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Chimeric porcine reproductive and respiratory syndrome virus containing shuffled multiple envelope genes confers cross-protection in pigs

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Running title: Shuffled PRRSV confers cross-protection

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The extensive genetic diversity of porcine reproductive and respiratory syndrome virus (PRRSV) strains is a major obstacle for vaccine development. We previously demonstrated that chimeric PRRSVs in which a single envelope gene (ORF3, ORF4, ORF5 or ORF6) was shuffled via DNA shuffling had an improved heterologous cross-neutralizing ability. In this study, we incorporate all of the individually-shuffled envelope genes together in different combinations into an infectious clone backbone of PRRSV MLV Fostera® PRRS. Five viable progeny chimeric viruses were rescued, and their growth characteristics were characterized in vitro. In a pilot pig study, two chimeric viruses (FV-SPDS-VR2,FV-SPDS-VR5) were found to induce cross-neutralizing antibodies against heterologous strains. A subsequent vaccination/challenge study in 72 pigs revealed that chimeric virus FV-SPDS-VR2 and parental virus conferred partial cross-protection when challenged with heterologous strains NADC20 or MN184B. The results have important implications for future development of an effective PRRSV vaccine that confers heterologous protection.

Key words: Porcine reproductive and respiratory syndrome virus (PRRSV); envelope genes; DNA shuffling; vaccines; cross-protection; heterologous strains.
**Introduction**

RNA viruses have high mutation rates mainly due to the low fidelity of viral RNA-dependent RNA polymerases (Arnold et al., 2005; Vignuzzi et al., 2008). This rapid evolution due to high mutation rate usually leads to the generation of genetically and antigenically variable virus strains in the field, which can hinder the development of effective vaccines. Porcine reproductive and respiratory syndrome virus (PRRSV), the causative agent of one of the most economically important global swine diseases, PRRS, has an extremely high mutation rate (Lunney et al., 2010; Murtaugh et al., 2010; Snijder et al., 2013). Genetically diverse field strains of PRRSV have been constantly emerging over the past two decades since its initial isolation from pigs in 1989 (Murtaugh et al., 2010; Shi et al., 2010a). PRRSV is currently classified into two distinct genotypes, type 1 and type 2. Within type 2, it is further subdivided into at least 9 distinct genetic lineages (Shi et al., 2010a; Shi et al., 2010b). It is estimated that the PRRSV mutation rate in the field is approximately $10^{-2}$/site/year, which is higher than any of other known RNA viruses ($10^{-3}$ to $10^{-5}$/site/year) (Hanada et al., 2005). The extensive heterogeneity of PRRSV presents challenges for the efficacy of current commercial vaccines, which are uniformly based on a single virus strain. Consequently the current vaccines generally confer only limited or partial cross-protection against heterologous PRRSV strains (Kimman et al., 2009; Li et al., 2014; Murtaugh and Genzow, 2011).

PRRSV utilizes a discontinuous transcription strategy to synthesize a nested set of subgenomic mRNAs (sg mRNAs) which possess the same 5'-UTRs and 3'-UTRs as the genomic RNAs (Pasternak et al., 2006). Replicase ORF1a and ORF1ab, via ribosomal
frameshift-mediated translational reprogramming, generate nonstructural proteins (nsps) that
direct viral genome replication and sg mRNA synthesis (Fang and Snijder, 2010). The
structural protein-encoding region generates glycoprotein GP2 (encoded by ORF2a), the
envelope proteins E (ORF2b), GP3 (ORF3), GP4 (ORF4), GP5 (ORF5), membrane protein
M (ORF6), nucleocapsid protein N (ORF7) and recently identified small hydrophobic protein
ORF5a (ORF5a) (Firth et al., 2011; Johnson et al., 2011; Snijder et al., 2013). These
structural proteins are critically important to the viral life cycle and for inducing neutralizing
antibodies.

GP5, the major envelope glycoprotein, contains 3-4 N-linked glycosylation sites and
neutralizing epitopes which may induce protective immunity (Ansari et al., 2006; Ostrowski
et al., 2002; Wei et al., 2012a). In viral particles, GP5 and M proteins form heterodimers,
which interact with cellular heparin sulfate contributing to virus entry into cells (Van
Breedam et al., 2010). GP5 is also one of the most variable structural proteins showing only
about 85% nucleotide sequence identity among type 2 PRRSV strains and about 62% identity
between type 1 and type 2 PRRSV (Kappes et al., 2013; Music and Gagnon, 2010; Nelsen et
al., 1999; Rowland et al., 1999). The GP5 has been extensively studied as a target for PRRSV
vaccine development (Kimman et al., 2009; Murtaugh and Genzow, 2011). Minor envelope
proteins (GP2, GP3, GP4 and E) form oligomeric complexes containing abundant N-linked
glycosylation sites and have been shown to induce neutralizing antibodies (Costers et al.,
2010; Lee and Yoo, 2006; Wei et al., 2012b). Recent studies have shown that the minor
envelope proteins play an important role in determining cell tropism by interacting with the
cellular receptor CD163 (Das et al., 2010; Tian et al., 2012) Therefore, for the rational design
of an effective vaccine, both major and minor PRRSV envelope proteins should be considered.

Molecular breeding through DNA shuffling accelerates gene evolution in vitro by mimicking the natural recombination process in vivo (Crameri et al., 1998; Stemmer, 1994). Compared to natural recombination, DNA shuffling rapidly generates recombinants with desired phenotypes in vitro (Dupuy et al., 2009; Patten et al., 1997). In the traditional DNA shuffling approach, a set of target gene fragments derived from parents are digested with DNase I to produce a pool of short DNA fragments, which are then reassembled through PCR amplification to generate a library of recombinants (Soong et al., 2000; Zhou et al., 2013). Recombinants with desired properties can then be screened from the library. The DNA shuffling approach has been successfully used to produce more stable and high-yield murine leukemia virus strains, and to broaden cross-neutralizing activities against dengue viruses (Apt et al., 2006; Powell et al., 2000). In our previous studies, by using molecular breeding through DNA shuffling, we have individually shuffled each of the GP3, GP4, GP5, and M genes of PRRSV (Ni et al., 2013; Zhou et al., 2013; Zhou et al., 2012). Numerous chimeric viruses were rescued and characterized. For example, chimeric virus DS722 (GP5 shuffled) showed an attenuated phenotype based on its pathogenicity (Ni et al., 2013). Chimeric viruses GP3TS22 (GP3 shuffled), GP4TS14 (GP4 shuffled), and MTS57 (M shuffled) displayed improved cross-neutralizing activities against heterologous virus strains in vitro (Zhou et al., 2013; Zhou et al., 2012).

In this present study, we hypothesize that integration of these single envelope gene-shuffled sequences together in different combinations into the genomic backbone of an
infectious clone based on a commercial PRRSV vaccine virus will generate novel chimeric viruses that confer cross-protection against heterologous virus challenges. Therefore, in this study we first generated a panel of chimeric viruses containing shuffled multiple envelope proteins in different combinations based on the genomic backbone of Fostera® PRRS, a commercial PRRSV vaccine virus. The shuffled chimeric viruses were successfully rescued and characterized for their growth characteristics in monkey kidney cell lines MARC-145 and ATCC CRL11171. An immunogenicity study in 21 pigs identified two chimeric viruses, FV-SPDS-VR2 and FV-SPDS-VR5, that both elicited significantly higher levels of cross-neutralizing antibodies compared to the parental virus. Importantly, in a vaccination/challenge study in 72 pigs, the chimeric virus FV-SPDS-VR2 and the parental virus were demonstrated to confer cross-protection when vaccinated pigs were challenged with heterologous strains NADC20 or MN184B.

Results

Rescue of chimeric viruses with shuffled multiple envelope genes

In our previous studies, we have successfully generated four single envelope gene-shuffled chimeric PRRSV strains, in the genomic backbone of the virulent VR2385 PRRSV strain, in which the envelope genes (ORF3-6) were each individually shuffled. (Ni et al., 2013; Zhou et al., 2013; Zhou et al., 2012). Importantly, we demonstrated that these single envelope gene-shuffled chimeric viruses (GP3TS22, GP4TS14, and MTS57) induced higher levels of cross-neutralizing antibodies against heterologous virus strains than that of the backbone virus in vitro, or displayed an attenuated phenotype (DS722) while still inducing protection in
vivo. Both major and minor envelope proteins of PRRSV play important roles in inducing protective immune responses, in the present study we hypothesized that combining the shuffled chimeric sequences of the envelope genes from each of the four single-gene shuffled chimeric viruses into one single mosaic virus would induce maximal cross-protection against heterologous strains. To achieve this objective, we integrated the chimeric sequences of all shuffled envelope protein genes into a single PRRSV strain in the genomic backbone of the vaccine virus Fostera® PRRS (FV). Five full-length chimeric plasmid clones (FV-SPDS, FV-SPDS-FV5, FV-SPDS-FV25, FV-SPDS-VR2, and FV-SPDS-VR5) were constructed (Fig. 1), and the authenticity of these chimeric clones was verified by DNA sequencing of the final constructs.

Following transfection of the full-length chimeric clones into BHK-21 cells, supernatants were harvested after two days (P0 virus) and used to inoculate fresh MARC-145 cells. At 3 to 4 days post-inoculation, CPE was observed in inoculated MARC-145 cells, which were subsequently confirmed by IFA using PRRSV N-specific monoclonal antibody, indicative of the production of viable progeny virions and rescue of infectious chimeric viruses (Fig. 2A). To further confirm that the rescued progeny viruses originated from the respective transfected chimeric virus clones, the ORFs 2-6 were amplified from each of the P3 viruses by RT-PCR and sequenced. Sequence analyses revealed that the ORFs 2-6 of each rescued progeny virus contained the shuffled ORFs 2-6 gene sequences, as originally engineered at the level of the full-length virus clones. The results demonstrated the successful generation of viable chimeric virus progeny with multiple shuffled envelope genes.
Growth characteristics of the chimeric viruses containing multiple shuffled envelope genes in MARC-145 and ATCC CRL11171 cells

In order to use these shuffled chimeric viruses as potential candidates for a modified live-attenuated vaccine (MLV), they must replicate well in cell cultures. Therefore, we investigated the growth characteristics of the multiple envelope gene-shuffled chimeric viruses in MARC-145 and ATCC CRL11171 cells. Using P3 viruses, MARC-145 cells were infected with the shuffled and parental viruses at an MOI of 0.1 to evaluate the growth kinetics. With the exception of chimera FV-SPDS-FV25, all other shuffled chimeric viruses displayed similar growth kinetics to the parental virus FV (Fig. 3A). The shuffled chimeric viruses FV-SPDS-FV5 and FV-SPDS-VR5 showed similar peak titers to parental virus FV ($\approx 2.5 \times 10^6$TCID$_{50}$/ml), while the FV-SPDS ($\approx 4.0 \times 10^5$TCID$_{50}$/ml) and FV-SPDS-VR2 ($\approx 6.3 \times 10^5$TCID$_{50}$/ml) had slightly lower peak titers ($< 1 \log_{10}$) compared to FV. The chimera FV-SPDS-VR2 showed an accelerated replication rate, reaching the peak virus titer earlier ($\approx 12$ h) than other viruses.

To further characterize the growth kinetics of the shuffled chimeric viruses, we also tested virus replication in the cell line ATCC CRL11171, a monkey kidney cell line. Cells were inoculated with P0 chimeric viruses. CPE appeared from 3 to 4 days post-infection, and was verified by IFA using PRRSV-specific antibody (Fig. 2B). In growth kinetics, both shuffled chimeric and parental viruses (P3) replicated well in ATCC CRL11171 cells (Fig. 3B). There was no significant difference in peak infectious virus titers between MARC-145 and ATCC CRL11171 (Fig. 3C), although the time points at peak virus titers for most chimeric viruses in ATCC CRL11171 cells were about 12 h later compared to that in MARC-145 cells (Fig.
Similarly, we observed that the chimera FV-SPDS-FV25 displayed an impaired growth whereas chimera FV-SPDS-VR2 had an accelerated growth rate. Collectively, the results showed that these multiple envelope gene-shuffled chimeric viruses replicated in both MARC-145 and ATCC CRL11171 cells to relatively high titers that are sufficient for use as a MLV.

Two shuffled chimeric viruses, FV-SPDS-VR2 and FV-SPDS-VR5, induced cross-neutralizing antibodies against heterologous virus strains

To screen for potential viable candidate vaccine strains, we conducted a small pilot animal study by experimentally infecting groups of 3 pigs with one of the five shuffled chimeric viruses or the parental virus to generate antisera specific to each virus. The six groups of pigs inoculated with shuffled viruses or parental virus seroconverted within 14 or 21 dpi and remained seropositive through the end of the study (Fig. 4), while the negative control pigs remained seronegative, indicating that all the five multiple envelope gene-shuffled viruses replicated in vivo and elicited immune responses in pigs. One pig in FV group had a very low S/P value compared to the other two pigs, and thus making the overall mean value of FV group lower than other groups. This was likely due to the individual difference caused by the small numbers of pigs (3) used in this pilot study.

To assess whether the shuffled chimeric viruses can induce cross-neutralizing antibodies against heterologous PRRSV strains, an SVN assay was performed using serum samples collected at 49 dpi from pigs against parental virus FV as well as five heterologous PRRSV strains belonging to different genetic lineages (Zhou et al., 2013; Zhou et al., 2012). In the
SVN assay against the parental virus FV (Fig. 5A), the mean NA titers of the FV-SPDS-FV5 (5.6) and FV-SPDS-FV25 (5.2) groups were slightly lower than that of the FV group (6.0), while the NA titers of the FV-SPDS, FV-SPDS-VR2, FV-SPDS-VR5 groups were much lower than that of the FV group, likely due to the fact that these three shuffled chimeric viruses contain less envelope protein sequences derived from FV.

For the SVN against the heterologous strain VR2430 (Fig. 5B), most shuffled virus groups reached mean titers of about 4.0, but there was no significant difference compared to the FV group (4.1). However, the mean NA titer of the FV-SPDS-FV25 group (2.6) was significantly lower than that of the FV group ($P=0.0018$). For the SVN against PRRSV strain VR2385, a different sublineage of lineage 5 relative to strain VR2430 (Fig. 5C), the FV-SPDS-VR2 and FV-SPDS-VR5 groups had mean NA titers of 1.7 and 3.1, respectively, while the other groups displayed low (<1.0) or undetectable titers. For SVN against the heterologous strain NADC20 (Fig. 5D), the mean NA titers of the FV-SPDS group (2.0) and FV-SPDS-VR2 group (4.0) were significantly higher than that of the FV group (0.8). For SVN against the heterologous strain MN184B (Fig. 5E), all groups had low (<1.0) or undetectable NA titers.

For SVN against the heterologous strain FL12, most serum samples had undetectable NA titers (data not shown).

The composite NA titers were generated by combining the individual NA titers against five respective heterologous strains (VR2430, VR2385, NADC20, MN184B, FL12), and analyzed to evaluate the cross-neutralizing ability for each of the five chimeric viruses (Fig. 5F). Chimeras FV-SPDS-VR2 ($P=0.0115$) and FV-SPDS-VR5 ($P=0.0057$) inoculated pigs displayed significantly higher NA titers against heterologous PRRSV strains compared to FV...
inoculated pigs, although the two groups had relatively large error bars due to different titers against different heterologous strain. This suggested that two of shuffled viruses, FV-SPDS-VR2 and FV-SPDS-VR5, had an improved ability to induce cross-neutralizing antibodies in vitro against heterologous PRRSV strains.

One shuffled chimeric virus, FV-SPDS-VR2, conferred cross-protection against challenge with heterologous PRRSV strains

Since two of the shuffled chimeric viruses, FV-SPDS-VR2 and FV-SPDS-VR5, induced cross-neutralizing antibodies, these two chimeric viruses were subsequently selected for a vaccination/challenge efficacy study in pigs (Table 1). Two heterologous strains NADC20 (lineage 9) and MN184B (lineage 1) were used as challenge viruses. When challenged with NADC20 or MN184B, 7 pigs in the non-vaccinated/NADC20 challenged group and 4 pigs in the non-vaccinated/MN184B challenged group developed high body temperatures (>104.5°F) (Fig. 6). However, only 1-2 pigs in the shuffled chimeric virus- or parental FV-vaccinated groups developed high temperatures when challenged with NADC20 (Fig. 6A). Only 2 pigs in the FV-SPDS-VR2-vaccinated group and 3 pigs in the FV-SPDS-VR5-vaccinated group developed fevers after challenge with MN184B, while 6 pigs displayed fevers in the FV-vaccinated/MN184B-challenged group (Fig. 6B).

At necropsy, both FV- and FV-SPDS-VR2-vaccinated groups showed significantly decreased gross lung lesion scores compared to the non-vaccinated control group when challenged with either NADC20 or MN184B (Fig. 7A, 7B). For the NADC20 challenge, the FV-vaccinated group had a lower mean gross lung lesion score than the FV-SPDS-VR2- and
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FV-SPDS-VR5-vaccinated groups (Fig. 7A). For the MN184B challenge, the FV-SPDS-VR2-vaccinated group had a numerically lower mean gross lung score than the FV-vaccinated group (FV-SPDS-VR2: 11; FV: 19), although the difference was not statistically significant ($P=0.1879$, Fig. 7B). For microscopic lung lesions, both FV- and the two shuffled chimeric viruses-vaccinated groups had significantly lower scores than the non-vaccinated control group when challenged with NADC20 (Fig. 7C), but not with MN184B (Fig. 7D).

For the pigs challenged with NADC20, both FV- and chimeric viruses-vaccinated groups had significantly decreased levels of serum viral RNA copies at 7 dpc compared to the non-vaccinated control group (Fig. 8A). The mean serum PRRSV viral RNA copy number of the FV-SPDS-VR2-vaccinated group ($6.3 \times 10^4$ copies/ml) was numerically lower than that of the FV-vaccinated group ($2.0 \times 10^6$ copies/ml), although the difference was not statistically significant ($P=0.0841$). Similarly, at 14 dpc (Fig. 8B), the mean serum viral RNA copies of the FV ($1.3 \times 10^5$ copies/ml) and FV-SPDS-VR2 ($5.0 \times 10^4$ copies/ml) vaccinated groups were significantly decreased when compared to the non-vaccinated control group ($5.0 \times 10^6$ copies/ml). Also, the viral RNA loads in the lung tissues of FV- and chimeric viruses-vaccinated groups at 14 dpc were significantly decreased when compared to the non-vaccinated control group (Fig. 8C). Three pigs in the FV-SPDS-VR2-vaccinated group and 1-3 pigs in the FV-vaccinated group were detected negative for viral RNA in serum samples or lung tissues (Fig. 8A-C).

For the pigs challenged with MN184B, at 7 dpc, the serum viral RNA loads showed a significant decrease in groups vaccinated with FV ($P=0.0311$) or with FV-SPDS-VR2...
(P=0.0297), but not with FV-SPDS-VR5 (P=0.1124), when compared to the non-vaccinated control group (Fig. 8D). Similarly, at 14 dpc (Fig. 8E), significant decreases in serum viral RNA loads were observed in FV-vaccinated pigs (P=0.0047), FV-SPDS-VR2-vaccinated pigs (P=0.0004), and FV-SPDS-VR5-vaccinated pigs (P=0.0125) compared to the control pigs. However, for the virus RNA loads in the lung at 14 dpc (Fig. 8F), neither FV- nor chimeric viruses-vaccinated groups had a statistically significant decrease compared to the control pigs. Also, the mean viral RNA load of the FV-SPDS-VR2-vaccinated group (6.3×10^8 copies/gram) was numerically lower than that of the FV-vaccinated group (1.6×10^10 copies/gram), although the difference was not statistically significant (P=0.1175).

The chimeric FV-SPDS-VR2 and FV-SPDS-VR5 viruses were genetically stable in vivo

To investigate whether the multiple envelope genes-shuffled chimeric viruses FV-SPDS-VR2 and FV-SPDS-VR5 are genetically stable in vivo, the chimeric viruses were recovered from serum samples of infected pigs at 14 dpi. ORFs 2-6 were amplified by RT-PCR and sequenced. Sequence analyses revealed that the recovered viruses had 99.9% nucleotide sequence and 100% amino acids identity to the original virus inocula, indicating the genetic stability of the two shuffled viruses in pigs.

Discussion

Novel strategies for developing universal cross-protective vaccines have been explored for a number of antigenically highly-variable viruses such as influenza virus and HIV based on highly conserved antigens (Almeida et al., 2012; Neirynck et al., 1999; Pica and Palese,
For PRRSV, which is also genetically and antigenically highly variable, thus far there is no vaccine that can provide sufficient cross-protection against all heterologous strains (Li et al., 2014; Martelli et al., 2009). Therefore, enhancing cross-protection is an important task but also the biggest challenge for the development of the next generation of PRRSV vaccines (Binjawadagi et al., 2014; Huang and Meng, 2010; Kimman et al., 2009). The heterogenic nature of the virus coupled with the fact that current vaccines are all based on a single virus strain explain why the current commercial vaccines are not fully effective in protecting against genetically diverse field strains of PRRSV. It is clear that heterogeneity needs to be considered when designing the next generation PRRSV vaccines, or PRRSV will remain difficult to control.

Molecular breeding through DNA shuffling directs the evolution of a virus *in vitro* at an accelerated rate, thus making it a powerful tool to rapidly generate virus strains with desired properties (Crameri et al., 1998; Stemmer, 1994). For example, a chimeric dengue virus antigen produced by DNA shuffling induced tetravalent cross-neutralizing antibodies against four different serotypes of Dengue viruses (Apt et al., 2006). In our previous studies (Ni et al., 2013; Zhou et al., 2013; Zhou et al., 2012), by using DNA shuffling, we successfully produced several single envelope gene shuffled chimeric PRRSV viruses in the genetic backbone of virulent strain VR2835. These shuffled viruses had an improved cross-neutralizing activity against heterologous PRRSV strains *in vitro*. Therefore, we reason that combining these single envelope gene-shuffled viruses with demonstrated cross-neutralizing activities into one “mosaic virus” may generate a candidate vaccine virus that will induce superior heterologous cross-protection than those single envelope
gene-shuffled viruses. Therefore, in this present study, by utilizing an infectious clone of the commercial vaccine Fostera® PRRS as the genomic backbone, we integrated the shuffled chimeric sequences from each of the single envelope gene-shuffled viruses (GP3TS22, GP4TS14, DS722, and MTS57) into one composite virus containing multiple shuffled envelope genes in various combinations. We successfully constructed and rescued five viable multiple envelope gene-shuffled chimeric viruses that could serve as potential vaccine candidates (Fig. 1). The growth kinetics and cross-neutralizing activities of the five chimeric viruses, and the heterologous cross-protective ability and vaccine efficacy of the two most promising chimeric viruses were investigated in this study.

The five rescued chimeric viruses containing multiple shuffled envelope genes in different combinations (Fig. 1) replicated well in two cell lines, MARC-145 and ATCC CRL11171, suggesting that the gene shuffling did not significantly impair the growth of most of the resulting chimeric viruses. The shuffled chimeric viruses caused PRRSV-specific CPE and produced high virus yields (up to 10⁶ TCID₅₀/ml) in both MARC-145 and ATCC CRL11171 cells, thus making these chimeric viruses viable candidates for further development into MLVs.

Importantly, we demonstrated that these chimeric viruses induced robust immune responses in pigs as evidenced by the detection of high levels of anti-PRRSV N antibodies in infected pigs (Fig. 4). Most of the NA titers tested in this study were consistent with our previous studies, suggesting a good reproducibility of the SVN assay (Zhou et al., 2013; Zhou et al., 2012). Chimera FV-SPDS-VR5 induced significantly higher cross-neutralizing antibodies against heterologous strain VR2385, and chimera FV-SPDS-VR2 induced
significantly higher cross-neutralizing antibodies against NADC20. However, against the heterologous strains MN184B and FL12, the NA titers of the parental virus FV and all the 5 chimeric viruses were very low (<1), and this was somewhat in contrast to our previous studies in which most NA titers were above 1 (Zhou et al., 2013; Zhou et al., 2012). We speculated that this was largely due to the fact that the virulent strain VR2385 backbone, which was used in our previous studies, can elicit higher levels of neutralizing antibodies against MN184B and FL12 than the vaccine virus FV backbone used in this present study. The data also suggested that the nonstructural proteins may also play roles in inducing NAs, and thus should be considered in the future vaccine design. In general, the NA titer against a particular virus strain was related to the sequence components of the shuffled envelope proteins. For example, compared to other shuffled viruses, FV-SPDS-FV5- and FV-SPDS-FV25-infected pigs developed higher NA titers against FV because the GP2 and/or GP5 of the FV-SPDS-FV5 and FV-SPDS-FV25 viruses were derived from FV (Fig. 1, 5A). FV-SPDS-VR2- and FV-SPDS-VR5-infected pigs had higher NA titers against VR2385 because the GP2 or GP5 of the FV-SPDS-VR2 and FV-SPDS-VR5 viruses were derived from the VR2385 strain (Fig. 1, 5C), indicating that GP2 as well as GP5 are important in inducing neutralizing antibody, as GP2 is one of the viral attachment proteins interacting with cellular receptor CD163 (Das et al., 2010).

Although the role of NAs in PRRSV protection is somewhat controversial, the NA titer is still an important criterion used by many research groups in evaluating candidate vaccine efficacy, especially for rapid screening of potential vaccine candidates for subsequent challenge-protection study (Binjawadagi et al., 2014; Wang et al., 2013). It is clear from the
composite NA titers against all heterologous strains tested in the study that FV-SPDS-VR2 and FV-SPDS-VR5 induced significantly higher cross-neutralizing antibody titers than FV (Fig. 5F). Therefore, the FV-SPDS-VR2 and FV-SPDS-VR5 chimeric viruses were chosen for the subsequent cross-protective vaccine efficacy study in pigs. It is important to point out that the small pilot virus infection study in a small number of pigs (n=3) was meant to be an *in vivo* screening assay to identify a viable infectious virus that induces cross-neutralizing antibodies for the subsequent large vaccine challenge study in pigs. This pilot small pig study was simply to identify one chimeric virus that is infectious in pigs and induces cross-neutralizing antibodies.

The body temperature, gross and histological pathology of the lungs, and the viral RNA loads in serum and lung tissues after challenge are the most commonly used parameters for assessing PRRSV vaccine efficacy (Kimman et al., 2009; Martelli et al., 2009; Wang et al., 2013; Zuckermann et al., 2007). In general, more of the non-vaccinated/challenged pigs developed high body temperatures compared to the vaccinated/challenged pigs. For example, when challenged with NADC20 virus, only 1-2 pigs had a high body temperature in the FV- or chimeric viruses-vaccinated groups, while 7 pigs developed high body temperature in the unvaccinated/challenged control group (Fig. 6A). Importantly, both parental FV- and chimeric viruses-vaccinated groups showed significantly lower macroscopic and microscopic lung lesion scores (Fig. 7A, 7C) and lower viral RNA loads in sera and lung tissues (Fig. 8A-C) than the control group. Also, the FV-SPDS-VR2-vaccinated group had numerically lower (but not significantly lower) viral RNA loads relative to the FV-vaccinated group. Together, this data suggest that the two shuffled chimeric viruses and the parental virus...
provided good protection against heterologous PRRSV strain NADC20 challenge, and it can be hypothesized that the chimera FV-SPDS-VR2 may be well suited to control NADC20 viremia by virtue of its NADC20 genetic components.

We also included the highly virulent heterologous strain MN184B (lineage 1) as another challenge virus to measure the level of cross-protection, since no current commercial vaccines are based on lineage 1 viruses. When challenged with MN184B, 2 pigs in the FV-SPDS-VR2-vaccinated group and 3 pigs in the FV-SPDS-VR5-vaccinated group had high body temperature, while 6 pigs in FV-vaccinated group and 4 pigs in the control group developed high body temperatures (Fig. 6B), indicating that the candidate chimeric virus vaccines can reduce fever associated with PRRSV infection. FV- and FV-SPDS-VR2-vaccinated groups also showed significantly lower levels of macroscopic lung lesion scores (Fig. 7B) and serum viral RNA loads (Fig. 8D, 8E) compared to the control group. Also, the FV-SPDS-VR2-vaccinated group had low macroscopic lung lesion scores (Fig. 7B) and low viral RNA loads in lungs (Fig. 8F). Taken together, the data suggest that chimera FV-SPDS-VR2, and parental FV provided partial cross-protection against the highly-virulent heterologous strain MN184B. It was noted that neither parental FV nor chimera FV-SPDS-VR2 induced a high level of NA titers based on SVN assay (Fig. 5E). Therefore, cellular immune cytokines such as interferon-gamma might also be involved in the protection. Unfortunately, no significant level of interferon gamma was detectable in sera at 0 dpc and 14 dpc, and the lack of fresh PBMCs (not collected in this study) prevented us from performing further cytokine testings in this study.

In summary, in this study we successfully generated five multiple envelope genes-shuffled
chimeric viruses in the genomic backbone of a commercial PRRSV vaccine virus FV. The rescued chimeric viruses replicated well in two cell lines and produced robust immune responses in pigs. One shuffled chimeric virus, FV-SPDS-VR2, and its parental FV induced partial cross-protection when challenged with two heterologous strains NADC20 and MN184B. We demonstrated in this study that simultaneous multiple envelope gene-shuffling of PRRSV is a viable approach to generate potential vaccine candidates that possess cross-protective ability and may lead to novel vaccines with enhanced cross-protection against diverse PRRSV strains.

Materials and methods

Cells and viruses

BHK-21 cells were cultured in Dulbecco’s Minimal Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Two different clones of monkey kidney cell line MA104, MARC-145 and ATCC CRL11171 (Halbur et al., 1995; Kim et al., 1993; Meng et al., 1996), were cultured in low glucose-supplemented DMEM with 10% FBS, and maintained in low glucose DMEM with 2% FBS for virus propagation. The cells were cultured in an incubator at 37°C with 5% CO₂. An infectious cDNA clone of the commercial vaccine virus Fostera® PRRS in a DNA-launched plasmid format (pFV) was chemically synthesized based on the complete genome sequence of the vaccine virus. As a positive control, Fostera-like virus (FV) was generated by direct transfection of cells with pFV, and evaluated in parallel with gene-shuffled chimeric viruses. Fostera® PRRS is derived from strain P129 (Accession no. AF494042), which is a lineage 8 PRRSV based on its ORF5
sequence. PRRSV strains from distinct genetic lineages of genotype 2 including VR2430 (lineage 5, accession no. JX050225), VR2385 (lineage 5, accession no. JX044140), NADC20 (lineage 9, accession no. JX044140), MN184B (lineage 1, accession no. DQ176020), and FL12 (lineage 8, accession no. AY545985) were propagated, titrated and stored at -80°C before use (Ni et al., 2013; Shi et al., 2010b).

Construction of chimeric virus clones containing shuffled multiple envelope genes

In our previous studies, based on the genomic backbone of the PRRSV strain VR2385, we generated several single envelope gene-shuffled chimeric viruses that possess in vitro cross-neutralizing activities against heterologous PRRSV strains or in vivo attenuated pathogenicity: GP3TS22 (shuffled ORF3), GP4TS14 (shuffled ORF4), DS722 (shuffled ORF5), and MTS57 (shuffled ORF6). In order to design a vaccine with maximal cross-protection against heterologous strains, we incorporated, in different combinations, the shuffled multiple envelope genes into the genomic backbone of the vaccine virus infectious clone pFV. Briefly, based on the envelope gene sequences of the four single gene-shuffled chimeric viruses (GP3TS22, GP4TS14, DS722, and MTS57) and the predicted structural topology of the PRRSV envelope proteins (Dokland, 2010), we designed and synthesized a nucleotide sequence fragment, designated SPDS, which contains the ORFs 2 through 6. For the SPDS sequence, the GP2 ectodomain-encoding region was derived from strain MN184B, GP3 from chimera GP3TS22, GP4 from chimera GP4TS14, and GP5 from chimera DS722. The entire M sequence was derived from chimera MTS57. The non-ectodomain regions of GP2 through 5 were from pFV. The overlapping sequences of ORFs 2-3 were derived from
chimera GP3TS22, and the overlapping sequences of ORFs 3-4 were from chimera GP4TS14.

The genomic organizations of the chimeric virus constructs are depicted in Fig. 1.

By using the *Afe* I and *Spe* I restriction enzyme sites engineered in the synthesized fragment SPDS, the ORFs 2-6 were introduced into the genomic backbone of pFV, to generate the chimeric virus designated FV-SPDS. Subsequently, based on the chimeric clone FV-SPDS, we constructed four other chimeric virus clones: FV-SPDS-FV5 (ORF5 derived from FV), FV-SPDS-FV25 (ORF2 and ORF5 from FV), FV-SPDS-VR2 (ORF2 from VR2385), and FV-SPDS-VR5 (ORF5 from VR2385) through fusion PCR as described elsewhere (Ni et al., 2013; Tian et al., 2012). Briefly, two flanking fragments amplified from pFV were fused to the corresponding target ORFs to form hybrid fragments. By utilizing the restriction enzyme sites in the flanking fragments, the hybrid fragments were then cloned into the pFV backbone to generate the four chimeric virus clones (Fig. 1). All the chimeric virus constructs were verified by nucleotide sequencing.

**In vitro transfection to rescue chimeric viruses**

Plasmid DNAs from full-length DNA-launched chimeric virus infectious clones were isolated using the QIAprep Spin Miniprep kit, and quantified using Nanodrop. Fresh BHK-21 cells in a 6-well plate at approximately 60–80% confluency were transfected with 2 µg of plasmid DNA per well using Lipofectamine LTX and Plus Reagent kit (Invitrogen) according to the manufacturer’s instructions, followed by incubation at 37°C with 5% CO₂. At 48 h post-transfection, cell culture supernatants were harvested and designated as passage 0 (P0) viruses.
**Indirect immunofluorescence assay (IFA)**

At 48 h post-inoculation, cells were washed twice with phosphate-buffered saline (PBS) and fixed in cold methanol for 15 min. After washing with PBS, the fixed cells were blocked in 1% bovine serum albumin (BSA) at room temperature for 30 min, washed with PBS and then incubated with anti-PRRSV N monoclonal antibody SDOW17 (Rural Technologies, Inc.) at 37°C for 2 h. After extensive washing with PBS, the cells were incubated with fluorescein isothiocyanate (FITC) or Alexa Fluor 594-conjugated goat anti-mouse IgG for 1 h at 37°C. After washing with PBS, fluorescent signals were visualized using an Olympus inverted fluorescence microscope fitted with a digital camera.

**Virus growth kinetics assay**

To investigate the growth properties of the shuffled chimeric viruses in MARC-145 and ATCC CRL11171 cells, a multiple-step growth curve analysis was conducted. Briefly, MARC-145 or ATCC CRL11171 cells in 6-well plates were infected with each of the shuffled chimeric viruses as well as parental FV virus (passage P3) at a low multiplicity of infection (MOI) of 0.1. At 12, 24, 36, 48, 60, 72, 84, 96, 108, and 120 h post-infection (hpi), 200 µl of each cell culture supernatant was collected, and wells were replenished with the same volume of fresh culture medium. Virus titrations were performed in MARC-145 or ATCC CRL11171 cells in 96-well plates with fresh cells that were inoculated with 10-fold serial virus dilutions (4 replicates per dilution, 100 µl/well) for 1 h, after which the cells were washed with PBS and then incubated in low glucose DMEM with 2% FBS in a humidified atmosphere.
CO₂ incubator. Presence of a cytopathic effect (CPE) was determined at 7 days post-inoculation. Viral titers were calculated using the Reed-Muench method and expressed as the 50% tissue culture infectious dose per milliliter (TCID₅₀/ml). Three independent experiments were carried out for each virus.

**Experimental design for a small pilot chimeric virus infection study in pigs to generate antibodies against each of the five chimeric viruses**

In order to screen for potential candidate viable chimeric virus strains for the subsequent vaccine efficacy testing, and to determine the viability of the multiple envelope gene-shuffled chimeric viruses in pigs, we conducted a small pilot *in vivo* pig infection study with the 5 chimeric viruses (FV-SPDS, FV-SPDS-FV5, FV-SPDS-FV25, FV-SPDS-VR2, and FV-SPDS-VR5). A total of 21 pigs were divided into 7 groups of 3 pigs each, and each group was inoculated with one of the 5 chimeras. Weekly serum samples were collected from each pig for a total of 7 weeks. The sera were tested for PRRSV neutralizing antibodies against homologous and heterologous strains as well as PRRSV antibody responses using the IDEXX HerdChek® X3 ELISA kit according to the protocol provided by the manufacturer. All experiments involving animals were conducted in compliance with national legislation and subject to review by both Virginia Tech and Iowa State University Institutional Animal Care and Use Committee (IACUC).

**Serum virus neutralization assay to evaluate cross-neutralizing activities**

The neutralizing antibody (NA) titers against homologous and heterologous PRRSV strains
were determined by a serum virus neutralization (SVN) assay essentially as previously described (Zhou et al., 2012). Briefly, 2-fold diluted serum samples collected at 49 days post-inoculation (dpi) from each pig were mixed with an equal volume of individual homologous (FV) or heterologous (VR2430, VR2385, NADC20, MN184B and FL12) virus at an infectious titer of $2 \times 10^3$ TCID$_{50}$/ml and incubated at 37°C for 1 h. The mixtures were then dispensed onto ATCC CRL11171 cells in 96-well plates and incubated for 1 h at 37°C. After washing with PBS, the cells were maintained in DMEM with 2% FBS. At approximately 20 hpi, the cells were assayed by IFA for virus infection. The NA titers were expressed as the highest dilution that showed at least 90% reduction in the number of fluorescent foci compared to antisera from negative control pigs. Three independent tests were performed for each serum sample.

**Experimental design for a vaccine efficacy and cross-protection study in pigs**

Based on the cross-neutralizing activities of the sera from pigs infected with each of the 5 chimeric viruses, we selected two chimeric viruses (FV-SPDS-VR2 and FV-SPDS-VR5) for a vaccine efficacy study in pigs. Briefly, a total of 72 PRRSV-negative piglets at 3 weeks of age were divided into 9 groups of 8 piglets per group. Piglets in each group were vaccinated with one of the two shuffled chimeric viruses (FV-SPDS-VR2, or FV-SPDS-VR5), parental virus (FV) derived from the pFV infectious clone, or PBS as shown in Table 1. Serum samples were collected from each pig prior to vaccination and weekly thereafter. At 42 days post-vaccination, the pigs were challenged with two heterologous virus strains NADC20 or MN184B as shown in Table 1. At 14 days post-challenge (dpc), all pigs were necropsied for
gross pathological and histopathological lesion evaluation. Serum samples at 7 dpc and 14 dpc as well as the samples of lung tissues at 14 dpc were used to quantify PRRSV viral RNA loads. The body temperatures from all pigs were also monitored every 2 days after challenge.

Quantitation of viral RNA loads in sera and lung tissues

Viral RNAs were extracted from serum samples at 7 and 14 dpc using ZR Viral RNA kit (ZYMO RESEARCH, USA) according to the protocol provided by the manufacturer. Total RNAs from samples of lung tissues were extracted using TRI Reagent (MRC). The RNA standard used for the RT-qPCR was derived from in vitro transcription of a PRRSV full-length cDNA clone pACYC-VR2385 by mMESSAGE mMACHINE T7 kit (Ambion). The PRRSV RNA copy numbers in sera or lung tissues were quantified by a SYBR green-based quantitative PCR using SuperScript III Platinum SYBR Green One-Step RT-qPCR kit (Invitrogen) with a protocol recommended by the manufacturer. The primer set (realtime2F/2R, binds to the conserve region of ORF7) used in the RT-qPCR assay was previously validated elsewhere (Ni et al., 2013; Ni et al., 2014). The RT-qPCR assay was conducted in a CFX96 real-time (RT) PCR system (Bio-Rad). Each reaction was performed in triplicate.

Gross pathology and histopathology evaluation

All pigs were humanely euthanized by intravenously overdose injection of pentobarbital (Fatal-Plus, Vortech Pharmaceutical Ltd., Dearborn, MI). At necropsy, the lungs were evaluated for gross pathology, and subsequently five sections of lung tissues were collected,
fixed in 10% neutral-buffered formalin and processed for histopathology evaluation. Fresh lung tissues were also collected and stored at -80°C for quantification of viral RNA loads by RT-qPCR.

The criteria for evaluating the gross pathology and histopathology have been well established and described previously (Halbur et al., 1995; Ni et al., 2013). For gross pathology evaluation, the total amount of lung lesions affected by pneumonia of each pig (0-100% of the lung affected by visible pneumonia) was scored by a veterinary pathologist (PGH) who was blinded to the treatment status of pigs. The scoring system is based on the approximate volume that each lung lobe contributes to the entire lung: the right cranial lobe (10%), right middle lobe (10%), cranial part of the left cranial lobe (10%), caudal part of the left cranial lobe (10%), the accessory lobe (5%), and right and left caudal lobes (27.5% each).

The microscopic lung lesions were evaluated and scored independently by two veterinary pathologists (TO and PGH) who were blinded to the treatment status. The scores based on the presence and severity of interstitial pneumonia ranging from 0 to 6 (0, normal; 1, mild multifocal; 2, mild diffuse; 3, moderate multifocal; 4, moderate diffuse; 5, severe multifocal; 6, severe diffuse). The mean of the two scores obtained for each pig was used as the final value.

Statistical analyses

The Student’s t test (unpaired) was used to evaluate the differences ($P<0.05$) between the samples in the two groups. The data were analyzed using GraphPad Prism (version 5.0).
Acknowledgments

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Conflict of interest statement:

Jay G. Calvert and Douglas S. Pearce are employees of Zoetis Inc, which funded this research project that was conducted at the Meng lab at Virginia Tech. There is no other apparent conflict of interest.

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### Table 1. Experimental design for the cross-protective vaccine efficacy study in pigs

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of pigs</th>
<th>Vaccination at 0 dpi</th>
<th>Challenge at 42 dpi</th>
<th>No. of pigs at necropsy (14dpc)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>(1.0×10^4.0 TCID_{50}/pig)</td>
<td>(1.0×10^5.0 TCID_{50}/pig)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>8</td>
<td>FV-SPDS-VR2</td>
<td>NADC20</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>FV-SPDS-VR2</td>
<td>MN184B</td>
<td>7\textsuperscript{a}</td>
</tr>
<tr>
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<td>8</td>
<td>FV-SPDSVR5</td>
<td>NADC20</td>
<td>8</td>
</tr>
<tr>
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<td>MN184B</td>
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<td>9</td>
<td>8</td>
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<td>PBS</td>
<td>8</td>
</tr>
</tbody>
</table>

\textsuperscript{a} One piglet died from an unrelated cause before challenge.
**Figure captions**

**FIG. 1.** Schematic diagrams of the organization of multiple envelope genes of backbone FV virus and 5 shuffled chimeric PRRSVs. The genes derived from the backbone FV virus are depicted with open rectangles. The predicted ectodomains of the envelope genes are represented by gray bars with numbers showing the start and ending nucleotide positions. Each pattern in the shuffled genes represents one of the six individual heterologous donor parental virus strains, which are shown at the bottom.

**FIG. 2.** Successful rescue and replication of multiple envelope gene-shuffled chimeric viruses. Two days post-transfection of BHK-21 cells with the vaccine virus FV backbone and shuffled chimeric virus clones, the P0 virus supernatants were harvested and used to inoculate fresh MARC-145 (A) or ATCC CRL11171 (B). MARC-145 or ATCC CRL11171 cells were fixed at 48 h post-inoculation, and immunostained by IFA with anti-PRRSV N monoclonal antibody (SDOW17).

**FIG. 3.** Growth kinetics and characteristics of multiple envelope gene-shuffled chimeric viruses in MARC-145 and ATCC CRL11171 cells. The parental FV and the rescued shuffled chimeric viruses were passaged in MARC-145 or ATCC CRL11171 cells to P3, and the P3 virus was then used to infect fresh MARC-145 (A) or ATCC CRL11171 (B) cells at an MOI of 0.1. The culture supernatants were collected at indicated time points. Infectious viral titers were determined and calculated using the Reed-Muench method. Three independent experiments were carried out for each virus. The open symbols were used to
represent the chimeric viruses that were not selected for the subsequent challenge study. (C)

Peak virus titers in MARC-145 and ATCC CRL11171 cells.

FIG. 4. Kinetics of anti-PRRSV antibody response in pigs experimentally infected with each of the five shuffled chimeric viruses as well as the parental virus FV. The anti-PRRSV N antibody titers at indicated time points were detected using the IDEXX HerdChek® X3 ELISA kit. The level of antibody was expressed as a sample/positive (S/P) value ratio. The dash line shows the cutoff threshold (S/P value ≥0.4). Each plot represents the mean value of 3 pigs in one group at that time point.

FIG. 5. Neutralizing antibody (NA) titers induced by each of the five chimeric viruses in pigs against homologous and heterologous PRRSV strains. At 49 dpi, the sera from pigs experimentally infected with FV and the 5 chimeric viruses were collected to determine the NA titers using ATCC CRL11171 cells by SVN assay. The NA titers against parental strain FV (A), and heterologous strains VR2430 (B), VR2385 (C), NADC20 (D), and MN184B (E) were expressed as the highest dilution ($2^n$) that showed a 90% or above reduction in the number of fluorescent foci compared to that of serum from negative control group. The NA titers against FL12 were not shown because most samples had undetectable NA titers. (F) The composite NA titers against five heterologous virus strains (VR2430, VR2385, NADC20, MN184B, FL12). The composite titers were generated by combining the individual NA titers against five respective heterologous PRRSV strains. Three independent experiments were performed for each test, and the error bars indicate standard errors. The $P$ values are shown if
one shuffled virus group displayed significant higher NA titers than that of parental FV group (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

**FIG. 6. Kinetics of body temperature of pigs in the vaccine efficacy and challenge study.**

Pig body temperatures were measured every 2 days after challenge with NADC20 (A) or MN184B (B). The numbers in brackets represent the number of pigs which developed a body temperature above 104.5°F after challenge.

**FIG. 7. Macroscopic and microscopic lung lesion scores at 14 dpc.** At necropsy, the lung tissues were scored for macroscopic lesions by a veterinary pathologist (A, B). The lung tissues were also fixed in neutral formalin for histological examination of microscopic lung lesion scores by two independent veterinary pathologists and the mean of two scores were used as the final value (C, D). Each plot represents the value of one pig, and the error bars indicate standard errors. Significant differences are indicated with asterisks (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

**FIG. 8. Viral RNA loads in sera and lung tissues after challenge with two heterologous strains.** The PRRSV RNA copy numbers in sera at 7 dpc (A, D) and 14 dpc (B, E), and in lung tissues at 14 dpc (C, F) were determined using quantitative RT-PCR. The detection limit is 1000 (3 log10) RNA copies per ml or gram. The samples under detection limit were considered as negative, and calculated as 2 log10 copy number. Each sample was tested in three separate reactions. Each plot represents the mean viral RNA copy number of three
separate tests of one pig, and the error bars indicate standard errors. Significant differences are indicated with asterisks (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).
FIG 2

A

FV
FV-SPDS
FV-SPDS-FV5
FV-SPDS-FV25
FV-SPDS-VR2
FV-SPDS-VR5
Mock

B

FV
FV-SPDS
FV-SPDS-FV5
FV-SPDS-FV25
FV-SPDS-VR2
FV-SPDS-VR5
Mock
FIG 3

A. MARC-145

B. ATCC CRL11171

C. Virus peak titer (log₁₀ TCID₅₀/ml)

Virus titer (log₁₀ TCID₅₀/ml)

Virus titer (log₁₀ TCID₅₀/ml)

Virus peak titer (log₁₀ TCID₅₀/ml)
FIG 5

A. NA against FV
B. NA against VR2430
C. NA against VR2385
D. NA against NADC20
E. NA against MN184B
F. Composite NA titer against heterologous strains
**FIG 6**

A. **NADC20 challenge**

- PBS/PBS: (0/8)
- PBS: (7/8)
- FV: (2/8)
- FV-SPDS-VR2: (1/8)
- FV-SPDS-VR5: (2/8)

B. **MN184B challenge**

- PBS/PBS: (0/8)
- PBS: (4/8)
- FV: (6/8)
- FV-SPDS-VR2: (2/7)
- FV-SPDS-VR5: (3/8)
FIG 8

A) NADC20 challenge

B) NADC20 challenge

C) NADC20 challenge

D) MN184B challenge

E) MN184B challenge

F) MN184B challenge

7 dpc

14 dpc

14 dpc

PBS/PBS
PBS
FV
FV-SPDS-VR2
FV-SPDS-VR5

P=0.0841
P=0.1058
*** P=0.0006
** P=0.0073
* P=0.0141

P=0.3558
P=0.0621
* P=0.0173
P=0.0663
* P=0.0244

P=0.4804
* P=0.0414
* P=0.0194
* P=0.0166
* P=0.0145

P=0.4614
P=0.2785
* P=0.0297
P=0.1124
* P=0.0311

P=0.3093
P=0.2398
*** P=0.0004
* P=0.0125
** P=0.0047

P=0.1175
P=0.3382
P=0.1427
P=0.3661
P=0.4331
P=0.1427

log_{10} RNA copy number/ml serum

log_{10} RNA copy number/ml serum

log_{10} RNA copy number/gram lung

log_{10} RNA copy number/gram lung

log_{10} RNA copy number/ml serum

log_{10} RNA copy number/ml serum

log_{10} RNA copy number/gram lung