immunofluorescence assay. To study the reactivity of the viruses to different PRRSV-specific monoclonal antibodies, MARC-145 cells were mock infected or infected with PRRSV-CON and wild-type FL12. At 48 h postinfection (p.i.), cells were washed twice with phosphate-buffered saline (PBS) (pH 7.4) and then fixed with 4% paraformaldehyde for 20 min at room temperature. After two washes in PBS, the cells were permeabilized with PBS containing 0.1% Triton X-100 for 15 min at room temperature. Next, the cells were incubated with PRRSV-specific MAbs for 1 h at room temperature, followed by 3 washes in PBS. Finally, the cells were incubated with anti-mouse Alexa Fluor 488-conjugated antibody for 1 h at room temperature. After 3 washes in PBS, cells were observed under an inverted fluorescence microscope.

Virus neutralization assay. A virus neutralization assay was done with MARC-145 cells, using a fluorescent-focus neutralization assay described previously (52). Neutralization titers were expressed as the reciprocal of the highest dilution that showed a ≥90% reduction in the number of fluorescent foci presenting in the control wells.

***In vitro* infectivity assay.** Immortalized PAM 3D4/31 (41), PK-15, BHK-21, and HeLa cells were separately plated into 24-well plates. Approximately 24 h later, cells in each well were infected with $2 \times 10^{4.0}$ 50% tissue culture infective doses ($TCID_{50}$) of PRRSV-CON or PRRSV strain FL12. Forty-eight hours after infection, the expression of viral nucleocapsid protein was examined by using an indirect-immunofluorescence assay as 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 PRRSV-CON or FL12 at a multiplicity of infection (MOI) of 0.01. At different time points postinfection, the 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 (53).

Assessment of virus virulence in pigs. A total of 18 PRRSV-seronegative, 3-week-old pigs were purchased from the UNL research farm. The pigs were randomly assigned into 3 treatment groups, with 6 pigs per group. Each treatment group was housed in a separate room in the biosecurity level 2 (BL-2) animal research facilities at the UNL. After 1 week of acclimation, pigs in group 1 were injected with PBS to serve as normal controls. Pigs in groups 2 and 3 were inoculated intramuscularly with $10^{5.0}$ $TCID_{50}$ of PRRSV-CON and PRRSV strain FL12, respectively. Rectal temperature was measured daily from days −1 to 13 p.i. Blood samples were collected periodically, and serum samples were extracted and stored at −80°C for evaluation of viremia levels and seroconversion. Viremia levels were quantitated by the Animal Disease Research and Diagnostic Laboratory, South Dakota State University, using a commercial quantitative reverse transcription-PCR (qRT-PCR) kit (Tetracore Inc., Rockville, MD). Results were reported as $\log_{10}$ copies per milliliter. For statistical purposes, samples that had undetectable levels of viral RNA were assigned a value of 0 $\log_{10}$ copies/ml. Seroconversion was evaluated by using the Idexx PRRS X3 Ab test (Idexx Laboratories Inc., Westbrook, ME). At day 14 p.i., pigs were humanely sacrificed and necropsied. Gross and microscopic lung lesions were evaluated by a pathologist in a blind manner, according to a method described previously (54).

Assessment of heterologous protection in pigs. Two sets of immunization/challenge experiments were conducted. Three-week-old PRRSV-seronegative pigs were obtained from the UNL research farm and were accommodated in BL-2 animal facilities at the UNL. Each set of experiments consisted of 3 groups of 6 weaning pigs. Pigs in group 1 served as nonimmunization controls, whereas those in groups 2 and 3 were immunized by infection with either PRRSV-CON or PRRSV strain FL12 at a dose of $10^{4.0}$ $TCID_{50}$ per pig, intramuscularly. At day 52 postimmunization, all control and immunized animals were challenged with a selected heterologous PRRSV field isolate at a challenge dose of $10^{5.0}$ $TCID_{50}$ per pig, intramuscularly. Parameters of protection included growth performance, viremia, and viral load in tissues. To measure growth performance, each pig was weighed immediately before challenge infection and at 15 days postchallenge (p.c.), and the average daily weight gain (ADWG) was calculated for 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°C. Viremia levels were quantitated by the Animal Disease Research and Diagnostic Laboratory, South Dakota State University, using a commercial qRT-PCR kit (Tetracore Inc., Rockville, MD). Results were reported as $\log_{10}$ copies per milliliter. For statistical purposes, samples that had undetectable levels of viral RNA were assigned a value of 0 $\log_{10}$ copies/ml. To quantitate levels of viral load in tissues, pigs were humanely sacrificed and necropsied at 15 days p.c. Samples of tonsil, lung, mediastinal lymph node, and inguinal lymph node were snap-frozen in liquid nitrogen immediately after collection and stored in a freezer at −80°C. Tissue samples were homogenized in TRIzol reagent (Life Technologies, Carlsbad, CA) at a ratio of 300 mg tissue to 3 ml TRIzol reagent. Total RNA was extracted by using the RNeasy minikit (Qiagen, Valencia, CA) according to the manufacturer's instruction. RNA concentrations were quantified by using a NanoDropND-1000 instrument (NanoDrop Technologies, Wilmington, DE) and adjusted to a final concentration of 200 ng/μl. Two different types of RT-PCR kits were used for quantitation of the viral load in tissues: (i) a commercial qRT-PCR kit (Tetracore, Rockville, MD) that detects total viral RNA resulting from primary infection and from challenge infection and (ii) differential qRT-PCR kits developed in-house that selectively detect viral RNA from challenge infection only. Design and validation of the differential qRT-PCR kit are presented in the Appendix. Five microliters of each RNA sample (equivalent to 1 μg RNA) was used for each qRT-PCR. Results were reported as $\log_{10}$ copies per microgram of total RNA. For statistical purposes, samples that had undetectable viral RNA levels were assigned a value of 0 log RNA copies/1 μg of total RNA.

Statistical analysis. Each pig was considered an experimental unit and a random effect. Data were analyzed as a completely randomized design by using the MIXED procedure in SAS (SAS Institute Inc., Cary, NC). All means are presented as least-squares means and standard errors of means (SEM). Data were considered significant when the P value was ≤0.05. Viremia data were analyzed with repeated measures by using the statistical model including treatment, time, and their interaction as fixed effects.

Nucleotide sequence accession number. The complete sequence of PRRSV-CON was submitted to GenBank under accession number KT894735.

RESULTS

Design of a consensus genome of type 2 PRRSV. We were able to obtain a set of 59 nonredundant, full-genome sequences of type 2 PRRSVs. Pairwise genetic distances among these 59 PRRSV genome sequences range from 0.1% to 17.8%. Phylogenetic analysis revealed that these 59 PRRSV full-genome sequences can be divided into 4 subgroups (Fig. 1A), with the mean nucleotide dis-

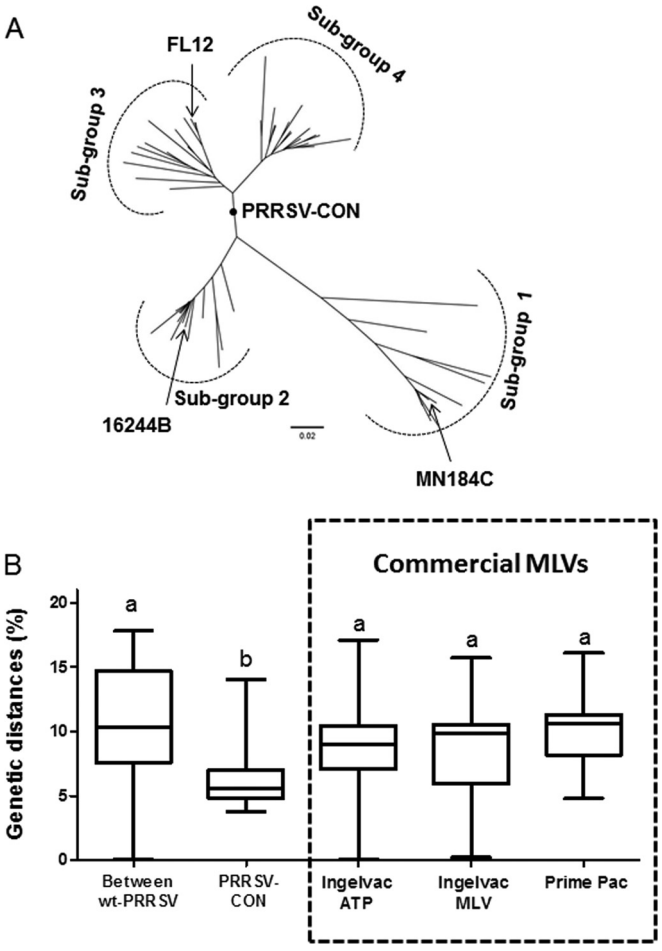

FIG 1 Phylogenetic analysis of full-genome sequences of type 2 PRRSVs. (A) Phylogenetic tree constructed from a set of 59 type 2 PRRSV full-genome sequences, together with a consensus sequence (PRRSV-CON) derived from these 59 PRRSV genomes. Bar represents nucleotide substitutions per site. Locations of the PRRSV strains involved in the cross-protection experiments are indicated by arrows. The phylogenetic tree with tip labels is presented in Fig. S1 in the supplemental material. (B) Pairwise nucleotide distances between wild-type PRRSVs, between wild-type PRRSV and the PRRSV-CON strain, and between wild-type PRSSV and different PRRS vaccine strains. The lower and upper boundaries of the box indicate the 25th and 75th percentiles, respectively. The solid line in the box represents the median. Whiskers above and below the box indicate the minima and maxima of the data. Letters on top of the whiskers indicate statistical differences.

tances 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 PRRSV-CON and wild-type PRRSV are also significantly shorter than the distances between the type 2 PRRS vaccine strains and wild-type PRRSV (Fig. 1B). Based on these data, we hypothesized that a vaccine formulated based on PRRSV-CON 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 cDNA 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 strain (44, 46, 51). Visible CPE was readily observed ~4 days after MARC-145 cells were transfected with the RNA transcripts generated from the PRRSV-CON cDNA clone. The resultant PRRSV-CON strain reacted with different PRRSV-specific monoclonal antibodies, including antibodies against the GP5, M, and N proteins (Fig. 2B). Importantly, PRRSV-CON was neutralized by a PRRSV-specific hyperimmune antibody with an endpoint titer equivalent to that of PRRSV strain FL12 (Fig. 2C). PRRSV-CON replicated efficiently in cell culture compared with the reference wild-type PRRSV strain FL12 (46). As shown in Fig. 2D, no significant difference in growth kinetics was observed between PRRSV-CON and FL12. Furthermore, PRRSV-CON produced larger plaques than did FL12 (Fig. 2E). Naturally occurring PRRSV has a very restricted cell tropism. Inside its natural host, the virus replicates mainly in macrophages residing in lung and lymphoid organs (55). *In vitro*, the virus is propagated mainly in primary PAMs (41) and in the monkey kidney cell line MA-104 and its derivatives MARC-145 and CL-2621 (40). Interestingly, the virus does not infect immortalized cell lines derived from pigs, such as the PK-15 and immortalized PAM 3D4/31 cell lines, presumably due to the absence of the CD163 receptor (41, 56, 57). We asked if the synthetic PRRSV-CON strain shows any alterations of cell tropism. To address this question, we investigated virus infectivity in different cell lines, including immortalized PAM 3D4/31 (41), PK-15, BHK-21, and HeLa cells. Similarly to PRRSV strain FL12, PRRSV-CON does not infect any of the cell lines tested (data not shown), indicating that the synthetic virus maintains the same cell tropism as that of naturally occurring PRRSV.

Synthetic PRRSV-CON is highly virulent. To characterize the pathogenicity of PRRSV-CON in pigs, an animal experiment with 3 groups of weaned (3-week-old) pigs was performed. Pigs in group 1 were injected with PBS to serve as normal controls. Pigs in groups 2 and 3 were inoculated intramuscularly with $10^{5.0}$ $TCID_{50}$ of PRRSV-CON and FL12, respectively. PRRSV strain FL12 was included in this study for comparative purposes because its pathogenicity in pigs has been extensively characterized in our laboratories (46). After infection, the PRRSV-CON and FL12 groups displayed significantly higher rectal temperature than did the PBS group (Fig. 3A). There was no difference in rectal temperature between the PRRSV-CON group and the FL12 group. Pigs infected with PRRSV-CON had the same kinetics and magnitude of viremia as those of pigs infected with PRRSV strain FL12 (Fig. 3B). All pigs in the PRRSV-CON and FL12 groups seroconverted by day 10 postinfection (p.i.). The level of antibody response in the PRRSV-CON group was slightly lower than that in the FL12 group (Fig. 3C). At necropsy (14 days p.i.), pigs in the PRRSV-CON group displayed a level of lung lesions similar to those in pigs in the FL12 group (Fig. 3D and E). Collectively, the results of this experiment indicate that the synthetic PRRSV-CON strain displays the same level of virulence as that of PRRSV strain FL12.

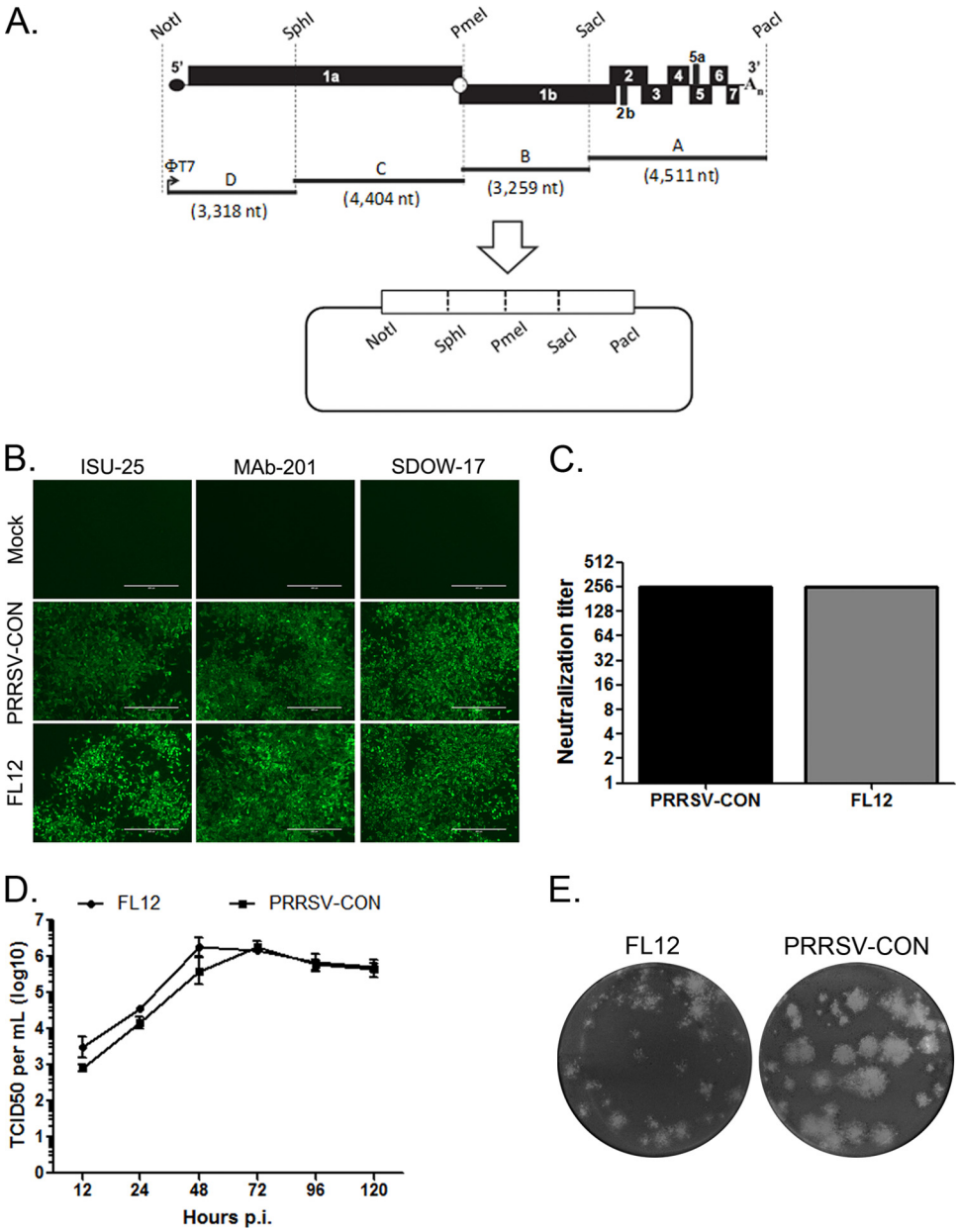

FIG 2 Generation and *in vitro* characterization of synthetic PRRSV-CON. (A) Strategy to construct the PRRSV-CON full-genome cDNA clone. At the top is a schematic representation of the PRRSV genome, together with the restriction enzyme sites used for cloning purposes. The horizontal black lines, with the letters A to D on top, represent the DNA fragments that were synthesized. The numbers inside the parentheses below the lines indicate the length (in nucleotides [nt]) of each corresponding fragment. ΦT7 represents the T7 RNA polymerase promoter. Individual DNA fragments of the genome were sequentially inserted into the shuttle vector (shown at the bottom) in the order of fragment A to fragment D. (B) Reactivity of the indicated PRRSV strains with different PRRSV-specific monoclonal antibodies. ISU-25 is anti-GP5, MAb-201 is anti-M protein, and SDOW-17 is anti-N protein. (C) Susceptibility to neutralization by a hyperimmune 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.

PRRSV-CON confers exceptional levels of heterologous protection. Two sets of immunization (by infection)/challenge experiments were conducted to evaluate the cross-protective capacity of PRRSV-CON. 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 PRRSV strain MN184C, which belongs to subgroup 1 in the phylogenetic tree (Fig. 1A). During the period of infection up to 15 days postchallenge (p.c.), pigs in the PRRSV-CON and FL12 groups had better ADWG than did those in the PBS group (Fig. 5A). There was no statistical difference between the PRRSV-CON and 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

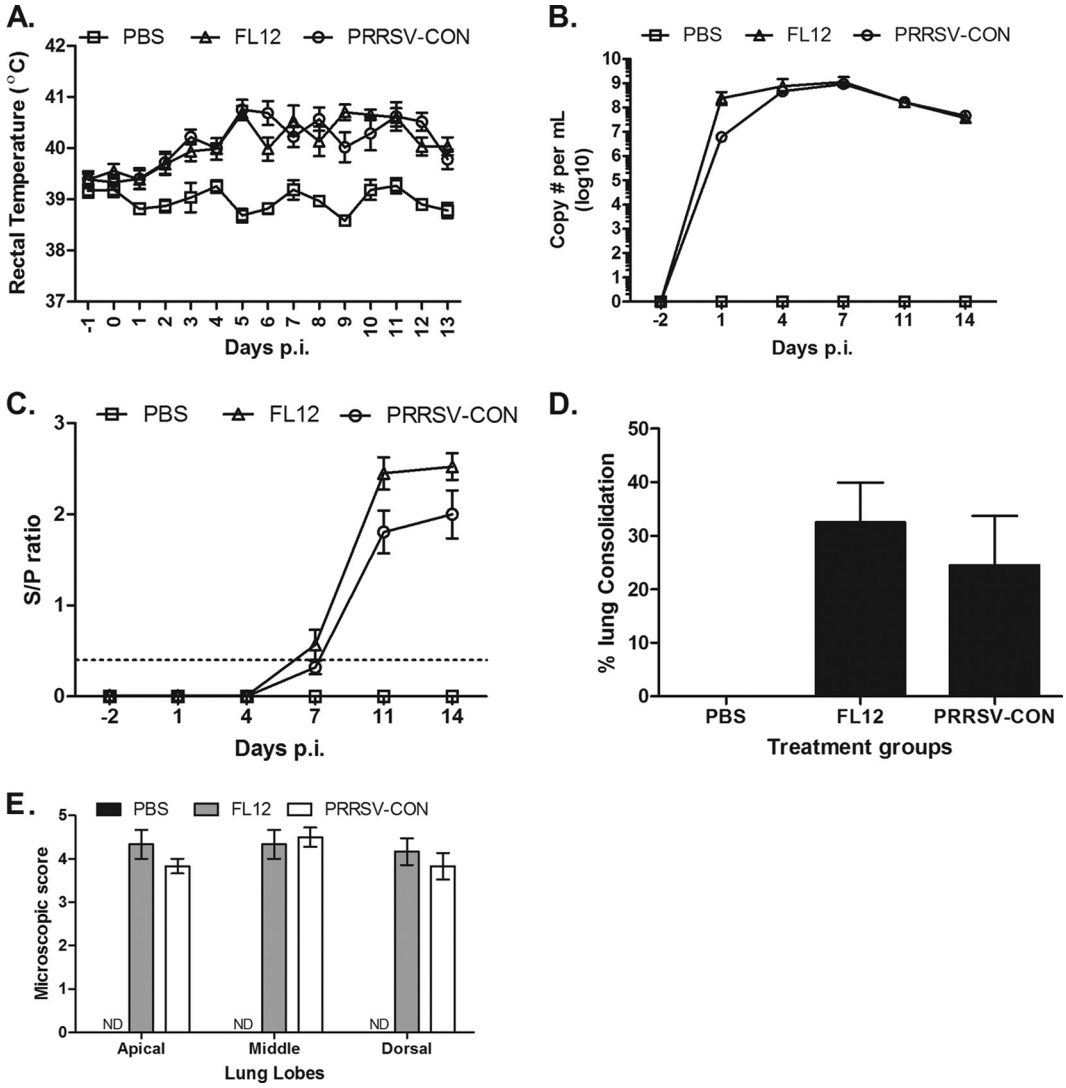

FIG 3 PRRSV-CON is highly virulent. (A) Rectal temperature measured daily from days −1 to 13 p.i. (B) Viremia levels determined by a commercial, universal qRT-PCR kit (Tetracore Inc., Rockville, MD). (C) Levels of antibody response after inoculation, determined by an Idexx enzyme-linked immunosorbent assay. The horizontal dotted line indicates the cutoff of the assay. (D) Gross lung lesions evaluated at necropsy. (E) Microscopic lung lesions. ND, not determined.

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.

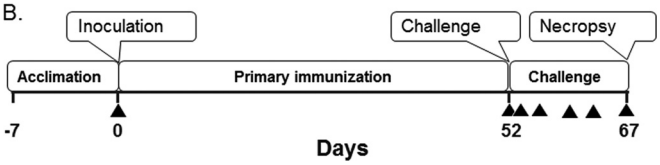

FIG 4 Experimental design to evaluate levels of cross-protection. (A) Treatment groups together with the corresponding PRRSV strains used for primary infection and challenge infection. (B) Chronology of cross-protection experiments. Triangles indicate blood sampling dates.

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). In 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 those 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 qRT-PCR kit (Tetracore, Rockville, MD) that detects total viral RNA resulting from primary infection (immunization) and from challenge infection. The results for total viral RNA are presented in Fig. 5C. The PRRSV-CON and FL12 groups had significantly lower levels of total viral RNA than did 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 qRT-

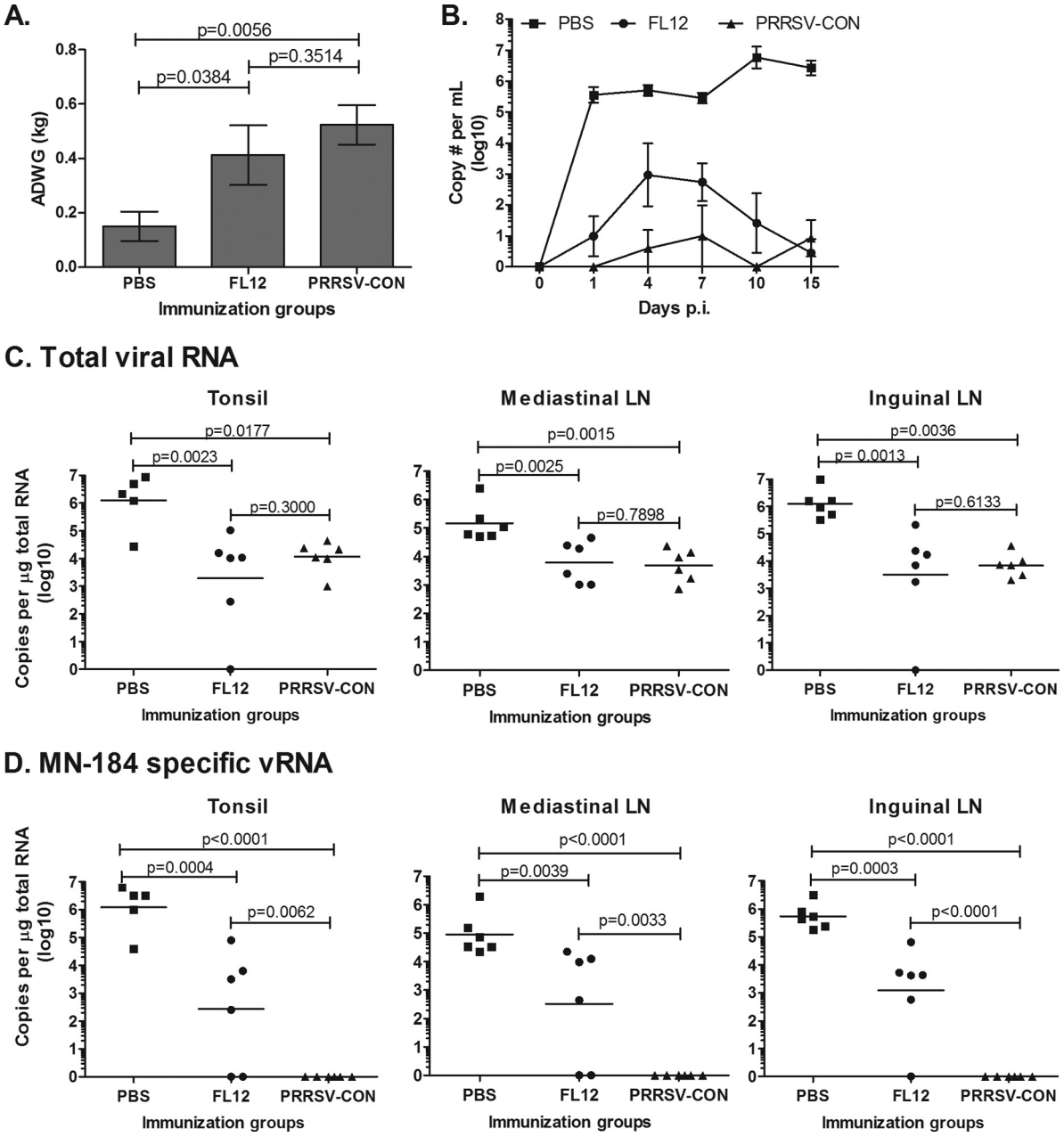

FIG 5 Cross-protection against PRRSV strain MN184C. (A) Average daily weight gain (ADWG) within 15 days p.c. (B) Viremia levels after challenge infection determined by a commercial qRT-PCR kit (Tetracore, Rockville, MD). (C) Total viral RNA levels in different tissues collected at 15 days p.c. as determined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

PCR kit to specifically quantitate the levels of challenge virus-specific RNA (e.g., MN184C-specific qRT-PCR kit). As shown in Fig. 5D, all pigs in the PBS group carried the MN184C-specific RNA in their tissues. Four pigs in the FL12 group had the MN184C-specific RNA in their tonsil and mediastinal lymph node, whereas 5 pigs in this group had 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 PRRSV-CON conferred a significantly better level of cross-protection against challenge with PRRSV strain MN184C than did PRRSV strain FL12.

In the second immunization/challenge experiment, we evaluated the level of cross-protection against PRRSV strain 16244B, which falls within subgroup 2 in the phylogenetic tree (Fig. 1A). During the period up to 15 days p.c., the PRRSV-CON group had a higher ADWG than did the PBS and FL12 groups (Fig. 6A). In contrast, the FL12 group did not exhibit a statistical difference in 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

TABLE 1 Levels of viremia after challenge infection with MN184C[a]

Treatment group and pig	Level of viremia (log_{10} copies/ml of serum) at postchallenge day:					
	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 for group	0.00	5.55	5.70	5.45	6.77	6.43
SD for group	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 for group	0.00	0.00	0.60	1.00	0.00	0.93
SD for group	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 for group	0.00	0.99	2.97	2.73	1.41	0.45
SD for group	0.00	1.58	2.50	1.50	2.35	1.09

[a] Samples that contained undetectable levels of viral RNA are assigned a value of 0 log_{10} copies/ml of serum.

all time points tested. Two out of five pigs in the PRRSV-CON group (pigs 442 and 445) did not resolve viremia at day 50 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 period up to day 15 p.c. (Table 2). In contrast, all pigs in the FL12 group resolved viremia by day 50 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 those 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 qRT-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 did the PBS group for 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 qRT-PCR kit to specifically quantify the levels of 16244B-specific RNA in tissues. The design and validation of the 16244B-specific qRT-PCR kit are presented in Fig. S1 in the supplemental material. All pigs in the PBS and FL12 groups carried the 16244B-specific RNA in their tissues (Fig. 6D). In contrast, only 1 pig in the PRRSV-CON group carried 16244B-specific RNA in its inguinal lymph node, while the remaining 4 pigs in this group did not carry 16244B-specific RNA in any of the tissues tested. Collectively, the results of this immunization/challenge experiment demonstrate that the synthetic PRRSV-CON strain conferred better protection against challenge infection with PRRSV strain 16244B than did PRRSV strain FL12.

Genetic stability of the PRRSV-CON strain 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. In total, there were 5 nucleotide 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 nucleotide changes resulted in amino acid changes. The nucleotide change in the overlapping region between ORF3 and ORF4 led to an 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 traditional molecular engineering approaches. This leads to the emergence of a new branch in the field of virus research, termed synthetic virology (58). A number of synthetic viruses have been generated by *de novo* synthesis of the viral genomes in the absence of natural viral templates (59–65). These synthetic viruses provide powerful tools for studying viral biology and pathogenesis as well as for rational design of novel vaccines (59, 63, 66–68). 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. The generation of "centralized" vaccine immunogens has been proven to be an effective method to reduce the genetic distances between the vaccine immunogen and the contemporary virus strains circulating in the field, thereby expanding vaccine coverage (31, 32). Thus far, centralized vaccine immunogens are commonly generated based on the amino acid sequences of selected viral proteins (34–37, 69). 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, PRRSV-CON confers significantly broader levels of het-

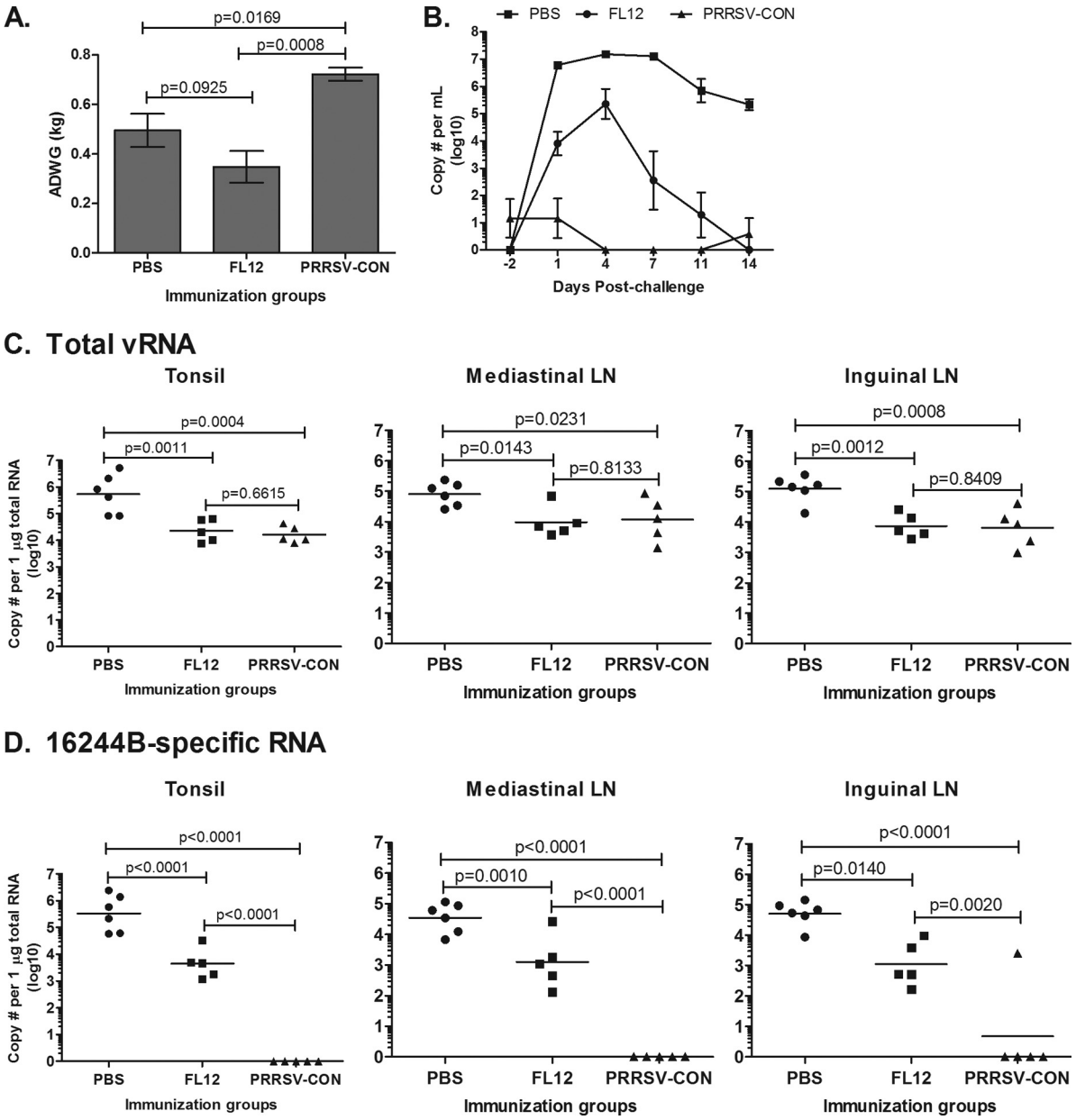

FIG 6 Cross-protection against PRRSV strain 16244B. (A) ADWG within 15 days p.c. (B) Viremia levels after challenge infection determined by a commercial qRT-PCR kit (Tetracore, Rockville, MD). (C) Total viral RNA levels in different tissues collected at day 15 p.c. as determined by a commercial qRT-PCR kit (Tetracore, Rockville, MD). (D) 16244B-specific RNA levels as determined by a differential qRT-PCR kit developed in-house.

erologous protection against divergent PRRSV strains than does a wild-type PRRSV strain.

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

As has been observed for HIV-1, the genetic distances between 2 clades of the group M envelope proteins can be up to 30%. A vaccine based on a single consensus envelope sequence can elicit significantly broader cross-clade cellular immune responses than could a vaccine based on a naturally occurring envelope sequence (34, 37). PRRSV is classified into 2 major types: type 1 and type 2. There is very limited cross-protection between type 1 and type 2 PRRSV strains (17, 18, 70). Genetically, type 1 and type 2 PRRSVs share ~65% sequence identity (20, 21). It is possible that a syn-

TABLE 2 Levels of viremia after challenge infection with 16244B[a]

Group and pig	Level of viremia (log_{10} copies/ml) at postchallenge day: 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 for group	0.00	6.78	7.18	7.11	5.84	5.34
SD for group	0.00	0.18	0.30	0.21	1.06	0.50
Group 2 (immunized by infection with PRRSV-CON)						
435	Removed from expt on day 23 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 for group	1.16	1.16	0.00	0.00	0.00	0.59
SD for group	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 expt on day 14 after primary infection					
Mean for group	0.00	3.90	5.35	2.55	1.29	0.00
SD for group	0.00	0.95	1.23	2.39	1.84	0.00

[a] Samples that contained undetected levels of viral RNA are assigned a value of 0 log_{10} copies/ml of serum. Pig 435 (group 2) and pig 543 (group 3) were removed from the experiment due to lameness in their limbs.

TABLE 3 Genetic stability of PRRSV-CON at 21 days p.i.

Nucleotide position	ORF(s)	Nucleotide change	Amino acid change
12883	3	A→G	Synonymous
13440	3 and 4	C→T	ORF3, Ala→Val; ORF4, synonymous
14280	5	G→A	Arg→Lys
14311	5	C→T	Synonymous
14703	6	T→C	Synonymous

thetic PRRSV strain whose genome is centralized between type 1 and type 2 would be able to provide equal protection against both types of PRRSV. The availability of such a PRRS vaccine would be extremely beneficial for the control and eradication of the disease, especially in areas where both types of PRRSV cocirculate.

The viral load in tissue samples collected after challenge infection is an important parameter to evaluate the protective efficacy of a PRRS vaccine candidate. Currently, the tissue viral load is usually quantified through the use of a commercial qRT-PCR kit or through titration on a permissive cell line such as MARC-145 cells. The use of these 2 methods will not allow precise quantification of the tissue viral load resulting from challenge infection in cases where pigs are immunized with a replicating vaccine (with either with MLV vaccines or virulent PRRSV strains) (71). This is because PRRSV can persist in infected animals for an extended period of time (72, 73). At the time of tissue collection for evaluation of viral load, the pigs that are immunized by infection with a live PRRSV may still carry the PRRSV strain that is used for immunization in their lymphoid tissues. Consequently, the tissue samples will possibly contain 2 populations of PRRSV: one from immunization and the other from challenge infection. Neither the commercial qRT-PCR kits nor titration on MARC-145 cells can differentiate the viral strain used for primary infection from the PRRSV strain used for challenge infection. In the present study, we used differential qRT-PCR kits to specifically quantitate the amount of viral RNA resulting from challenge infection. Through the use of these differential qRT-PCR kits, we demonstrate that pigs previously infected with the PRRSV-CON strain carried undetectable levels of challenge PRRSV strains, while those infected with FL12 showed only lower levels of challenge viral RNA (Fig. 5D and 6D).

Of the 59 full-genome sequences that were used in this study to design the PRRSV-CON genome, only 3 sequences were from live-attenuated PRRSV strains. The remaining 56 sequences were from wild-type PRRSV strains/isolates. Therefore, it is expected that PRRSV-CON should display the virulent phenotype of wild-type PRRSV strains. Obviously, PRRSV-CON must be inactivated or attenuated before it can be used as a vaccine in pigs. Both KV vaccines and MLV vaccines are being used in the field. MLV vaccines are commonly developed by successively passaging virulent PRRSV strains in nonnatural host cell lines. Recently, molecular approaches have been used to attenuate virulent PRRSV strains (74, 75). Several studies have demonstrated that MLV vaccines are far more effective than KV vaccines (10, 11). Even so, there are swine producers who prefer to use KV vaccines rather than MLV vaccines because of the concern that MLV vaccines might revert to virulence. It is highly possible that the killed PRRSV-CON vaccine

TABLE A1 Primers and probe used in the differential RT-PCR kit for quantitation of PRRSV strain MN184C-specific RNA[a]

Primer or probe	Sequence (5′→3′)	Binding site (positions)
Forward primer (sense)	AGCTGGCATTCTTGAGACAT	14871–14891
Reverse primer (antisense)	AGGTGACTTAGAGGCACAATATC	14935–14957
Probe (sense)	AGGATGTGTGGTGAATGGCACTGA	14908–14932

[a] See GenBank accession no. EF488739.

TABLE A2 Primers and probe used in the differential RT-PCR kit for quantitation of PRRSV strain 16244B-specific RNA[a]

Primer or probe	Sequence (5′→3′)	Binding site (positions)
Forward primer (sense)	GGCTGGCATTCTTGAGGCAT	15262–15282
Reverse primer (antisense)	CACGGTCGCCCTAATTGAATA	15348–15369
Probe (antisense)	CAGTGCCATTCACCACACATTCTTCC	15297–15323

[a] See GenBank accession no. AF046869.

may confer better levels of cross-protection than the KV vaccine made of naturally occurring PRRSV strains.

The mechanisms by which PRRS vaccines confer protection remain poorly understood (71). Passive-immunization studies using both reproductive models and respiratory models have demonstrated that neutralizing antibodies (NAbs) can protect pigs against infection with a virulent PRRSV strain provided that sufficient amounts of NAbs are present in pigs prior to challenge infection (42, 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 gamma interferon (IFN-γ)-producing cells have 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 is 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 PRRSV-CON 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 involved 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 an outstanding level of heterologous protection. This synthetic PRRSV strain could be an excellent candidate for the formulation of the next generation of PRRS vaccines with improved levels of heterologous protection. In addition, this synthetic PRRSV strain will provide us a unique tool and gold standard to investigate the mechanisms of cross-protection.

TABLE A3 Specificity of the MN184-specific RT-PCR kit

No. of RNA copies/reaction	Cycle threshold for MN184C[a]
5×10^1	38.93
5×10^2	34.68
5×10^3	31.54
5×10^4	28.05
5×10^5	24.67

[a] Cycle threshold for FL12 and PRRSV-CON were not detected.

TABLE A4 Specificity of the 16244B-specific RT-PCR kit

No. of RNA copies/reaction	Cycle threshold for 16244B[a]
5×10^1	40.00
5×10^2	36.27
5×10^3	33.92
5×10^4	29.99
5×10^5	26.55

[a] Cycle threshold for FL12 and PRRSV-CON were not detected.

APPENDIX

Design and validation of differential RT-PCR kits for quantification of challenge virus RNA in tissue samples. Two differential real-time RT-PCR kits for the specific detection and quantification of MN184C-specific and 16244B-speficic viral RNA in tissue samples were developed according to the TaqMan hydrolysis probe method. Specific primers and probes used for differential RT-PCR are presented in Tables A1 and A2. All primers and probes were synthesized by Sigma-Aldrich (Woodland, TX). Real-time RT-PCRs were performed with 25-μl 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 follows: 1 cycle at 50°C for 10 min, 1 cycle at 95°C for 2 min, and 40 cycles at 95°C for 15 s and 60°C for 60 s. Two sets of viral RNA templates with known copy numbers were used to establish the standard curves from which the RNA copy numbers 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

TABLE A5 Comparison between the MN184C-specific RT-PCR kit and the commercial RT-PCR kit

Tissue type	Pig	No. of copies/μg total RNA ($\log_{10}$) determined by: 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 lymph node	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 lymph node	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
Total (mean ± SD)		5.77 ± 0.82	5.56 ± 0.80

TABLE A6 Comparison between the 16244B-specific RT-PCR kit and the commercial RT-PCR kit

Tissue type	Pig	No. of copies/μg total RNA (log_{10}) determined by: 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 lymph node	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 lymph node	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
Total (mean ± SD)		5.25 ± 0.63	4.92 ± 0.68

stocks by using the QIAamp viral RNA minikit (Qiagen, Valencia, CA). Viral genome copies in each of these RNA samples were quantified by using a commercial RT-PCR kit (Tetracore), according to the manufacturer's instructions. After that, these viral RNA samples were diluted to different concentrations, ranging from 10^1 copies per μl to 10^5 copies per μl. Five microliters of each dilution of these viral RNA samples was used for differential RT-PCRs. Data demonstrating the specificities 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 carry viral RNA of the viral strains used for challenge infection only. In general, the viral RNA copy numbers quantitated by using the differential RT-PCR kits were ~0.2 to 0.3 logs lower than the copy numbers quantitated by the commercial RT-PCR kits (Tables A5 and A6).

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