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ABSTRACT

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*

130 *vivo*. Both major and minor envelope proteins of PRRSV play important roles in inducing
131 protective immune responses, in the present study we hypothesized that combining the
132 shuffled chimeric sequences of the envelope genes from each of the four single-gene shuffled
133 chimeric viruses into one single mosaic virus would induce maximal cross-protection against
134 heterologous strains. To achieve this objective, we integrated the chimeric sequences of all
135 shuffled envelope protein genes into a single PRRSV strain in the genomic backbone of the
136 vaccine virus Fostera® PRRS (FV). Five full-length chimeric plasmid clones (FV-SPDS,
137 FV-SPDS-FV5, FV-SPDS-FV25, FV-SPDS-VR2, and FV-SPDS-VR5) were constructed
138 (Fig. 1), and the authenticity of these chimeric clones was verified by DNA sequencing of the
139 final constructs.

140 Following transfection of the full-length chimeric clones into BHK-21 cells, supernatants
141 were harvested after two days (P0 virus) and used to inoculate fresh MARC-145 cells. At 3 to
142 4 days post-inoculation, CPE was observed in inoculated MARC-145 cells, which were
143 subsequently confirmed by IFA using PRRSV N-specific monoclonal antibody, indicative of
144 the production of viable progeny virions and rescue of infectious chimeric viruses (Fig. 2A).
145 To further confirm that the rescued progeny viruses originated from the respective transfected
146 chimeric virus clones, the ORFs 2-6 were amplified from each of the P3 viruses by RT-PCR
147 and sequenced. Sequence analyses revealed that the ORFs 2-6 of each rescued progeny virus
148 contained the shuffled ORFs 2-6 gene sequences, as originally engineered at the level of the
149 full-length virus clones. The results demonstrated the successful generation of viable
150 chimeric virus progeny with multiple shuffled envelope genes.
151

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 (≈$2.5\times10^{6}$$TCID_{50}$/ml), while the FV-SPDS (≈$4.0\times10^{5}$$TCID_{50}$/ml) and FV-SPDS-VR2 (≈$6.3\times10^{5}$$TCID_{50}$/ml) had slightly lower peak titers (<1 log10) compared to FV. The chimera FV-SPDS-VR2 showed an accelerated replication rate, reaching the peak virus titer earlier (≈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.

174 3A, 3B). Similarly, we observed that the chimera FV-SPDS-FV25 displayed an impaired
175 growth whereas chimera FV-SPDS-VR2 had an accelerated growth rate. Collectively, the
176 results showed that these multiple envelope gene-shuffled chimeric viruses replicated in both
177 MARC-145 and ATCC CRL11171 cells to relatively high titers that are sufficient for use as a
178 MLV.
179
180 *Two shuffled chimeric viruses, FV-SPDS-VR2 and FV-SPDS-VR5, induced cross-neutralizing*
181 *antibodies against heterologous virus strains*
182 To screen for potential viable candidate vaccine strains, we conducted a small pilot animal
183 study by experimentally infecting groups of 3 pigs with one of the five shuffled chimeric
184 viruses or the parental virus to generate antisera specific to each virus. The six groups of pigs
185 inoculated with shuffled viruses or parental virus seroconverted within 14 or 21 dpi and
186 remained seropositive through the end of the study (Fig. 4), while the negative control pigs
187 remained seronegative, indicating that all the five multiple envelope gene-shuffled viruses
188 replicated *in vivo* and elicited immune responses in pigs. One pig in FV group had a very low
189 S/P value compared to the other two pigs, and thus making the overall mean value of FV
190 group lower than other groups. This was likely due to the individual difference caused by the
191 small numbers of pigs (3) used in this pilot study.
192 To assess whether the shuffled chimeric viruses can induce cross-neutralizing antibodies
193 against heterologous PRRSV strains, an SVN assay was performed using serum samples
194 collected at 49 dpi from pigs against parental virus FV as well as five heterologous PRRSV
195 strains belonging to different genetic lineages (Zhou et al., 2013; Zhou et al., 2012). In the

196 SVN assay against the parental virus FV (Fig. 5A), the mean NA titers of the FV-SPDS-FV5
197 (5.6) and FV-SPDS-FV25 (5.2) groups were slightly lower than that of the FV group (6.0),
198 while the NA titers of the FV-SPDS, FV-SPDS-VR2, FV-SPDS-VR5 groups were much
199 lower than that of the FV group, likely due to the fact that these three shuffled chimeric
200 viruses contain less envelope protein sequences derived from FV.

201 For the SVN against the heterologous strain VR2430 (Fig. 5B), most shuffled virus groups
202 reached mean titers of about 4.0, but there was no significant difference compared to the FV
203 group (4.1). However, the mean NA titer of the FV-SPDS-FV25 group (2.6) was significantly
204 lower than that of the FV group (P=0.0018). For the SVN against PRRSV strain VR2385, a
205 different sublineage of lineage 5 relative to strain VR2430 (Fig. 5C), the FV-SPDS-VR2 and
206 FV-SPDS-VR5 groups had mean NA titers of 1.7 and 3.1, respectively, while the other
207 groups displayed low (<1.0) or undetectable titers. For SVN against the heterologous strain
208 NADC20 (Fig. 5D), the mean NA titers of the FV-SPDS group (2.0) and FV-SPDS-VR2
209 group (4.0) were significantly higher than that of the FV group (0.8). For SVN against the
210 heterologous strain MN184B (Fig. 5E), all groups had low (<1.0) or undetectable NA titers.
211 For SVN against the heterologous strain FL12, most serum samples had undetectable NA
212 titers (data not shown).

213 The composite NA titers were generated by combining the individual NA titers against five
214 respective heterologous strains (VR2430, VR2385, NADC20, MN184B, FL12), and analyzed
215 to evaluate the cross-neutralizing ability for each of the five chimeric viruses (Fig. 5F).
216 Chimeras FV-SPDS-VR2 (P=0.0115) and FV-SPDS-VR5 (P=0.0057) inoculated pigs
217 displayed significantly higher NA titers against heterologous PRRSV strains compared to FV

218 inoculated pigs, although the two groups had relatively large error bars due to different titers
219 against different heterologous strain. This suggested that two of shuffled viruses,
220 FV-SPDS-VR2 and FV-SPDS-VR5, had an improved ability to induce cross-neutralizing
221 antibodies *in vitro* against heterologous PRRSV strains.
222
223 *One shuffled chimeric virus, FV-SPDS-VR2, conferred cross-protection against challenge*
224 *with heterologous PRRSV strains*
225 Since two of the shuffled chimeric viruses, FV-SPDS-VR2 and FV-SPDS-VR5, induced
226 cross-neutralizing antibodies, these two chimeric viruses were subsequently selected for a
227 vaccination/challenge efficacy study in pigs (Table 1). Two heterologous strains NADC20
228 (lineage 9) and MN184B (lineage 1) were used as challenge viruses. When challenged with
229 NADC20 or MN184B, 7 pigs in the non-vaccinated/NADC20 challenged group and 4 pigs in
230 the non-vaccinated/MN184B challenged group developed high body temperatures (>104.5°F)
231 (Fig. 6). However, only 1-2 pigs in the shuffled chimeric virus- or parental FV-vaccinated
232 groups developed high temperatures when challenged with NADC20 (Fig. 6A). Only 2 pigs
233 in the FV-SPDS-VR2-vaccinated group and 3 pigs in the FV-SPDS-VR5-vaccinated group
234 developed fevers after challenge with MN184B, while 6 pigs displayed fevers in the
235 FV-vaccinated/MN184B-challenged group (Fig. 6B).
236 At necropsy, both FV- and FV-SPDS-VR2-vaccinated groups showed significantly
237 decreased gross lung lesion scores compared to the non-vaccinated control group when
238 challenged with either NADC20 or MN184B (Fig. 7A, 7B). For the NADC20 challenge, the
239 FV-vaccinated group had a lower mean gross lung lesion score than the FV-SPDS-VR2- and

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 (6.3×10^4 copies/ml) was numerically lower than that of the FV-vaccinated group (2.0×10^6 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 (1.3×10^5 copies/ml) and FV-SPDS-VR2 (5.0×10^4 copies/ml) vaccinated groups were significantly decreased when compared to the non-vaccinated control group (5.0×10^6 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,

2013). 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

306 gene-shuffled viruses. Therefore, in this present study, by utilizing an infectious clone of the
307 commercial vaccine Fostera® PRRS as the genomic backbone, we integrated the shuffled
308 chimeric sequences from each of the single envelope gene-shuffled viruses (GP3TS22,
309 GP4TS14, DS722, and MTS57) into one composite virus containing multiple shuffled
310 envelope genes in various combinations. We successfully constructed and rescued five viable
311 multiple envelope gene-shuffled chimeric viruses that could serve as potential vaccine
312 candidates (Fig. 1). The growth kinetics and cross-neutralizing activities of the five chimeric
313 viruses, and the heterologous cross-protective ability and vaccine efficacy of the two most
314 promising chimeric viruses were investigated in this study.

315 The five rescued chimeric viruses containing multiple shuffled envelope genes in different
316 combinations (Fig. 1) replicated well in two cell lines, MARC-145 and ATCC CRL11171,
317 suggesting that the gene shuffling did not significantly impair the growth of most of the
318 resulting chimeric viruses. The shuffled chimeric viruses caused PRRSV-specific CPE and
319 produced high virus yields (up to 10^6 $TCID_{50}$/ml) in both MARC-145 and ATCC CRL11171
320 cells, thus making these chimeric viruses viable candidates for further development into
321 MLVs.

322 Importantly, we demonstrated that these chimeric viruses induced robust immune
323 responses in pigs as evidenced by the detection of high levels of anti-PRRSV N antibodies in
324 infected pigs (Fig. 4). Most of the NA titers tested in this study were consistent with our
325 previous studies, suggesting a good reproducibility of the SVN assay (Zhou et al., 2013;
326 Zhou et al., 2012). Chimera FV-SPDS-VR5 induced significantly higher cross-neutralizing
327 antibodies against heterologous strain VR2385, and chimera FV-SPDS-VR2 induced

328 significantly higher cross-neutralizing antibodies against NADC20. However, against the
329 heterologous strains MN184B and FL12, the NA titers of the parental virus FV and all the 5
330 chimeric viruses were very low (<1), and this was somewhat in contrast to our previous
331 studies in which most NA titers were above 1 (Zhou et al., 2013; Zhou et al., 2012). We
332 speculated that this was largely due to the fact that the virulent strain VR2385 backbone,
333 which was used in our previous studies, can elicit higher levels of neutralizing antibodies
334 against MN184B and FL12 than the vaccine virus FV backbone used in this present study.
335 The data also suggested that the nonstructural proteins may also play roles in inducing NAs,
336 and thus should be considered in the future vaccine design. In general, the NA titer against a
337 particular virus strain was related to the sequence components of the shuffled envelope
338 proteins. For example, compared to other shuffled viruses, FV-SPDS-FV5- and
339 FV-SPDS-FV25-infected pigs developed higher NA titers against FV because the GP2 and/or
340 GP5 of the FV-SPDS-FV5 and FV-SPDS-FV25 viruses were derived from FV (Fig. 1, 5A).
341 FV-SPDS-VR2- and FV-SPDS-VR5-infected pigs had higher NA titers against VR2385
342 because the GP2 or GP5 of the FV-SPDS-VR2 and FV-SPDS-VR5 viruses were derived
343 from the VR2385 strain (Fig. 1, 5C), indicating that GP2 as well as GP5 are important in
344 inducing neutralizing antibody, as GP2 is one of the viral attachment proteins interacting with
345 cellular receptor CD163 (Das et al., 2010).

346 Although the role of NAs in PRRSV protection is somewhat controversial, the NA titer is
347 still an important criterion used by many research groups in evaluating candidate vaccine
348 efficacy, especially for rapid screening of potential vaccine candidates for subsequent
349 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

372 provided good protection against heterologous PRRSV strain NADC20 challenge, and it can
373 be hypothesized that the chimera FV-SPDS-VR2 may be well suited to control NADC20
374 viremia by virtue of its NADC20 genetic components.

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

393 In summary, in this study we successfully generated five multiple envelope genes-shuffled

394 chimeric viruses in the genomic backbone of a commercial PRRSV vaccine virus FV. The
395 rescued chimeric viruses replicated well in two cell lines and produced robust immune
396 responses in pigs. One shuffled chimeric virus, FV-SPDS-VR2, and its parental FV induced
397 partial cross-protection when challenged with two heterologous strains NADC20 and
398 MN184B. We demonstrated in this study that simultaneous multiple envelope gene-shuffling
399 of PRRSV is a viable approach to generate potential vaccine candidates that possess
400 cross-protective ability and may lead to novel vaccines with enhanced cross-protection
401 against diverse PRRSV strains.
402

403 **Materials and methods**

404 *Cells and viruses*

405 BHK-21 cells were cultured in Dulbecco's Minimal Essential Medium (DMEM)
406 supplemented with 10% fetal bovine serum (FBS). Two different clones of monkey kidney
407 cell line MA104, MARC-145 and ATCC CRL11171 (Halbur et al., 1995; Kim et al., 1993;
408 Meng et al., 1996), were cultured in low glucose-supplemented DMEM with 10% FBS, and
409 maintained in low glucose DMEM with 2% FBS for virus propagation. The cells were
410 cultured in an incubator at 37°C with 5% CO_2. An infectious cDNA clone of the commercial
411 vaccine virus Fostera® PRRS in a DNA-launched plasmid format (pFV) was chemically
412 synthesized based on the complete genome sequence of the vaccine virus. As a positive
413 control, Fostera-like virus (FV) was generated by direct transfection of cells with pFV, and
414 evaluated in parallel with gene-shuffled chimeric viruses. Fostera® PRRS is derived from
415 strain P129 (Accession no. AF494042), which is a lineage 8 PRRSV based on its ORF5

416 sequence. PRRSV strains from distinct genetic lineages of genotype 2 including VR2430
417 (lineage 5, accession no. JX050225), VR2385 (lineage 5, accession no. JX044140), NADC20
418 (lineage 9, accession no. JX069953), MN184B (lineage 1, accession no. DQ176020), and
419 FL12 (lineage 8, accession no. AY545985) were propagated, titrated and stored at -80°C
420 before use (Ni et al., 2013; Shi et al., 2010b).
421
422 *Construction of chimeric virus clones containing shuffled multiple envelope genes*
423 In our previous studies, based on the genomic backbone of the PRRSV strain VR2385, we
424 generated several single envelope gene-shuffled chimeric viruses that possess *in vitro*
425 cross-neutralizing activities against heterologous PRRSV strains or *in vivo* attenuated
426 pathogenicity: GP3TS22 (shuffled ORF3), GP4TS14 (shuffled ORF4), DS722 (shuffled
427 ORF5), and MTS57 (shuffled ORF6). In order to design a vaccine with maximal
428 cross-protection against heterologous strains, we incorporated, in different combinations, the
429 shuffled multiple envelope genes into the genomic backbone of the vaccine virus infectious
430 clone pFV. Briefly, based on the envelope gene sequences of the four single gene-shuffled
431 chimeric viruses (GP3TS22, GP4TS14, DS722, and MTS57) and the predicted structural
432 topology of the PRRSV envelope proteins (Dokland, 2010), we designed and synthesized a
433 nucleotide sequence fragment, designated SPDS, which contains the ORFs 2 through 6. For
434 the SPDS sequence, the GP2 ectodomain-encoding region was derived from strain MN184B,
435 GP3 from chimera GP3TS22, GP4 from chimera GP4TS14, and GP5 from chimera DS722.
436 The entire M sequence was derived from chimera MTS57. The non-ectodomain regions of
437 GP2 through 5 were from pFV. The overlapping sequences of ORFs 2-3 were derived from

438 chimera GP3TS22, and the overlapping sequences of ORFs 3-4 were from chimera GP4TS14.
439 The genomic organizations of the chimeric virus constructs are depicted in Fig. 1.
440 By using the *Afe* I and *Spe* I restriction enzyme sites engineered in the synthesized
441 fragment SPDS, the ORFs 2-6 were introduced into the genomic backbone of pFV, to
442 generate the chimeric virus designated FV-SPDS. Subsequently, based on the chimeric clone
443 FV-SPDS, we constructed four other chimeric virus clones: FV-SPDS-FV5 (ORF5 derived
444 from FV), FV-SPDS-FV25 (ORF2 and ORF5 from FV), FV-SPDS-VR2 (ORF2 from
445 VR2385), and FV-SPDS-VR5 (ORF5 from VR2385) through fusion PCR as described
446 elsewhere (Ni et al., 2013; Tian et al., 2012). Briefly, two flanking fragments amplified from
447 pFV were fused to the corresponding target ORFs to form hybrid fragments. By utilizing the
448 restriction enzyme sites in the flanking fragments, the hybrid fragments were then cloned into
449 the pFV backbone to generate the four chimeric virus clones (Fig. 1). All the chimeric virus
450 constructs were verified by nucleotide sequencing.
451
452 *In vitro transfection to rescue chimeric viruses*
453 Plasmid DNAs from full-length DNA-launched chimeric virus infectious clones were
454 isolated using the QIAprep Spin Miniprep kit, and quantified using Nanodrop. Fresh BHK-21
455 cells in a 6-well plate at approximately 60–80% confluency were transfected with 2 μg of
456 plasmid DNA per well using Lipofectamine LTX and Plus Reagent kit (Invitrogen) according
457 to the manufacturer's instructions, followed by incubation at 37°C with 5% CO_2. At 48 h
458 post-transfection, cell culture supernatants were harvested and designated as passage 0 (P0)
459 viruses.

460

461 *Indirect immunofluorescence assay (IFA)*

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

470

471 *Virus growth kinetics assay*

472 To investigate the growth properties of the shuffled chimeric viruses in MARC-145 and
473 ATCC CRL11171 cells, a multiple-step growth curve analysis was conducted. Briefly,
474 MARC-145 or ATCC CRL11171 cells in 6-well plates were infected with each of the
475 shuffled chimeric viruses as well as parental FV virus (passage P3) at a low multiplicity of
476 infection (MOI) of 0.1. At 12, 24, 36, 48, 60, 72, 84, 96, 108, and 120 h post-infection (hpi),
477 200 µl of each cell culture supernatant was collected, and wells were replenished with the
478 same volume of fresh culture medium. Virus titrations were performed in MARC-145 or
479 ATCC CRL11171 cells in 96-well plates with fresh cells that were inoculated with 10-fold
480 serial virus dilutions (4 replicates per dilution, 100 µl/well) for 1 h, after which the cells were
481 washed with PBS and then incubated in low glucose DMEM with 2% FBS in a humidified

482 CO_2 incubator. Presence of a cytopathic effect (CPE) was determined at 7 days
483 post-inoculation. Viral titers were calculated using the Reed-Muench method and expressed
484 as the 50% tissue culture infectious dose per milliliter ($TCID_{50}$/ml). Three independent
485 experiments were carried out for each virus.
486
487 *Experimental design for a small pilot chimeric virus infection study in pigs to generate*
488 *antibodies against each of the five chimeric viruses*
489 In order to screen for potential candidate viable chimeric virus strains for the subsequent
490 vaccine efficacy testing, and to determine the viability of the multiple envelope gene-shuffled
491 chimeric viruses in pigs, we conducted a small pilot *in vivo* pig infection study with the 5
492 chimeric viruses (FV-SPDS, FV-SPDS-FV5, FV-SPDS-FV25, FV-SPDS-VR2, and
493 FV-SPDS-VR5). A total of 21 pigs were divided into 7 groups of 3 pigs each, and each group
494 was inoculated with one of the 5 chimeras. Weekly serum samples were collected from each
495 pig for a total of 7 weeks. The sera were tested for PRRSV neutralizing antibodies against
496 homologous and heterologous strains as well as PRRSV antibody responses using the IDEXX
497 HerdChek® X3 ELISA kit according to the protocol provided by the manufacturer. All
498 experiments involving animals were conducted in compliance with national legislation and
499 subject to review by both Virginia Tech and Iowa State University Institutional Animal Care
500 and Use Committee (IACUC).
501
502 *Serum virus neutralization assay to evaluate cross-neutralizing activities*
503 The neutralizing antibody (NA) titers against homologous and heterologous PRRSV strains

504 were determined by a serum virus neutralization (SVN) assay essentially as previously
505 described (Zhou et al., 2012). Briefly, 2-fold diluted serum samples collected at 49 days
506 post-inoculation (dpi) from each pig were mixed with an equal volume of individual
507 homologous (FV) or heterologous (VR2430, VR2385, NADC20, MN184B and FL12) virus
508 at an infectious titer of 2×10^3 $TCID_{50}$/ml and incubated at 37°C for 1 h. The mixtures were
509 then dispensed onto ATCC CRL11171 cells in 96-well plates and incubated for 1 h at 37°C.
510 After washing with PBS, the cells were maintained in DMEM with 2% FBS. At
511 approximately 20 hpi, the cells were assayed by IFA for virus infection. The NA titers were
512 expressed as the highest dilution that showed at least 90% reduction in the number of
513 fluorescent foci compared to antisera from negative control pigs. Three independent tests
514 were performed for each serum sample.
515
516 *Experimental design for a vaccine efficacy and cross-protection study in pigs*
517 Based on the cross-neutralizing activities of the sera from pigs infected with each of the 5
518 chimeric viruses, we selected two chimeric viruses (FV-SPDS-VR2 and FV-SPDS-VR5) for
519 a vaccine efficacy study in pigs. Briefly, a total of 72 PRRSV-negative piglets at 3 weeks of
520 age were divided into 9 groups of 8 piglets per group. Piglets in each group were vaccinated
521 with one of the two shuffled chimeric viruses (FV-SPDS-VR2, or FV-SPDS-VR5), parental
522 virus (FV) derived from the pFV infectious clone, or PBS as shown in Table 1. Serum
523 samples were collected from each pig prior to vaccination and weekly thereafter. At 42 days
524 post-vaccination, the pigs were challenged with two heterologous virus strains NADC20 or
525 MN184B as shown in Table 1. At 14 days post-challenge (dpc), all pigs were necropsied for

526 gross pathological and histopathological lesion evaluation. Serum samples at 7 dpc and 14
527 dpc as well as the samples of lung tissues at 14 dpc were used to quantify PRRSV viral RNA
528 loads. The body temperatures from all pigs were also monitored every 2 days after challenge.
529
530 *Quantitation of viral RNA loads in sera and lung tissues*
531 Viral RNAs were extracted from serum samples at 7 and 14 dpc using ZR Viral RNA kit
532 (ZYMO RESEARCH, USA) according to the protocol provided by the manufacturer. Total
533 RNAs from samples of lung tissues were extracted using TRI Reagent (MRC). The RNA
534 standard used for the RT-qPCR was derived from *in vitro* transcription of a PRRSV
535 full-length cDNA clone pACYC-VR2385 by mMESSAGE mMACHINE T7 kit (Ambion).
536 The PRRSV RNA copy numbers in sera or lung tissues were quantified by a SYBR
537 green-based quantitative PCR using SuperScript III Platinum SYBR Green One-Step
538 RT-qPCR kit (Invitrogen) with a protocol recommended by the manufacturer. The primer set
539 (realtime2F/2R, binds to the conserve region of ORF7) used in the RT-qPCR assay was
540 previously validated elsewhere (Ni et al., 2013; Ni et al., 2014). The RT-qPCR assay was
541 conducted in a CFX96 real-time (RT) PCR system (Bio-Rad). Each reaction was performed
542 in triplicate.
543
544 *Gross pathology and histopathology evaluation*
545 All pigs were humanely euthanized by intravenously overdose injection of pentobarbital
546 (Fatal-Plus, Vortech Pharmaceutical Ltd., Dearborn, MI). At necropsy, the lungs were
547 evaluated for gross pathology, and subsequently five sections of lung tissues were collected,

548 fixed in 10% neutral-buffered formalin and processed for histopathology evaluation. Fresh
549 lung tissues were also collected and stored at -80°C for quantification of viral RNA loads by
550 RT-qPCR.

551 The criteria for evaluating the gross pathology and histopathology have been well
552 established and described previously (Halbur et al., 1995; Ni et al., 2013). For gross
553 pathology evaluation, the total amount of lung lesions affected by pneumonia of each pig
554 (0-100% of the lung affected by visible pneumonia) was scored by a veterinary pathologist
555 (PGH) who was blinded to the treatment status of pigs. The scoring system is based on the
556 approximate volume that each lung lobe contributes to the entire lung: the right cranial lobe
557 (10%), right middle lobe (10%), cranial part of the left cranial lobe (10%), caudal part of the
558 left cranial lobe (10%), the accessory lobe (5%), and right and left caudal lobes (27.5% each).
559 The microscopic lung lesions were evaluated and scored independently by two veterinary
560 pathologists (TO and PGH) who were blinded to the treatment status. The scores based on the
561 presence and severity of interstitial pneumonia ranging from 0 to 6 (0, normal; 1, mild
562 multifocal; 2, mild diffuse; 3, moderate multifocal; 4, moderate diffuse; 5, severe multifocal;
563 6, severe diffuse). The mean of the two scores obtained for each pig was used as the final
564 value.

565

566 *Statistical analyses*

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

569

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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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719

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

Group	No. of pigs	Vaccination at 0 dpi ($1.0\times10^{4.0}$ $TCID_{50}$/pig) with	Challenge at 42 dpi ($1.0\times10^{5.0}$ $TCID_{50}$/pig) with	No. of pigs at necropsy (14dpc)
1	8	FV-SPDS-VR2	NADC20	8
2	8	FV-SPDS-VR2	MN184B	7[a]
3	8	FV-SPDSVR5	NADC20	8
4	8	FV-SPDSVR5	MN184B	8
5	8	FV	NADC20	8
6	8	FV	MN184B	8
7	8	PBS	NADC20	8
8	8	PBS	MN184B	8
9	8	PBS	PBS	8

721 [a] One piglet died from an unrelated cause before challenge.

722

723 **Figure captions**

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

730

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

737

738 **FIG. 3. Growth kinetics and characteristics of multiple envelope gene-shuffled chimeric**
739 **viruses in MARC-145 and ATCC CRL11171 cells**. The parental FV and the rescued
740 shuffled chimeric viruses were passaged in MARC-145 or ATCC CRL11171 cells to P3, and
741 the P3 virus was then used to infect fresh MARC-145 (A) or ATCC CRL11171 (B) cells at
742 an MOI of 0.1. The culture supernatants were collected at indicated time points. Infectious
743 viral titers were determined and calculated using the Reed-Muench method. Three
744 independent experiments were carried out for each virus. The open symbols were used to

745 represent the chimeric viruses that were not selected for the subsequent challenge study. (C)
746 Peak virus titers in MARC-145 and ATCC CRL11171 cells.
747
748 **FIG. 4. Kinetics of anti-PRRSV antibody response in pigs experimentally infected with**
749 **each of the five shuffled chimeric viruses as well as the parental virus FV**. The
750 anti-PRRSV N antibody titers at indicated time points were detected using the IDEXX
751 HerdChek® X3 ELISA kit. The level of antibody was expressed as a sample/positive (S/P)
752 value ratio. The dash line shows the cutoff threshold (S/P value ≥0.4). Each plot represents
753 the mean value of 3 pigs in one group at that time point.
754
755 **FIG. 5. Neutralizing antibody (NA) titers induced by each of the five chimeric viruses in**
756 **pigs against homologous and heterologous PRRSV strains**. At 49 dpi, the sera from pigs
757 experimentally infected with FV and the 5 chimeric viruses were collected to determine the
758 NA titers using ATCC CRL11171 cells by SVN assay. The NA titers against parental strain
759 FV (A), and heterologous strains VR2430 (B), VR2385 (C), NADC20 (D), and MN184B (E)
760 were expressed as the highest dilution (2^n) that showed a 90% or above reduction in the
761 number of fluorescent foci compared to that of serum from negative control group. The NA
762 titers against FL12 were not shown because most samples had undetectable NA titers. (F)
763 The composite NA titers against five heterologous virus strains (VR2430, VR2385, NADC20,
764 MN184B, FL12). The composite titers were generated by combining the individual NA titers
765 against five respective heterologous PRRSV strains. Three independent experiments were
766 performed for each test, and the error bars indicate standard errors. The *P* values are shown if

767 one shuffled virus group displayed significant higher NA titers than that of parental FV group
768 (* P<0.05, ** P<0.01, *** P<0.001).
769
770 **FIG. 6. Kinetics of body temperature of pigs in the vaccine efficacy and challenge study.**
771 Pig body temperatures were measured every 2 days after challenge with NADC20 (A) or
772 MN184B (B). The numbers in brackets represent the number of pigs which developed a body
773 temperature above 104.5°F after challenge.
774
775 **FIG. 7. Macroscopic and microscopic lung lesion scores at 14 dpc**. At necropsy, the lung
776 tissues were scored for macroscopic lesions by a veterinary pathologist (A, B). The lung
777 tissues were also fixed in neutral formalin for histological examination of microscopic lung
778 lesion scores by two independent veterinary pathologists and the mean of two scores were
779 used as the final value (C, D). Each plot represents the value of one pig, and the error bars
780 indicate standard errors. Significant differences are indicated with asterisks (* P<0.05, **
781 P<0.01, *** P<0.001).
782
783 **FIG. 8. Viral RNA loads in sera and lung tissues after challenge with two heterologous**
784 **strains**. The PRRSV RNA copy numbers in sera at 7 dpc (A, D) and 14 dpc (B, E), and in
785 lung tissues at 14 dpc (C, F) were determined using quantitative RT-PCR. The detection limit
786 is 1000 (3 log10) RNA copies per ml or gram. The samples under detection limit were
787 considered as negative, and calculated as 2 log10 copy number. Each sample was tested in
788 three separate reactions. Each plot represents the mean viral RNA copy number of three

789 separate tests of one pig, and the error bars indicate standard errors. Significant differences
790 are indicated with asterisks (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

Fig. 1

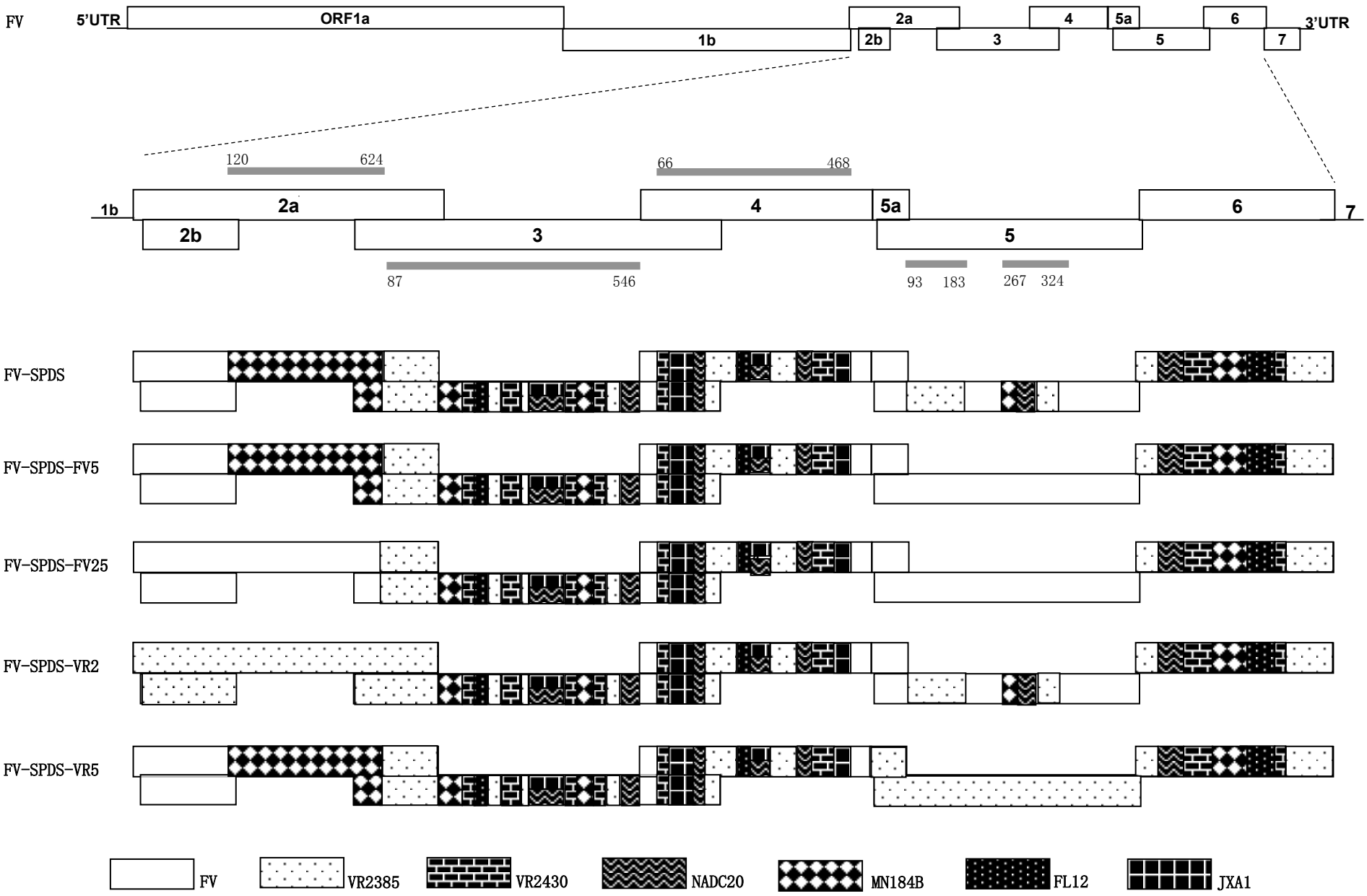

FV
5'UTR
ORF1a
1b
2a
2b
3
4
5a
5
6
7
3'UTR
120
624
66
468
87
546
93
183
267
324
FV-SPDS
FV-SPDS-FV5
FV-SPDS-FV25
FV-SPDS-VR2
FV-SPDS-VR5
FV
VR2385
VR2430
NADC20
MN184B
FL12
JXA1

FIG 2

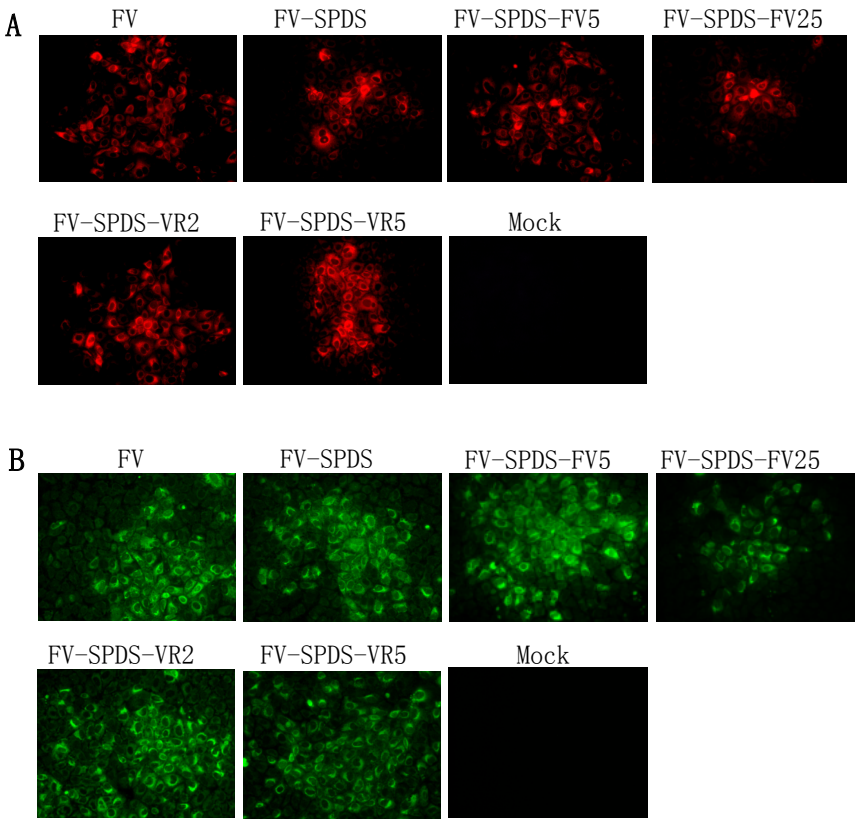

A
FV
FV-SPDS
FV-SPDS-FV5
FV-SPDS-FV25
FV-SPDS-VR2
FV-SPDS-VR5
Mock
B
FV
FV-SPDS
FV-SPDS-FV5
FV-SPDS-FV25
FV-SPDS-VR2
FV-SPDS-VR5
Mock

FIG 3

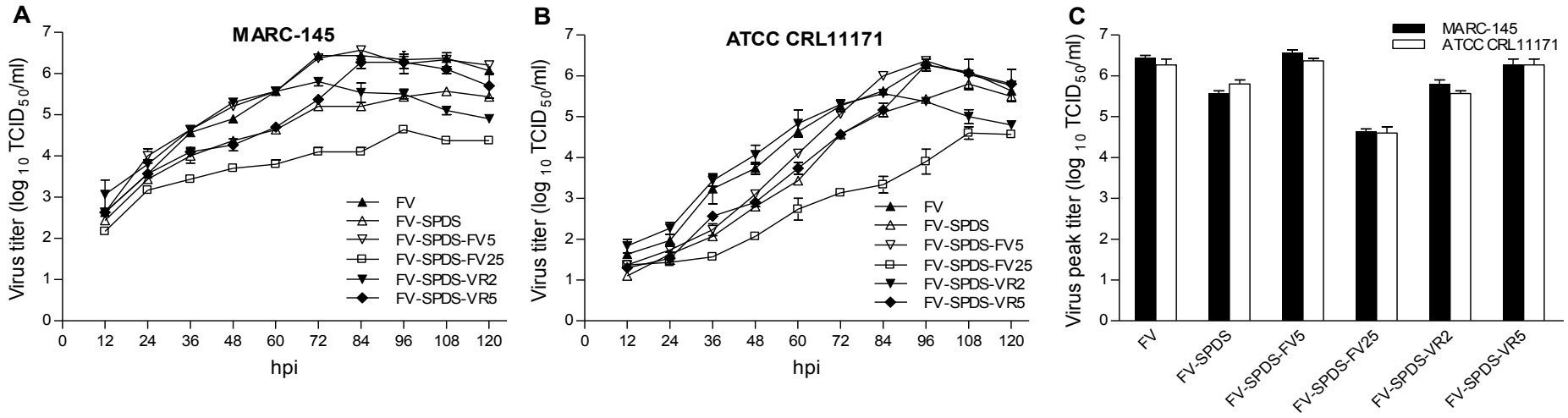

A
MARC-145
Virus titer (log 10 TCID50/ml)
hpi
FV
FV-SPDS
FV-SPDS-FV5
FV-SPDS-FV25
FV-SPDS-VR2
FV-SPDS-VR5
B
ATCC CRL11171
C
MARC-145
ATCC CRL11171
Virus peak titer (log 10 TCID50/ml)

FIG 4

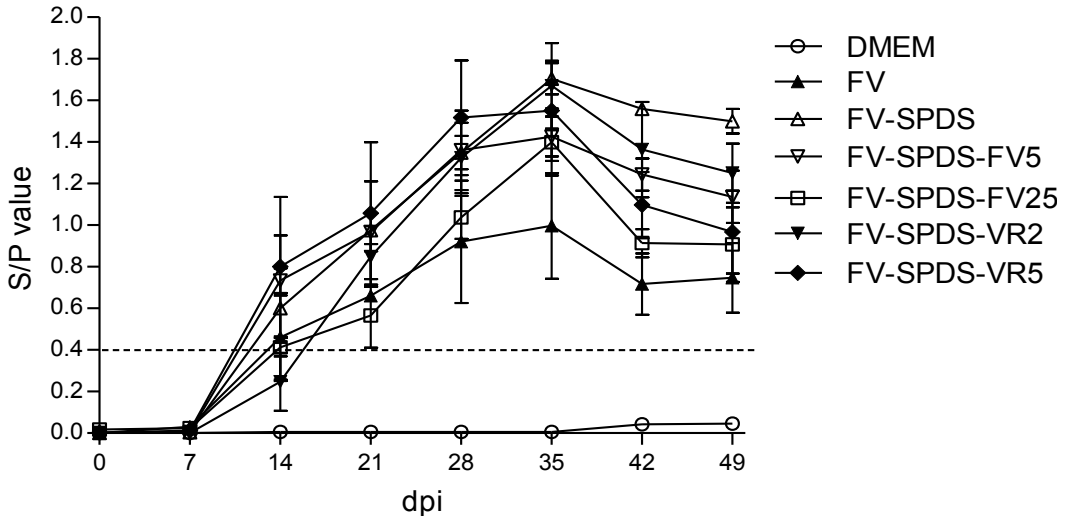

S/P value
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
7
14
21
28
35
42
49
dpi
DMEM
FV
FV-SPDS
FV-SPDS-FV5
FV-SPDS-FV25
FV-SPDS-VR2
FV-SPDS-VR5

FIG 5

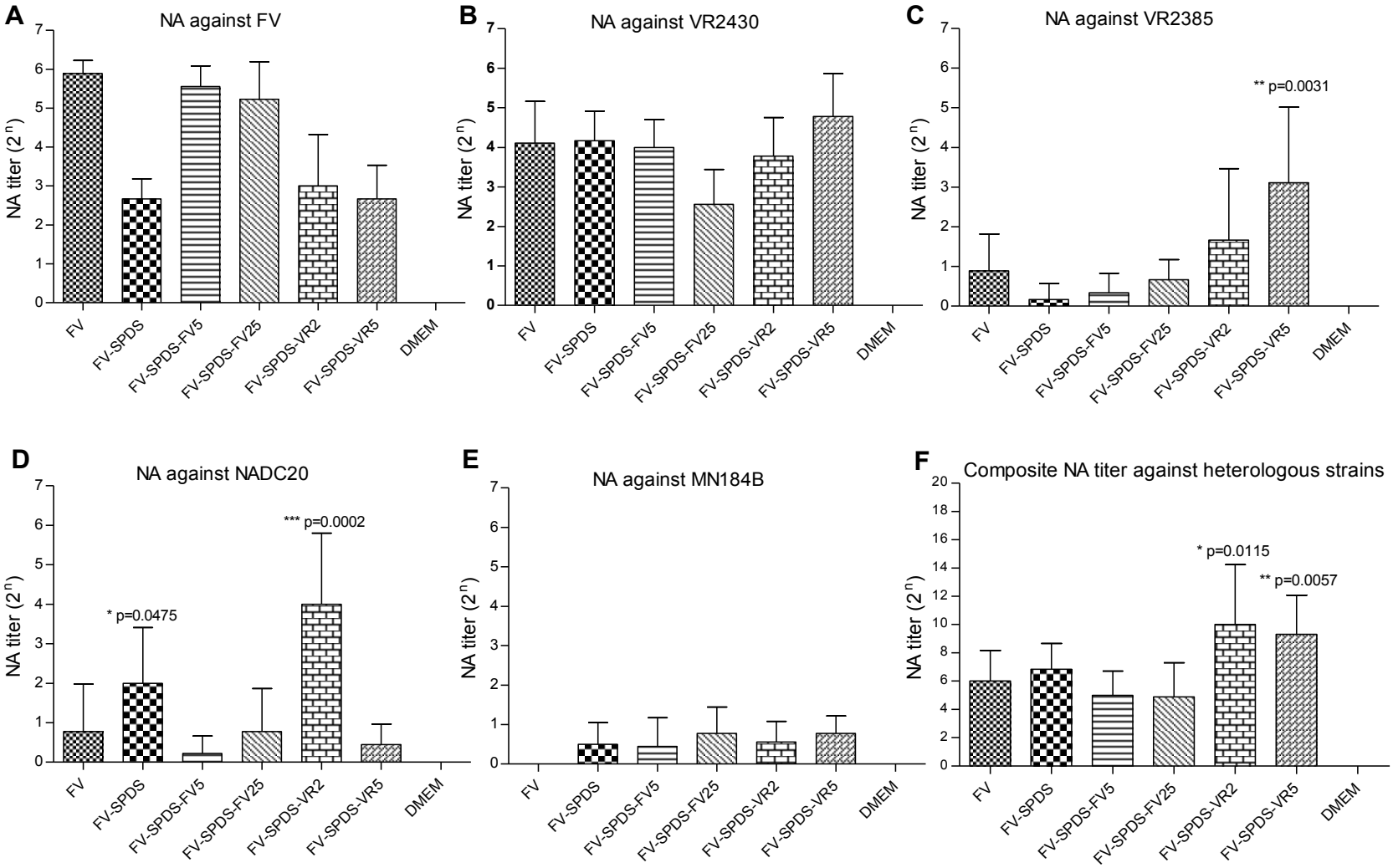

A
NA against FV
NA titer (2^n)
B
NA against VR2430
C
NA against VR2385
** p=0.0031
D
NA against NADC20
* p=0.0475
*** p=0.0002
E
NA against MN184B
F
Composite NA titer against heterologous strains
* p=0.0115
** p=0.0057
FV
FV-SPDS
FV-SPDS-FV5
FV-SPDS-FV25
FV-SPDS-VR2
FV-SPDS-VR5
DMEM

FIG 6

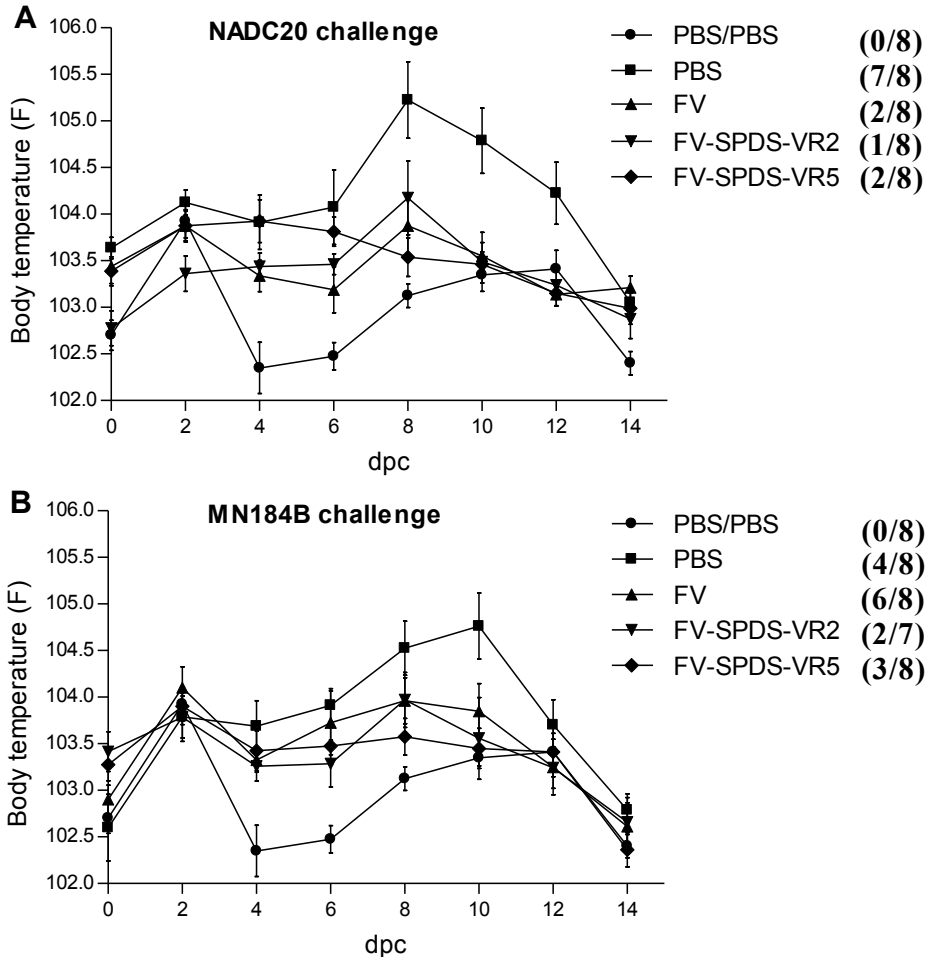

A
NADC20 challenge
Body temperature (F)
106.0
105.5
105.0
104.5
104.0
103.5
103.0
102.5
102.0
0
2
4
6
8
10
12
14
dpc
PBS/PBS (0/8)
PBS (7/8)
FV (2/8)
FV-SPDS-VR2 (1/8)
FV-SPDS-VR5 (2/8)
B
MN184B challenge
Body temperature (F)
106.0
105.5
105.0
104.5
104.0
103.5
103.0
102.5
102.0
0
2
4
6
8
10
12
14
dpc
PBS/PBS (0/8)
PBS (4/8)
FV (6/8)
FV-SPDS-VR2 (2/7)
FV-SPDS-VR5 (3/8)

FIG 7

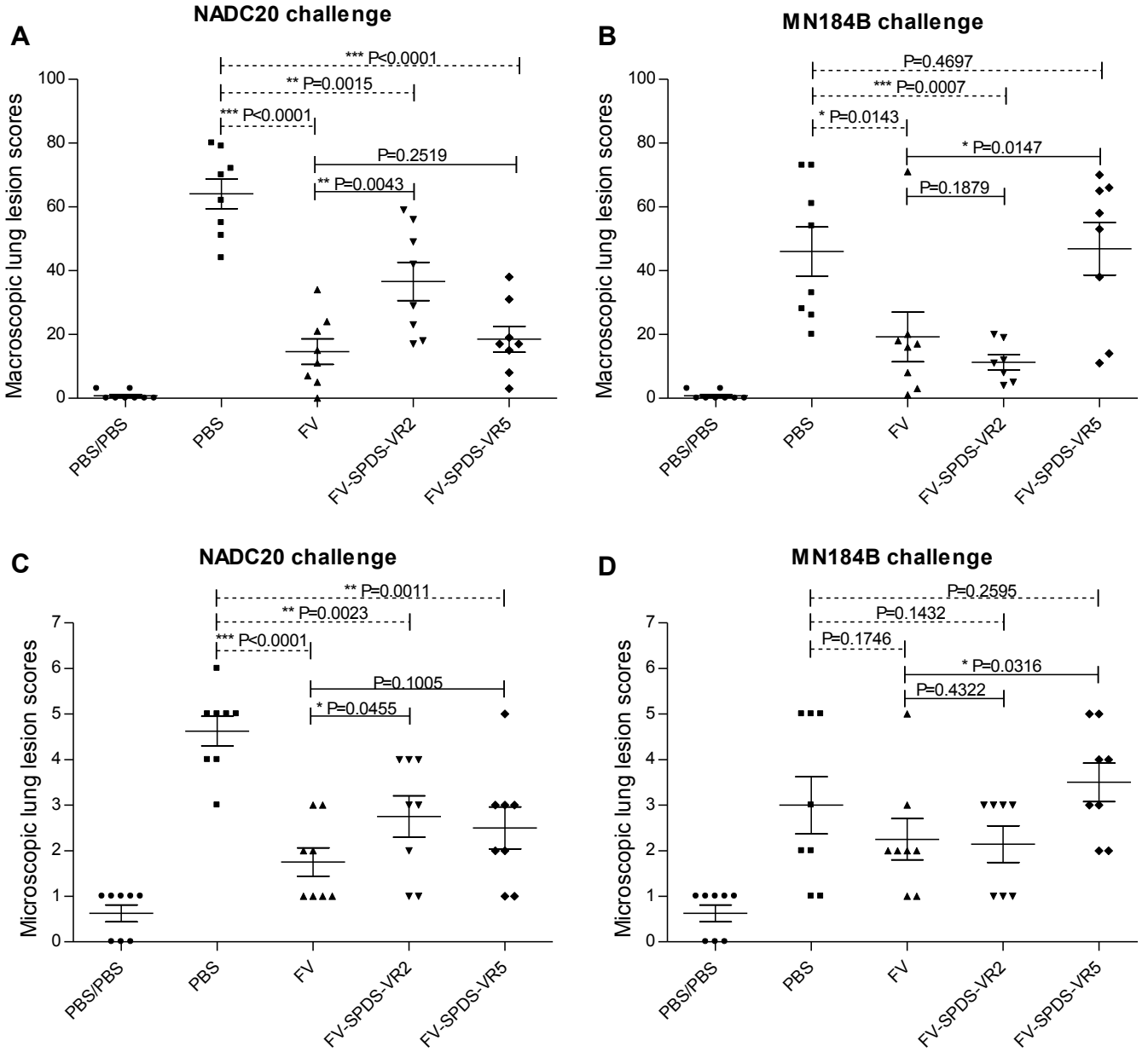
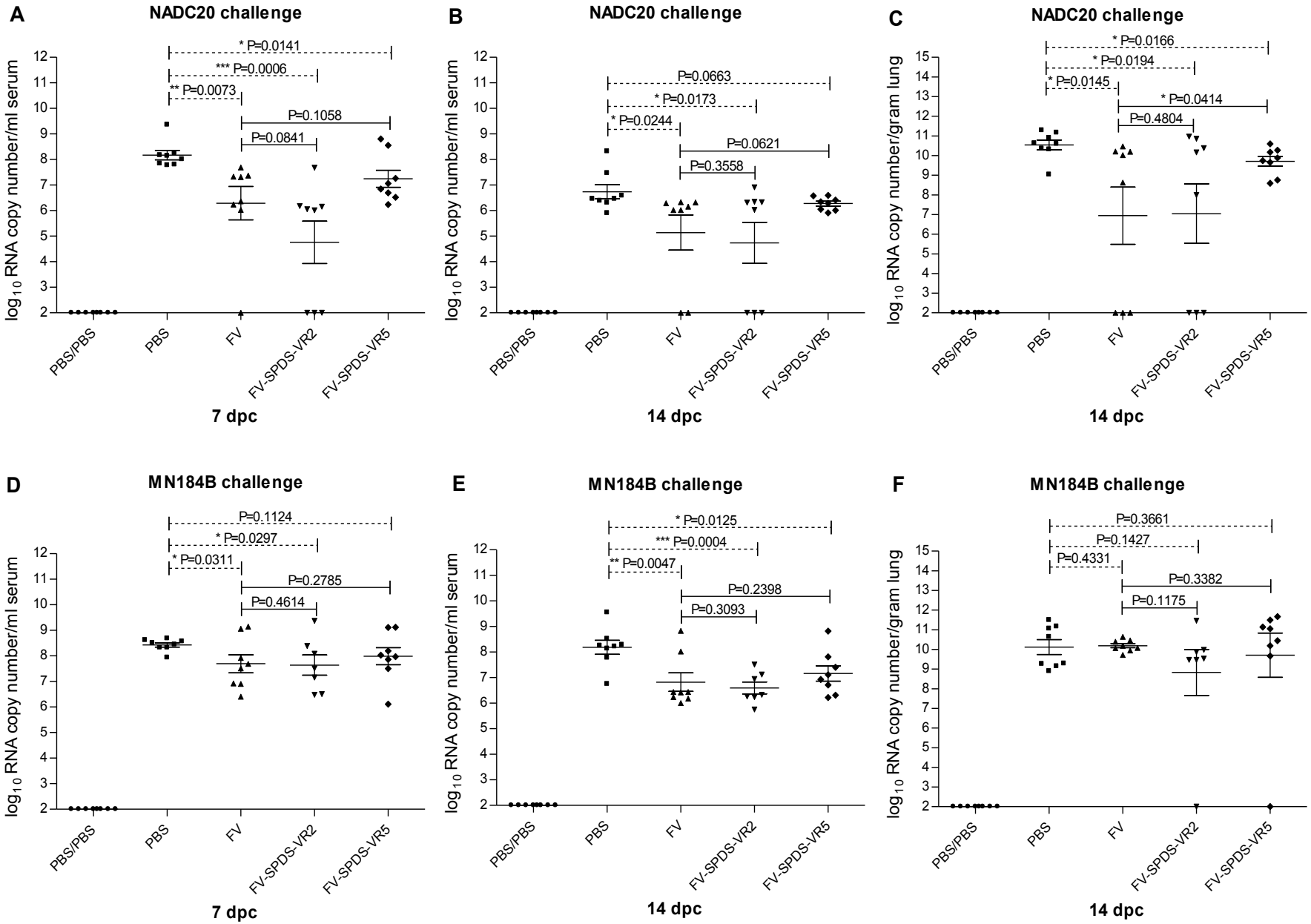

A
NADC20 challenge
Macroscopic lung lesion scores
*** P<0.0001
** P=0.0015
*** P<0.0001
P=0.2519
** P=0.0043
PBS/PBS
PBS
FV
FV-SPDS-VR2
FV-SPDS-VR5
B
MN184B challenge
Macroscopic lung lesion scores
P=0.4697
*** P=0.0007
* P=0.0143
* P=0.0147
P=0.1879
PBS/PBS
PBS
FV
FV-SPDS-VR2
FV-SPDS-VR5
C
NADC20 challenge
Microscopic lung lesion scores
** P=0.0011
** P=0.0023
*** P<0.0001
P=0.1005
* P=0.0455
PBS/PBS
PBS
FV
FV-SPDS-VR2
FV-SPDS-VR5
D
MN184B challenge
Microscopic lung lesion scores
P=0.2595
P=0.1432
P=0.1746
* P=0.0316
P=0.4322
PBS/PBS
PBS
FV
FV-SPDS-VR2
FV-SPDS-VR5

FIG 8


A
NADC20 challenge
log10 RNA copy number/ml serum
* P=0.0141
*** P=0.0006
** P=0.0073
P=0.1058
P=0.0841
PBS/PBS
PBS
FV
FV-SPDS-VR2
FV-SPDS-VR5
7 dpc
B
NADC20 challenge
log10 RNA copy number/ml serum
P=0.0663
* P=0.0173
* P=0.0244
P=0.0621
P=0.3558
PBS/PBS
PBS
FV
FV-SPDS-VR2
FV-SPDS-VR5
14 dpc
C
NADC20 challenge
log10 RNA copy number/gram lung
* P=0.0166
* P=0.0194
* P=0.0145
* P=0.0414
P=0.4804
PBS/PBS
PBS
FV
FV-SPDS-VR2
FV-SPDS-VR5
14 dpc
D
MN184B challenge
log10 RNA copy number/ml serum
P=0.1124
* P=0.0297
* P=0.0311
P=0.2785
P=0.4614
PBS/PBS
PBS
FV
FV-SPDS-VR2
FV-SPDS-VR5
7 dpc
E
MN184B challenge
log10 RNA copy number/ml serum
* P=0.0125
*** P=0.0004
** P=0.0047
P=0.2398
P=0.3093
PBS/PBS
PBS
FV
FV-SPDS-VR2
FV-SPDS-VR5
14 dpc
F
MN184B challenge
log10 RNA copy number/gram lung
P=0.3661
P=0.1427
P=0.4331
P=0.3382
P=0.1175
PBS/PBS
PBS
FV
FV-SPDS-VR2
FV-SPDS-VR5
14 dpc