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Enhancing heterologous protection in pigs vaccinated with chimeric porcine reproductive and respiratory syndrome virus containing the full-length sequences of shuffled structural genes of multiple heterologous strains

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Running title: Chimeric PRRSV vaccine for heterologous protection

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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) is the causative agent of arguably the most economically important global swine disease. The extensive genetic variation of PRRSV strains is a major obstacle for heterologous protection of current vaccines. Previously, we constructed a panel of chimeric viruses containing only the ectodomain sequences of DNA-shuffled structural genes of different PRRSV strains in the backbone of a commercial vaccine, and found that one chimeric virus had an improved cross-protection efficacy. In this present study, to further enhance the cross-protective efficacy against heterologous strains, we constructed a novel chimeric virus VR2385-S3456 containing the full-length sequences of shuffled structural genes (ORFs 3-6) from 6 heterologous PRRSV strains in the backbone of PRRSV strain VR2385. We showed that the chimeric virus VR2385-S3456 induced a high level of neutralizing antibodies in pigs against two heterologous strains. A subsequent vaccination and challenge study in 48 pigs revealed that the chimeric virus VR2385-S3456 conferred an enhanced cross-protection when challenged with heterologous virus strain NADC20 or a contemporary heterologous strain RFLP 1-7-4. The results suggest that the chimera VR2385-S3456 may be a good PRRSV vaccine candidate for further development to confer heterologous protection.

Key words: Porcine reproductive and respiratory syndrome virus (PRRSV); vaccine; DNA shuffling; cross-protection; heterologous strains; RFLP 1-7-4.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) causes an economically important global swine disease resulting in more than $660 million annual economic losses to the swine industry in the United States alone [1, 2]. As a single-strand positive-sense RNA virus, PRRSV has an extremely high mutation rate which is approximately 10^{-2}/site/year [3-6]. Field strains with extensive genetic variations have been frequently emerging since its initial isolation from pigs in 1989 [3, 7, 8]. These diverse populations of virus strains are classified into two distinct genotypes, type 1 and type 2, and at least 9 distinct genetic lineages can be subdivided within type 2 [7, 8]. The current commercially available vaccines only confer a limited level of cross-protection against heterologous PRRSV strains [9-11]. Therefore, an important objective for PRRSV control is to develop a universal vaccine that can provide better heterologous protection than the current available vaccines [12, 13].

One promising strategy to achieve this objective is to include protective immunogenic domains from different strains in the vaccine by molecular breeding of multiple heterologous strains through DNA shuffling. By mimicking natural recombination process *in vivo*, DNA shuffling can rapidly generate recombinants with desired phenotypes *in vitro* [14]. The DNA shuffling approach has been successfully used to generate desired phenotypes of viruses such as Murine leukemia virus strains, Dengue virus, and Venezuelan equine encephalitis virus [15-17].

The PRRSV genome encodes at least eight structural proteins, most of which are important for protective immunity [18, 19]. The major envelope glycoprotein GP5 has been extensively studied as a target for PRRSV vaccine development since it contains neutralizing epitopes

and contributes to virus entry into cells through heterodimers formed with membrane protein (M). GP5 has also been showed to be responsible for PRRSV virulence [20-24]. Minor envelope glycoproteins (GP2, GP3, GP4) also induce neutralizing antibodies and play important roles in cell entry by interacting with the cellular receptor CD163 [25-28]. Therefore, for the rational design of a broadly cross-protective vaccine, both major and minor PRRSV envelope proteins should be considered.

Previously, we have individually shuffled each of the GP3, GP4, GP5, and M genes in the backbone of PRRSV virulent strain VR2385 through DNA shuffling [29-31]. We identified chimeric viruses with improved cross-neutralizing activities against heterologous virus strains *in vitro*. Furthermore, we demonstrated that, when the ectodomain sequences of these individually-shuffled structural genes were assembled into the backbone of a commercial vaccine (Fostera® PRRS), the resulting chimeria FV-SPDS-VR2 conferred improved heterologous protection [32].

In this present study, we hypothesized that inclusion of the full-length, not just the ectodomain, sequences of each individually-shuffled structural genes in the backbone of PRRSV strain VR2385, which is the original backbone used to screen for individual chimeras with improved cross-neutralizing activities, would further improve the heterologous protection. Therefore, a novel chimeric virus VR2385-S3456 containing the full-length sequences of all shuffled structural genes in the backbone of VR2385 strain was generated in this study, and shown to induce cross-protection in pigs challenged with heterologous strain NADC20 and a contemporary heterologous strain RFLP 1-7-4.

2. Materials and methods

2.1. Cells and viruses

BHK-21 cells were cultured in Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Monkey kidney cell lines MARC-145 were cultured in low glucose-supplemented DMEM with 10% FBS, and maintained in low glucose DMEM with 2% FBS for virus propagation. A DNA-launched infectious clone of PRRSV strain VR2385, pIR-VR2385-CA, was constructed previously [33]. PRRSV strain VR2385 (lineage 5, accession no. JX044140) was originally isolated from a pig experiencing severe respiratory disease in Iowa [34, 35]. The attenuated PRRSV strain DS722, a derivative of VR2385 generated by DNA shuffling of the ORF5 gene, was constructed in our lab previously [30]. The PRRSV strain NADC20 (lineage 9, accession no. JX069953) was provided by Dr. Kelly Lager of USDA-National Animal Disease Center [8]. A contemporary PRRSV strain ISU2014016404 (referred to as "RFLP 1-7-4") belonging to lineage 1 with a pattern 1-7-4 based on restriction fragment length polymorphism (RFLP) pattern of the ORF5 gene was provided by Dr. Jianqiang Zhang of the Iowa State University [36].

2.2. Construction and rescue of chimeric virus

A nucleotide acid sequence fragment S3456 was designed and commercially synthesized (Integrated DNA Technologies, Coralville, Iowa). The S3456 fragment contains the full-length sequences of the shuffled ORF3 gene derived from chimera GP3TS22 [29], shuffled ORF4 gene derived from chimera GP4TS14 [31], shuffled ORF5 gene derived from chimera DS722 [30], and shuffled ORF6 (M) gene derived from chimera MTS57 [31]. The overlapping sequences between ORF3 and ORF4 were derived from chimera GP4TS14. The

inclusion of the shuffled ORF5 gene from chimera DS722 ensured that the resulting virus is attenuated, as the DS722 chimera with the shuffled ORF5 gene is attenuated in pigs [30]. By using the *BsrG* I and *Xba* I restriction enzyme sites engineered into the synthesized fragment S3456, the full-length sequences of the shuffled ORFs 3-6 were successfully introduced into the genomic backbone of a DNA-launched PRRSV infectious clone pIR-VR2385-CA, to create the final chimera designated VR2385-S3456 (**Fig. 1A**).

To rescue the chimeric virus VR2385-S3456, fresh BHK-21 cells seeded in a 6-well plate at approximately 60–80% confluency were transfected with 2 μg of the respective plasmid DNA per well using the Lipofectamine LTX and Plus Reagent kit (Invitrogen) according to the manufacturer's instructions. After 48 h incubation at 37°C with 5% CO_2, cell culture supernatants were harvested and designated as passage 0 (P0) virus.

2.3. Indirect immunofluorescence assay (IFA)

IFA with an anti-PRRSV antibody (SDOW17) was used to verify PRRSV infection in cells as described previously [28].

2.4. Virus growth kinetics and plaque morphology assay

To characterize the growth kinetics and properties of the rescued chimeric virus VR2385-S3456 *in vitro*, a multiple-step growth curve and plaque morphology assays were conducted in MARC-145 cells as described previously [28].

2.5. Experimental design for a cross-protection vaccine efficacy study in pigs

This study was approved by Virginia Tech Institutional Animal Care and Use Committee (approval number 16-127). A total of 48 PRRSV-negative piglets all at 3 weeks of age were randomly divided into 6 groups of 8 piglets per group regardless of the body weights. Piglets

132 in each group were vaccinated intramuscularly with the novel chimera VR2385-S3456, an
133 attenuated chimera DS722 containing only shuffled ORF5 [30], or PBS (**Table 1**). Serum
134 samples were collected from each pig prior to vaccination and weekly thereafter. At 49 days
135 post-vaccination (dpv), the pigs were challenged with one of two heterologous PRRSV
136 strains, NADC20 or RFLP 1-7-4 which share 92.9% and 88.2% nucleotide sequence identity
137 in the ORF5 gene with the VR2385-S3456, respectively. At 14 days post-challenge (dpc), all
138 pigs were euthanized and necropsied. Lung tissue samples were collected for gross pathology
139 and histopathology evaluation and quantification of PRRSV RNA load.

140 *2.6. Serum virus neutralization (SVN) assay*

141 The neutralizing antibody (NA) titers against heterologous strains NADC20 and RFLP
142 1-7-4 were determined by a SVN assay [29]. Briefly, two-fold diluted serum samples
143 collected at 49 dpv from each pig were mixed with an equal volume of respective test virus at
144 an infectious titer of 2×10^3 $TCID_{50}$/ml and incubated at 37°C for 1 h. The mixture was then
145 dispensed onto MARC-145 cells in 96-well plates (100 ml/well) and incubated for 1 h at
146 37°C. After washing with PBS once, the cells were maintained in DMEM (2% FBS) for 20 h.
147 The cells were fixed and stained for IFA to detect evidence of virus infection. The NA titers
148 were expressed as the highest dilution that showed at least 90% reduction in the number of
149 fluorescent foci compared to negative control serum. Three independent tests were performed
150 for each serum sample.

151 *2.7. Gross pathology and histopathology evaluation of lung tissues*

152 At necropsy, lungs were evaluated for visible gross lesions as described previously [35],
153 and subsequently five sections of lung tissues were collected, fixed in formalin and processed

for histopathology evaluation. The evaluation was conducted by a board-certified veterinary pathologist who was blinded to the treatment status. For the histopathology evaluation, the microscopic lung lesions were scored based on the presence and severity of interstitial pneumonia ranging from 0 to 4 (0, no microscopic lesions; 1, mild interstitial pneumonia; 2, moderate multifocal interstitial pneumonia; 3, moderate diffuse interstitial pneumonia; 4, severe interstitial pneumonia).

2.8. Quantitation of viral RNA loads in sera and lung tissues

Serum viral RNAs were extracted from serum samples at 49 dpv, 7 and 14 dpc using ZR Viral RNA kit (ZYMO RESEARCH, USA) following the manufacturer's protocol. Total RNAs from the lung tissue were extracted using TRI Reagent (MRC) following the manufacturer's protocol. The quantitation of PRRSV RNA copy number was conducted by RT-qPCR as described previously [30, 32].

2.9. Statistical analyses

The data were analyzed using GraphPad Prism (version 6.0). The viral titers were analyzed by the Student's t test (unpaired), and the other data were analyzed using one-way ANOVA followed by Tukey's multiple comparison test.

3. Results

3.1. Successful rescue of chimeric virus VR2385-S3456 containing the full-length sequences of shuffled ORFs 3-6 genes of multiple heterologous strains

Previously, we have successfully generated single envelope gene-shuffled chimeric PRRSV viruses (GP3TS22, GP4TS14, DS722 and MTS57), in the genomic backbone of a

virulent PRRSV strain VR2385, as well as a multiple genes-shuffled chimeric virus (FV-SPDS-VR2) in the backbone of commercial vaccine Fostera® PRRS [29-32]. In an attempt to further improve the efficacy of cross-protection, in this present study we included the full-length sequences, not just the ectodomain sequences, of all shuffled structural genes in the genomic backbone of PRRSV strain VR2385. The rationale is that additional cross-protective epitopes reside in regions other than the ectodomains. Also, the use of wild-type PRRSV VR2385 (instead of a MLV) would also enhance humoral and cell-mediated immune responses.

The DNA fragment S3456 representing the full-length sequences of shuffled ORFs3-6 derived from multiple chimeras with respective individually-shuffled gene was commercially synthesized (**Fig. 1A**). The S3456 was cloned into the backbone of a DNA-launched infectious clone pIR-VR2385-CA to produce a novel chimera VR2385-S3456. The authenticity of the chimeric clone VR2385-S3456 was verified by DNA sequencing.

Following transfection of BHK-21 cells with the full-length chimeric clone VR2385-S3456, supernatant was harvested two days post-transfection (P0 virus) and used to inoculate fresh MARC-145 cells. At four days post-inoculation, cytopathic effects (CPEs) were observed in inoculated cells. IFA using PRRSV N-specific monoclonal antibody confirmed that the CPEs were PRRSV-specific, thus indicative of the production of viable progeny viruses (**Fig. 1B**). To further confirm that the rescued virus indeed originated from the clone, the ORFs3-6 genes were amplified from the P3 viruses by RT-PCR and sequenced. Sequence data (not shown) confirmed that the ORFs3-6 of the rescued virus were identical to that of the original chimeric clone. Therefore, the results demonstrated the successful rescue of a viable novel

198 chimeric virus VR2385-S3456 containing the full-length sequences of the shuffled ORFs 3-6
199 genes.

200 *3.2. Chimeric virus VR2385-S3456 had a reduced growth ability phenotype in MARC-145*
201 *cells*

202 To evaluate the growth kinetics of the rescued VR2385-S3456, MARC-145 cells were
203 infected with the P3 virus of the VR2385-S3456 or VR2385 at an MOI of 0.1. The chimeric
204 virus VR2385-S3456 had an overall reduced growth ability when compared to VR2385 (**Fig.**
205 **1C**). Specifically, compared to parental virus VR2385, the chimera VR2385-S3456 had
206 significantly lower virus titers between 48 to 96 h post-inoculation (hpi). The peak virus titer
207 of the parental virus VR2385 was approximately 3.0×10^7 $TCID_{50}$/ml, whereas the peak titer
208 of the chimera VR2385-S3456 was about 3.0×10^5 $TCID_{50}$/ml, 100 times lower than the
209 parental virus. Compared to the parental virus, the chimeric virus formed smaller and turbid
210 plaques (**Fig. 1D**) in MARC-145 cells indicating a reduced rate of growth and spread to
211 adjacent cells. This is consistent with the observations on growth kinetics. Collectively, the
212 results showed that the chimeric virus VR2385-S3456 containing the full-length sequences of
213 the shuffled ORFs3-6 genes are viable and have a reduced growth ability in MARC-145 cells.

214 *3.3. Chimeric virus VR2385-S3456 induced higher cross-neutralizing antibodies against*
215 *heterologous virus strains NADC20 and RFLP 1-7-4*

216 To evaluate the efficacy of heterologous protection of the chimeric virus VR2385-S3456,
217 we conducted a vaccination/challenge study in pigs. Since the PRRSV strain VR2385 is a
218 virulent strain [35], a derivative of VR2385, chimera DS722, which contains a shuffled GP5
219 gene and is attenuated, was used as the control [30]. After vaccination, the anti-PRRSV

antibodies in each pig were monitored using the IDEXX HerdChek X3 ELISA kit. The data showed that all vaccinated pigs seroconverted at 14 dpv, while the negative control group pigs remained seronegative until after challenge (**Table 2**).

To investigate whether the chimeric virus induces cross-neutralizing antibodies against heterologous strains NADC20 and RFLP 1-7-4, an SVN assay was performed using serum samples collected at 49 dpv. When tested against the heterologous virus strain NADC20 (**Fig. 2A**), both DS722- and VR2385-S3456-vaccinated pigs produced a higher level of NA titers, and the VR2385-S3456 group (mean value 5.5) was significantly higher than the DS722 group (mean value 4.1). When tested against the heterologous virus strain RFLP 1-7-4 (**Fig. 2B**), both vaccinated groups produced NAs, and the VR2385-S3456 group (mean value 2.4) was significantly higher than the DS722 group (mean value 1.7). Overall, the NA titers against the RFLP 1-7-4 strain were not as high as those against the NADC20 strain. Nevertheless, the results suggested that the chimeric virus VR2385-S3456 induced cross-neutralizing antibodies against heterologous PRRSV strains NADC20 and RFLP 1-7-4.

3.4. Chimeric virus VR2385-S3456-vaccinated pigs had more average daily weight gain (ADWG) and less microscopic lung lesions after challenge

After challenge, three pigs in the PBS/NADC20 group, and two in the PBS/RFLP 1-7-4 group developed mild respiratory symptoms (**Table 2**). Prior to challenge, there was no statistically significant difference in ADWG between any vaccinated groups and PBS control group (**Fig. 3A**). After challenge with heterologous strain NADC20, both DS722- and VR2385-S3456-vaccinated pigs had more ADWG than the PBS control group (**Fig. 3B**). After challenge with heterologous strain RFLP 1-7-4, the VR2385-S3456-vaccinated pigs

had more ADWG than DS722-vaccinated and PBS control pigs (**Fig. 3B**).

For microscopic lung lesions, the VR2385-S3456-vaccinated group had lower lesion scores than the DS722-vaccinated group when challenged with NADC20 (**Fig. 3C**). The VR2385-S3456-vaccinated group had numerically lower microscopic lung lesion scores, although not statistically significant, than the DS722-vaccinated group when challenged with RFLP 1-7-4 (**Fig. 3D**). The gross lung lesion scores were not statistically different between groups (data not shown).

3.5. Chimeric virus VR2385-S3456-vaccinated pigs had reduced viral RNA loads in sera and lung tissues after challenge

Viral RNA loads in serum and lung are routinely used as a parameter for measuring virus replication level for PRRSV studies [30, 37, 38]. Only one pig in the VR2385-S3456/NADC20 group had a low but detectable PRRSV viremia (1.6×10^3 copies/ml) at 49 dpv (**Table 2**) indicating that most pigs had cleared the residual vaccine virus at the time of challenge. When challenged with NADC20, both DS722- and VR2385-S3456-vaccinated groups had significantly reduced levels of serum viral RNA copies at 7, 14 dpc compared to the non-vaccinated group (**Fig. 4A, B**). Compared to the DS722-vaccinated group, the VR2385-S3456-vaccinated group had a numerically lower viral RNA copies, although the difference was not significant. 62.5% (5/8) pigs in the DS722-vaccinated group, and 85.7% (6/7) pigs in the VR2385-S3456-vaccinated group were negative for viral RNAs in sera at 14 dpc. Also, the viral RNA loads in the lung tissues of both DS722- and VR2385-S3456-vaccinated groups were significantly decreased when compared to the non-vaccinated control group (**Fig. 4C**). The VR2385-S3456-vaccinated pigs

showed numerically lower viral RNA loads, although not significantly different, in lung tissues than the DS722-vaccinated group.

For pigs challenged with RFLP 1-7-4, both DS722- and VR2385-S3456-vaccinated groups had significantly decreased levels of viral RNA loads in sera (7, 14 dpc) and lung tissues (14 dpc) compared to the non-vaccinated control group (**Fig. 4D-4F**). Similarly, the VR2385-S3456-vaccinated group had a numerically lower viral RNA copy number than that of DS722-vaccinated group, although the difference was not statistically significant. 62.5% (5/8) of pigs in the DS722-vaccinated and 71.4% (5/7) of VR2385-S3456-vaccinated groups were negative for viral RNA loads in lung tissues at 14 dpc.

3.6. Chimeric virus VR2385-S3456 was *genetically stable in vitro and in vivo*

To investigate the genetic stability of the chimeric virus VR2385-S3456, the virus was serially passaged to P8 in MARC-145 cells. The ORFs3-6 sequences of the P8 virus showed a 99.9% nucleotide sequence identity to the P3 virus. The chimeric virus recovered from the serum samples of infected pigs at 14 dpv had 99.8% nucleotide sequence identity to that of the original virus.

4. Discussion

The extensive genetic and antigenic diversity of field PRRSV strains worldwide makes the current commercial vaccines, which are all based on a single virus strain, less effective in protection against diverse field strains [9, 11, 39]. Enhancing cross-protection is critically important but a major challenge for the development of the next generation PRRSV vaccines [10, 13, 40, 41]. To overcome this challenge, one strategy is to expand the antigenic coverage

286 of PRRSV vaccines [12]. Molecular breeding through DNA shuffling has been shown to be a
287 very promising approach to expand the antigenic coverage [15, 42, 43].

288 Previously, we successfully generated a chimeric virus FV-SPDS-VR2 containing only the
289 ectodomains of the shuffled structural genes (ORFs3-6) in the genomic backbone of a
290 commercial vaccine [32]. In this present study, we constructed a novel and improved
291 chimeric virus VR2385-S3456. Compared to FV-SPDS-VR2, the novel chimeric virus
292 VR2385-S3456 from this study has two key improvements: (1) It contains the full-length, not
293 just the ectodomains, sequences of each shuffled structural genes; (2) The full-length
294 sequences of the shuffled structural genes from multiple strains were cloned into the genomic
295 backbone of PRRSV strain VR2385, which was originally used to screen those single
296 gene-shuffled chimeras with significantly higher cross-neutralizing activities [29-31].

297 The vaccine efficacy of the novel chimera VR2385-S3456 was tested in a
298 vaccination/challenge pig model. The results showed that the novel chimera VR2385-S3456
299 conferred an enhanced cross-protection against heterologous virus strains NADC20 and
300 RFLP 1-7-4. In the parental virus control group, we used the chimera DS722 virus which
301 contains the shuffled GP5 gene. The DS722 has previously been shown to induce similar
302 immune protection compared to the wild-type virulent VR2385 but is attenuated in pigs [30].
303 After vaccination, both DS722- and VR2385-S3456-vaccinated pigs had similar ADWG to
304 the PBS control pigs, suggesting a good safety of both DS722 and VR2385-S3456. Although
305 cell-mediated immunity is also important for protection against PRRSV, neutralizing
306 antibodies is an important parameter to assess protection in pigs against PRRSV infection [32,
307 37, 40]. At 49 dpv, VR2385-S3456-vaccinated pigs produced higher NA titers against

heterologous strains NADC20 and RFLP 1-7-4. Based on the ORF5 sequences, the RFLP 1-7-4 strain used in this study as well as the strain MN184B both belong to genetic lineage 1 [8]. Protection against lineage 1 strains is important, as the lineage 1 virus is currently highly prevalent [38, 41]. Unfortunately all the chimeric viruses from previously studies induced only very low NA titers (<1) against the lineage 1 MN184B strain [32]. In this present study, we found that most of VR2385-S3456-vaccinated pigs (11/14) and some of DS722-vaccinated pigs (6/16) elicited relatively higher NA titers (>2) against the lineage 1 RFLP 1-7-4. It remains to be determined if the aforementioned two improvements of the novel chimera VR2385-S3456 are responsible for the observed higher NA titers against a lineage 1 virus strain.

When challenged with the heterologous strain NADC20, the VR2385-S3456-vaccinated pigs had an improved ADWG, lower microscopic lung lesion scores, reduced viral RNA loads in sera and lung tissues than the DS722-vaccinated or PBS control pigs. Importantly, most of VR2385-S3456-vaccinated pigs had cleared the viruses at necropsy. The data indicated that VR2385-S3456 conferred cross-protection against the heterologous strain NADC20.

When challenged with a contemporary lineage 1 virus RFLP 1-7-4 which is currently circulating in North America, similar results were observed, suggesting a good protection against the heterologous strain RFLP 1-7-4. We noticed that the chimera DS722 also provided a partial protection against the two heterologous strains, indicating that the shuffled GP5 gene, which is the same for chimera DS722 and chimera VR2385-S3456, may have played a role in cross-protection as well.

In summary, in this study we successfully generated a novel chimeric virus VR2385-S3456 which contains the full-length sequences of each shuffled structural genes of ORFs 3-6 in the genomic backbone of a PRRSV strain VR2385. The rescued chimeric virus had a reduced replication ability *in vitro*, induced relatively higher NA titers, and conferred an enhanced cross-protection in pigs against two heterologous virus strains. Therefore, the novel chimeric virus VR2385-S3456 is a good candidate for further development as a PRRSV vaccine.

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Conflicts of Interest Statement:

The authors declare no conflict of interest.

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476

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

Group	No. of pigs	Vaccination at 0 dpv[a] with	Challenge at 49 dpv[b] with	No. of pigs at necropsy (14 dpc)
1	8	PBS	NADC20	8
2	8	PBS	RFLP 1-7-4	8
3	8	DS722	NADC20	8
4	8	DS722	RFLP 1-7-4	8
5	8	VR2385-S3456	NADC20	7[c]
6	8	VR2385-S3456	RFLP 1-7-4	7[c]

478 [a] Dose: $1.0\times10^{4.0}$ $TCID_{50}$/pig. Route: intramuscular injection (IM).

479 [b] Dose: NADC20, $1.0\times10^{5.0}$ $TCID_{50}$/pig; RFLP 1-7-4, $5.0\times10^{4.0}$ $TCID_{50}$/pig. Route:

480 intramuscular injection (IM).

481 [c] One piglet died from an unrelated cause before challenge.

482 dpv=days post-vaccination; dpc=days post-challenge

483

484

485 **Table 2. Seroconversion, serum viral RNA loads, and clinical signs of pigs**

Group	No. of pigs seroconverted/total[a]							No. of pigs serum viral RNA at 49 dpv/total[b]	No. of pigs clinical sign (after challenge)/total
	7	14	21	28	35	42	49 dpv		
1	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	3/8
2	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	2/8
3	0/8	8/8	8/8	ND	ND	ND	8/8	0/8	0/8
4	0/8	8/8	8/8	ND	ND	ND	8/8	0/8	0/8
5	0/8	7/7	7/7	ND	ND	ND	7/7	1/7	0/7
6	0/8	7/7	7/7	ND	ND	ND	7/7	0/7	0/7

486 [a] Seroconversion was monitored using IDEXX HerdChek® X3 ELISA kit. ND, not done.

487 [b] Serum viral RNA load was determined by RT-qPCR.

488

489

Figure legends

FIG. 1. Construction and virological characteristics of a novel chimeric virus VR2385-S3456. (A). Schematic diagrams of the genomic organization of the genomic backbone VR2385 virus and the novel chimeric virus VR2385-S3456. The genes derived from the backbone VR2385 virus are depicted with open rectangles. Each pattern in the genes of the shuffled ORFs3-6 represents a single shuffled gene derived from one of the single gene-shuffled chimeric viruses (GP3TS22, GP4TS14, DS722, MTS57) respectively, which are shown at the bottom. **(B).** Two days post-transfection of BHK-21 cells with the VR2385 backbone as well as the novel chimeric virus clone, the P0 virus supernatants were harvested and used to inoculate fresh MARC-145 cells. Cells were fixed at 48 h post-inoculation, and immunostained by IFA with anti-PRRSV N monoclonal antibody (SDOW17). The parental virus VR2385 and the rescued novel chimeric virus VR2385-S3456 were passaged in MARC-145 cells to P3. **(C).** The P3 virus was used to infect fresh MARC-145 cells at an MOI of 0.1. The culture supernatants were collected at indicated time points. Infectious titers were determined and calculated using the Reed-Muench method. Three independent experiments were carried out for each virus. **(D).** Plaque morphology. The P3 viruses were used to infect MARC-145 cells, and then overlaid with medium containing 2% FBS and 1% low-melting-point agarose. Four days later, the cells were visualized by crystal violet staining.

FIG. 2. Neutralizing antibody (NA) titers induced by the novel chimeric virus VR2385-S3456 in pigs against heterologous PRRSV strains NADC20 and RFLP 1-7-4.

512 At 49 dpv, the sera from vaccinated and control pigs were collected to determine the NA
513 titers using MARC-145 cells by serum virus neutralization (SVN) assay. The NA titers were
514 expressed as the highest dilution (2^n) that showed a 90% or above reduction in the number of
515 fluorescent foci compared to that of negative control serum. **(A).** NA against a heterologous
516 strain NADC20. **(B).** NA against a contemporary heterologous strain RFLP 1-7-4. Each plot
517 represents the mean titer of three separate tests, and the error bars indicate standard errors. *P*
518 values were shown (** $P<0.01$, **** $P<0.0001$).
519
520 **FIG. 3. Average daily weight gain and microscopic lung lesions of vaccinated pigs.**
521 Average daily weight gain (ADWG) before (**A**) and after (**B**) virus challenge in vaccinated
522 pigs. The pigs were weighed at the time of vaccination (0 dpv), challenge (49 dpv) and
523 necropsy (14 dpc), respectively. For the evaluation of histological lung lesions, the lung
524 tissues were fixed in formalin and scored for histological lesions by a board-certified
525 veterinary pathologist (**C, D**). Each plot represents the value of one pig, and the error bars
526 indicate standard errors. Significant difference is indicated with asterisks (* $P<0.05$).
527
528 **FIG. 4. Viral RNA loads in sera and lung tissues after challenge with heterologous virus**
529 **strains NADC20 and RFLP 1-7-4**. PRRSV RNA copy numbers in sera at 7 dpc (**A, D**)
530 and 14 dpc (**B, E**), and in lung tissues at 14 dpc (**C, F**) were determined by RT-qPCR. The
531 detection limit is 3 log10 copies per ml (serum) or gram (lung tissue). Samples below the
532 detection limit were considered as negative, and calculated as 2 log10 copies for statistical
533 analysis. Each plot represents the mean viral RNA copy number of triplicate testing results of

534 one pig, and the error bars indicate standard errors. *P* values were shown (* *P*<0.05, **

535 *P*<0.01, *** *P*<0.001, **** *P*<0.0001).

Fig. 1

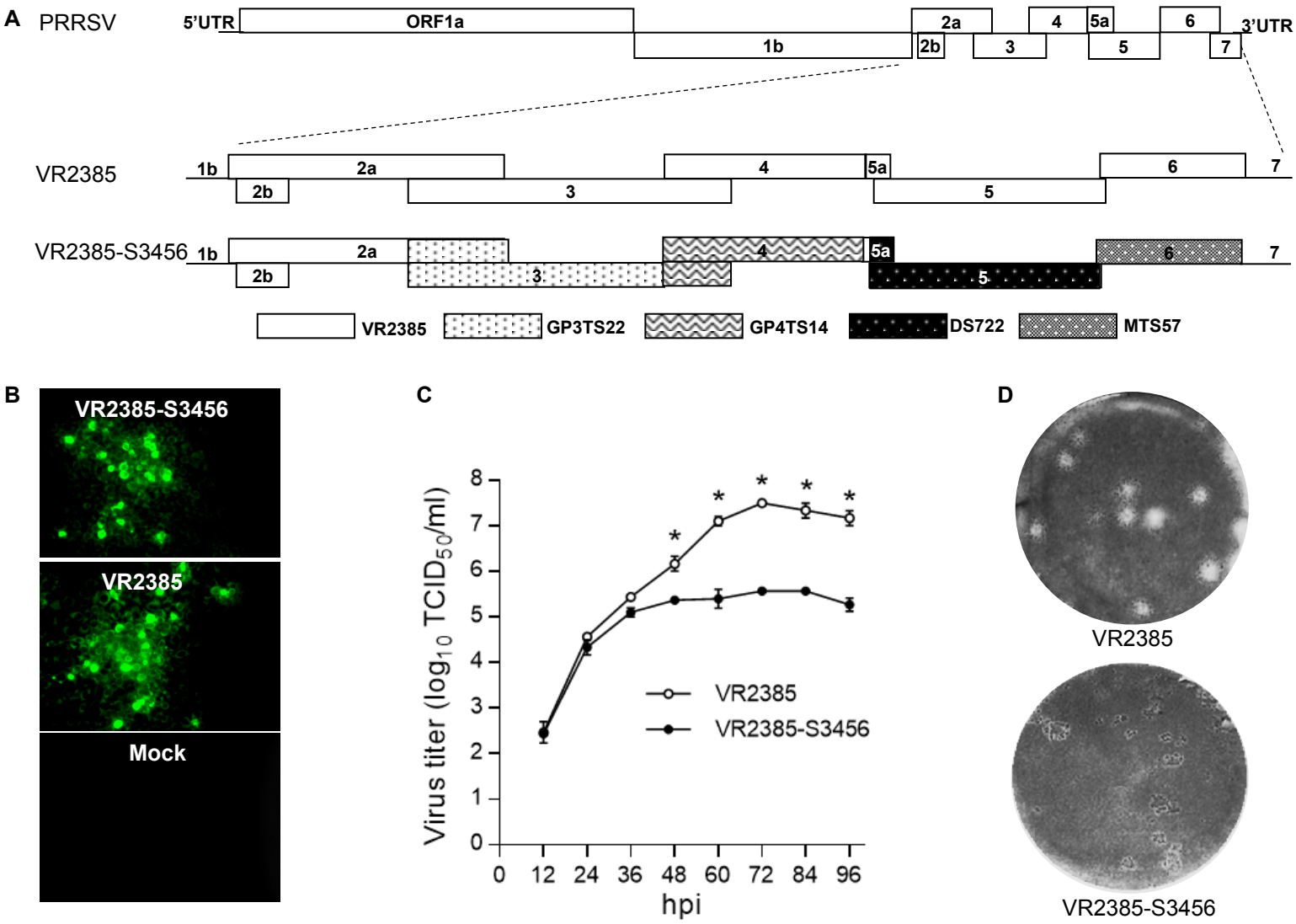

A
PRRSV
5'UTR
ORF1a
1b
2a
2b
3
4
5a
5
6
7
3'UTR
VR2385
VR2385-S3456
VR2385
GP3TS22
GP4TS14
DS722
MTS57
B
VR2385-S3456
VR2385
Mock
C
Virus titer (log10 TCID50/ml)
hpi
VR2385
VR2385-S3456
D
VR2385
VR2385-S3456

Fig. 2

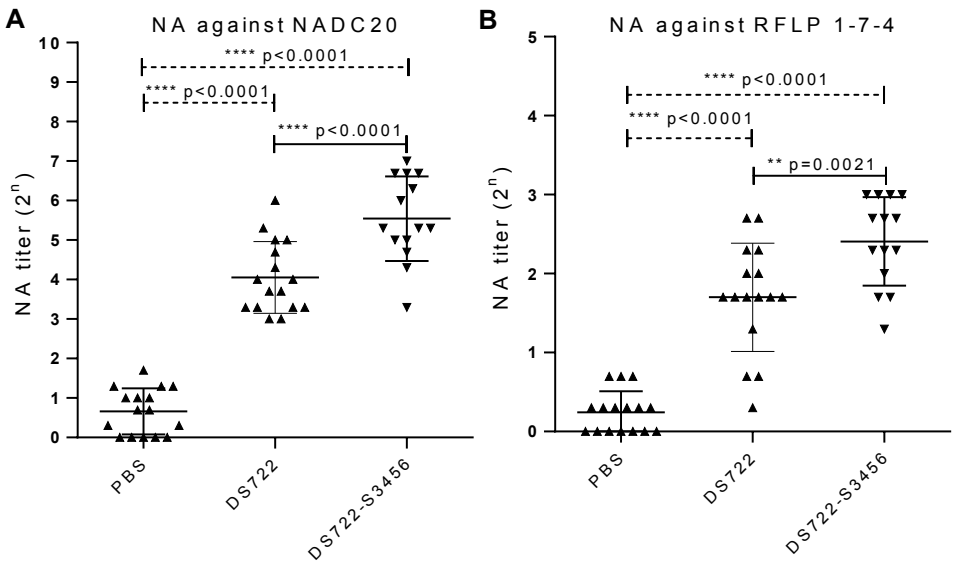

A
NA against NADC20
NA titer (2^n)
**** p<0.0001
**** p<0.0001
**** p<0.0001
PBS
DS722
DS722-S3456
B
NA against RFLP 1-7-4
NA titer (2^n)
**** p<0.0001
**** p<0.0001
** p=0.0021
PBS
DS722
DS722-S3456

Fig. 3

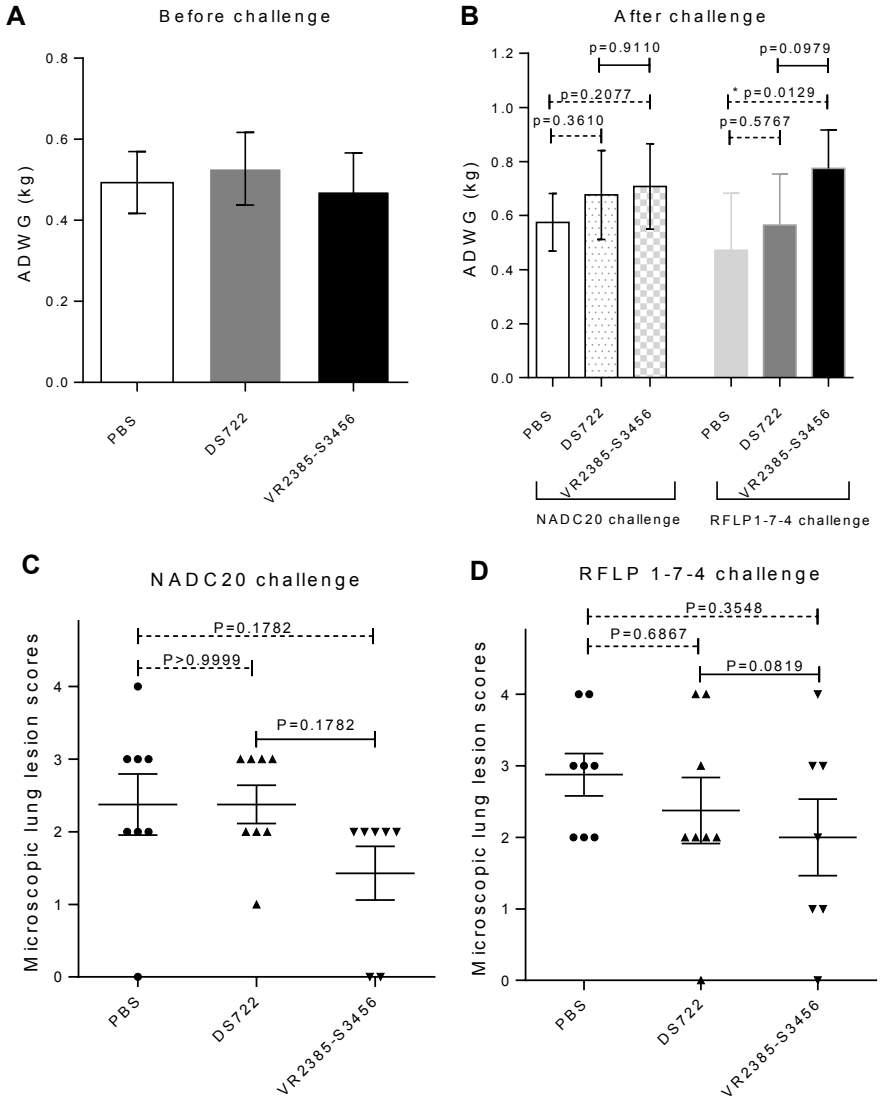
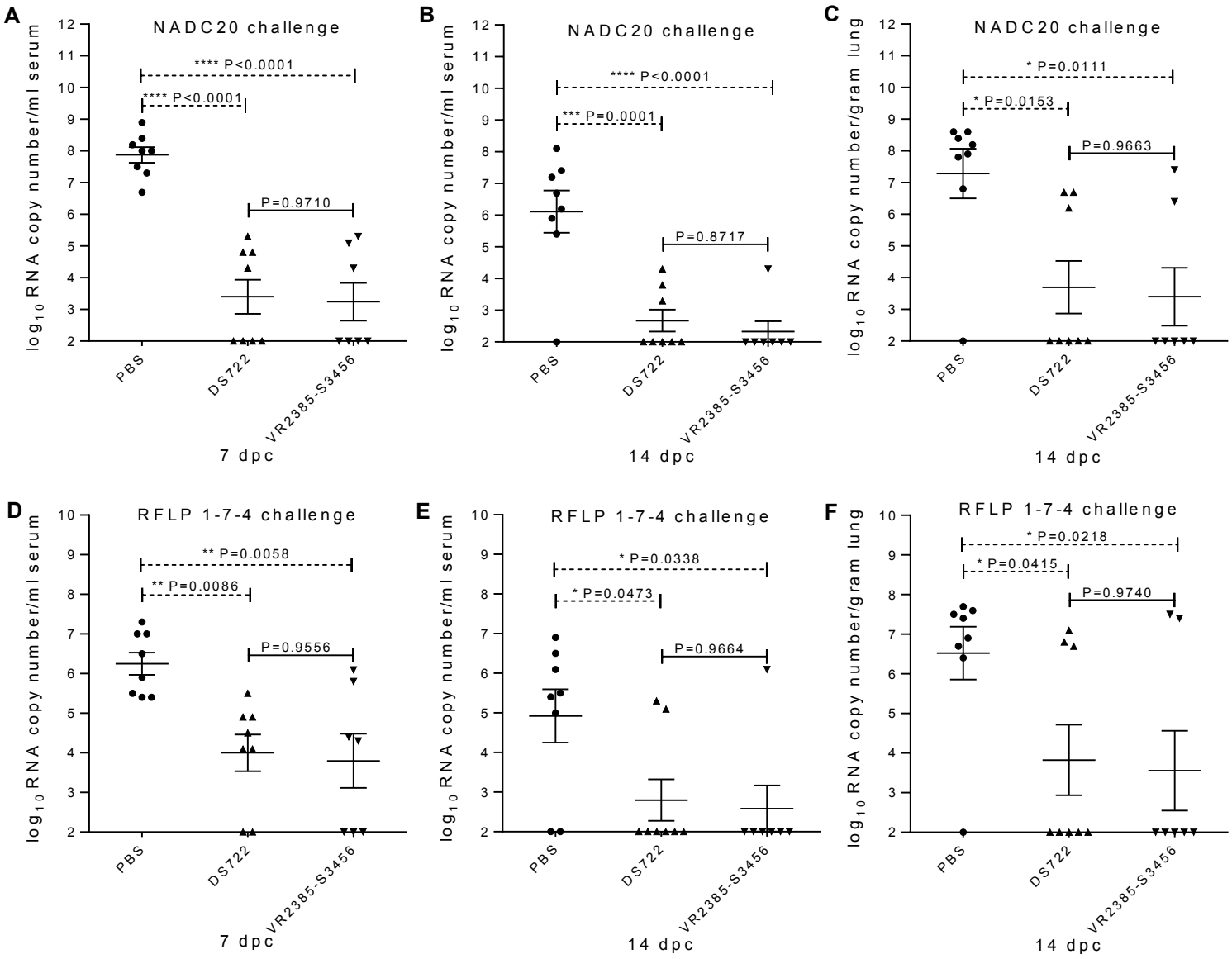

A
Before challenge
ADWG (kg)
0.0
0.2
0.4
0.6
0.8
PBS
DS722
VR2385-S3456
B
After challenge
ADWG (kg)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
p=0.9110
p=0.0979
p=0.2077
* p=0.0129
p=0.3610
p=0.5767
PBS
DS722
VR2385-S3456
PBS
DS722
VR2385-S3456
NADC20 challenge
RFLP1-7-4 challenge
C
NADC20 challenge
Microscopic lung lesion scores
0
1
2
3
4
P=0.1782
P>0.9999
P=0.1782
PBS
DS722
VR2385-S3456
D
RFLP 1-7-4 challenge
Microscopic lung lesion scores
0
1
2
3
4
P=0.3548
P=0.6867
P=0.0819
PBS
DS722
VR2385-S3456

Fig. 4


A
NADC20 challenge
log10 RNA copy number/ml serum
**** P<0.0001
**** P<0.0001
P=0.9710
PBS
DS722
VR2385-S3456
7 dpc
B
NADC20 challenge
log10 RNA copy number/ml serum
**** P<0.0001
*** P=0.0001
P=0.8717
PBS
DS722
VR2385-S3456
14 dpc
C
NADC20 challenge
log10 RNA copy number/gram lung
* P=0.0111
* P=0.0153
P=0.9663
PBS
DS722
VR2385-S3456
14 dpc
D
RFLP 1-7-4 challenge
log10 RNA copy number/ml serum
** P=0.0058
** P=0.0086
P=0.9556
PBS
DS722
VR2385-S3456
7 dpc
E
RFLP 1-7-4 challenge
log10 RNA copy number/ml serum
* P=0.0338
* P=0.0473
P=0.9664
PBS
DS722
VR2385-S3456
14 dpc
F
RFLP 1-7-4 challenge
log10 RNA copy number/gram lung
* P=0.0218
* P=0.0415
P=0.9740
PBS
DS722
VR2385-S3456
14 dpc