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Attenuation of Porcine Reproductive and Respiratory Syndrome Virus by Molecular Breeding of Virus Envelope Genes from Genetically Divergent Strains

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Molecular breeding via DNA shuffling can direct the evolution of viruses with desired traits. By using a positive-strand RNA virus, porcine reproductive and respiratory syndrome virus (PRRSV), as a model, rapid attenuation of the virus was achieved in this study by DNA shuffling of the viral envelope genes from multiple strains. The GP5 envelope genes of 7 genetically divergent PRRSV strains and the GP5-M genes of 6 different PRRSV strains were molecularly bred by DNA shuffling and iteration of the process, and the shuffled genes were cloned into the backbone of a DNA-launched PRRSV infectious clone. Two representative chimeric viruses, DS722 with shuffled GP5 genes and DS5M3 with shuffled GP5-M genes, were rescued and shown to replicate at a lower level and to form smaller plaques in vitro than their parental virus. An in vivo pathogenicity study revealed that pigs infected with the two chimeric viruses had significant reductions in viral-RNA loads in sera and lungs and in gross and microscopic lung lesions, indicating attenuation of the chimeric viruses. Furthermore, pigs vaccinated with the chimeric virus DS722, but not pigs vaccinated with DS5M3, still acquired protection against PRRSV challenge at a level similar to that of the parental virus. Therefore, this study reveals a unique approach through DNA shuffling of viral envelope genes to attenuate a positive-strand RNA virus. The results have important implications for future vaccine development and will generate broad general interest in the scientific community in rapidly attenuating other important human and veterinary viruses.
bred PRRSV by DNA shuffling of the GP5 genes of 7 and the
GP5-M genes of 6 genetically divergent strains of PRRSV. The
shuffled chimeric viruses were infectious in vitro and, most im-
portantly, attenuated in pigs. This represents the first report of
successful virus attenuation by a DNA-shuffling approach. Fur-
thermore, one shuffled chimeric virus elicited protection against
PRRSV challenge at a level similar to that of its parental virus in
pigs.

MATERIALS AND METHODS

Cells and viruses. BHK-21 and MARC-145 cells were grown at 37°C in
Dulbecco's minimum essential medium (DMEM) supplemented with
10% fetal bovine serum (FBS) and antibiotics. The North American type
2 PRRSV was systematically classified into 9 genetically distinct lineages
based on the ORF5 gene sequences of 8,624 PRRSV strains (16). To pro-
duce a chimeric virus by molecular breeding, a total of 7 genetically dif-
ferent strains of PRRSV, each representing a distinct genetic lineage or
sublineage in the phylogenetic tree (16), i.e., MN184B (lineage 1), VR2385
(lineage 5.1), VR2430 (lineage 5.2), S132 (lineage 6), Chinese highly
pathogenic strain JXA1 (lineage 8.7), FL-12 (lineage 8.9), and NADC20
(lineage 9), were selected for DNA shuffling in the study. The genetic
relationship of these selected strains of PRRSV used in DNA shuffling is
shown in a phylogenetic tree (Fig. 1). The GP5 gene sequences of VR2385
and FL-12 were amplified from the infectious clones pIR-VR2385-CA
(12) and pFL-12 (5), respectively. The GP5 gene sequences of the
other 4 PRRSV strains (MN184B, S132, JXA1, and NADC20) were com-
mercially synthesized (Genscript) based on the sequences in the GenBank
database.

DNA shuffling of the GP5 and GP5-M genes. For GP5 gene shuffling,
the GP5 genes from seven strains of PRRSV were mixed in equimolar
amounts with a total of 5 μg DNA and diluted in 50 μl of 50 mM Tris-HCl
(pH 7.4) and 10 mM MgCl₂. The mixture was incubated at 15°C for 2 min
with 0.15 U of DNase I (Sigma). DNA fragments 50 to 150 bp in size were purified from 2% agarose gels. The purified DNA fragments were subsequently added to the *Pfu* PCR mixture consisting of 1× *Pfu* buffer, 0.4 mM each deoxynucleoside triphosphate (dNTP), and 0.06 U *Pfu* polymerase. A PCR program without primers (95°C for 4 min; 35 cycles of 95°C for 30 s, 60°C for 30 s, 57°C for 30 s, 54°C for 30 s, 53°C for 30 s, 48°C for 30 s, 45°C for 30 s, 39°C for 30 s, and 72°C for 2 min; and finally, 72°C for 7 min) was performed to reassemble the digested DNA fragments. Subsequently, specific primers flanking the shuffled GP5 gene region, GP5trunc-F (5′-GGGAACA
GCAGCTCAAATTTACAG-3′) and GP5trunc-R (5′-AGGGGTAGCC
CGGAACCAT-3′), were used to amplify the assembled shuffling products.

Similar approaches were used to shuffle the GP5-M genes from 6 different strains of PRRSV. Unlike GP5 shuffling, strain S132 was not included in the GP5-M gene shuffling, since the M gene sequence strain S132 was not available. Primers GP5F (5′-ATGTTGGGGAAATGCTTGA
CCG-3′) and mfu3R (5′-GCCGCAATCGGATGAAAGCCTG-3′) were used to amplify the assembled shuffling products.

**Construction of chimeric PRRSV libraries.** The shuffled product libraries were cloned into a blunt-end vector, pCR-BLUNT, to assess the quality of the DNA shuffling. The recombination efficiency was analyzed by sequencing the shuffled genes from 30 randomly selected clones to delineate crossovers. The nucleotide changes among the parental strains served as markers to delineate the origin of each fragment between two proximate crossover sites incorporated in the shuffled product. The fragment between the crossover sites with the same nucleotide pattern as a particular parental strain was considered to be derived from that parental strain (Fig. 2 and 3). The shuffled products that contained segments derived from all parental viruses and that had a good number of crossovers were selected for the study. The GP5 clone DS722 and the GP5-M clone DSSM3 were ultimately selected from their respective libraries for the construction of chimeric viruses in the backbone of a DNA-launched PRRSV infectious clone, plR-VR2385-CA (38, 39). For cloning purpose, two flanking fragments amplified from plR-VR2385-CA containing the naturally occurring unique restriction sites Acl1 and XbaI, respectively, were fused to the corresponding shuffled products, and the fusion fragments were cloned into the DNA-launched infectious clone to produce chimeric viruses containing the shuffled GP5 or GP5-M gene. The amino acid differences in GP5 among the 7 parental virus strains and the two chimeric viruses (DS722 and DSSM3) are presented in Fig. 3.

**In vitro transfection and immunofluorescence assay (IFA).** To rescue the infectious chimeric PRRSV from the recombinant DNA-launched infectious clones, BHK-21 cells at 60% confluence in 6-well plates were transfected with 3 μg of chimeric PRRSV DNA using 8 μl of Lipofectamine LTX (Invitrogen) according to the manufacturer’s instructions. At 48 h posttransfection, the cell culture supernatant was harvested and analyzed.

**FIG 2** Schematic diagram of the shuffled chimeric GP5 gene sequences in two representative chimeras (DS722 and DSSM3). The parental virus sequences of the GP5 gene for the two chimeras are depicted schematically. The exact boundaries of crossovers are indicated with nucleotide position numbers relative to the GP5 gene. Each pattern represents the sequence derived from an individual parental virus strain. If two patterns are displayed in the same region, it indicates that the region contains sequences shared by two different parental strains.

**FIG 3** Alignment of the GP5 amino acid sequences among the seven parental virus strains and the two chimeras (DS722 and DSSM3). The GP5 sequence of the backbone virus, VR2385, is shown at the top. Only differences are indicated for other strains.
passaged onto MARC-145 cells. Two days later, the cells were washed with 0.05% phosphate-buffered saline (PBS)-Tween and fixed with 80% acetone (Sigma). The fixed cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG at 37°C for 1 h. After washing three times, the cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG at 37°C for 1 h. The stained cells were visualized with a Nikon Eclipse TE300 fluorescence microscope fitted with a digital camera (Nikon).

**Plaque assay.** Confluent monolayers of MARC-145 cells cultured in a 6-well plate were infected with 10-fold serially diluted viruses (10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶, and 10⁻⁷). After 1 h of incubation, the inoculum was removed and an agar overlay was applied to the cell monolayer. Plaques were stained with neutral red solution (Sigma) 4 days postinfection at 37°C. The wells containing 10 to 100 plaques in each plate were selected to measure the diameter of each plaque. Plaque morphology and size were compared between the parental virus VR2385 and the two chimeric viruses.

**Growth characterization of the chimeric viruses in vitro.** To analyze the growth characteristics of the chimeric viruses in vitro, a growth curve was performed in MARC-145 cells, as well as in porcine alveolar macrophages (PAMs). The PAMs were obtained by lung lavage of a piglet from a PRRSV-free university research herd. Confluent monolayers of MARC-145 or PAMs seeded in 96-well plates were infected with the parental virus VR2385 and two rescued chimeric viruses (DS722 and DS5M3) at the same multiplicity of infection (MOI) of 0.1. Both the infected MARC-145 cells and PAMs were harvested at 6, 12, 24, 36, 48, 60, and 72 h postinfection (p.i.), and an additional time point at 84 h p.i. was added for the infected MARC-145 cells. The titers of virus harvested at different time points were determined by IFA in MARC-145 cells and quantified as 50% tissue culture infective doses (TCID₅₀)/ml. All in vitro experiments were performed in triplicate.

**Pathogenicity study of the two chimeric viruses in specific-pathogen-free (SPF) pigs.** To determine and compare the virulence of the two chimeric viruses and the parental virus in pigs, we used a nursery pig respiratory disease model to assess the pathogenicity of PRRSV, since the nursery pig model has been widely used worldwide for evaluating PRRSV respiratory disease model to assess the pathogenicity of PRRSV, since the nursery pig model has been widely used worldwide for evaluating PRRSV. Considering the viral-RNA loads.

**Challenge-and-protection study in SPF pigs vaccinated with chimeric viruses.** A total of 72 SPF pigs at 3 weeks of age were divided into 8 groups of 9 pigs per group and vaccinated as shown in Table 2. At 35 days postvaccination (p.v.), pigs in each group were challenged with either the parental VR2385 virus or a heterologous NADC20 virus (Table 2). At 14 days postchallenge (p.c.), all pigs were necropsied, and gross pathological lung lesions were recorded and scored (35). Lung tissues were also collected for histological examination and viral-RNA load quantification. Real-time PCR to quantify viral-RNA loads in sera and lung tissues of pigs. To quantify viral-RNA loads in lung tissues, samples of lung tissues (500 mg) collected at each necropsy were homogenized in 10% (wt/vol) sterile PBS. The homogenates were centrifuged at 3,000 rpm at 4°C for 15 min, and the supernatants were used for quantification of PRRSV RNA. Total RNAs were extracted from weekly serum samples and homogenates of lung tissues using TRIzol Reagent (MRC) and used to synthesize cDNA using a Superscript II kit (Invitrogen). PRRSV genomes were quantified using a SYBR green-based quantitative PCR (qPCR). A pair of primers (forward primer, 5'-TTAAATATGACCAATTACACGGG-3'; reverse primer, 5'-TGCTCTGACTGTTT-3') were designed based on the conserved region in the nucleocapsid gene using Beacon software. The qPCR assay was conducted in a CFX96 real-time (RT) PCR system (Bio-Rad). The reactions were performed in a 20-μl PCR volume containing 10 μl SsoFast EvaGreen Supermix (Bio-Rad), 0.5 μl of each primer (10 μM), 5 μl of the template cDNA, and 4 μl of nuclease-free water. The cycling parameters included an initial denaturation at 95°C for 10 min, followed by 39 cycles of denaturation at 95°C for 10 s and annealing and extension at 58°C for 20 s. Dissociation curve analysis was performed using the instrument’s default setting immediately after each PCR run. Each reaction was measured in triplicate.

**Necropsy and gross pathology evaluation.** All pigs were humanely euthanized by intravenous pentobarbital overdose (Fatal-Plus; Vortech Pharmaceutical, Ltd., Dearborn, MI). Veterinary pathologists were blinded to the treatment status of the pigs for evaluation of gross lung lesions. The total amount of lung affected by pneumonia (0 to 100% of the lung affected by grossly visible pneumonia) was recorded for each pig at necropsy while blinded to the treatment status, as described previously (35). Briefly, the scoring system is based on the approximate volume that each lung lobe contributes to the entire lung: the right cranial lobe, right middle lobe, cranial part of the left cranial lobe; and caudal part of the left cranial lobe contribute 10% each to the total lung volume; the accessory lobe contributes 5%; and the right and left caudal lobes contribute 27.5% each (35). Five defined sections of lungs (35) were collected, immediately immersed in 10% neutral buffered formalin, and routinely processed for histological examination. In addition, fresh lung tissues were collected separately and immediately stored at −80°C for virological testing.

**Histopathology evaluation.** Microscopic lung lesions were evaluated independently by two veterinary pathologists (T.O. and P.G.H.) blinded to the treatment status. Lung sections were scored for 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) (35). If the results obtained by the two pathologists on a certain tissue differed, the mean of the two scores was calculated.

**Statistical analyses.** A two-tailed Student’s t test was used to evaluate the differences (P < 0.05) between the samples in the two groups for both in vitro and in vivo studies. The data were analyzed using GraphPad Prism (version 5.0).

**Nucleotide sequence accession numbers.** The GP5 sequences of the parental virus strains and the two shuffled chimeras were deposited in the

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Experimental design for comparative pathogenicity study to determine virulence of shuffled chimeric viruses in pigs</th>
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<td>Group</td>
<td>Inoculum</td>
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<tr>
<td>A</td>
<td>Parental VR2385</td>
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<td>B</td>
<td>Chimera DS722</td>
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<td>C</td>
<td>Chimera DS5M3</td>
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<th>Table 2</th>
<th>PRRSV challenge and protection study in pigs vaccinated with attenuated chimeric viruses or with parental virus</th>
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<td>Group</td>
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a The challenge virus dose for all viruses was 2 × 10⁵ TCID₅₀/pig.

b One pig died from an unrelated cause before the challenge.
RESULTS

Generation of infectious chimeric viruses containing well-shuffled GP5 or GP5-M genes from 7 and 6 genetically distinct strains of PRRSV, respectively. To generate GP5-shuffled chimeric viruses with good growth fitness, we selected 96 nucleotides (nt) from the 5’ end of the GP5 gene, including the signal peptide sequence and the 16-nt region overlapping the M gene, as well as the junction site sequence from 23 to 18 nt upstream of the M gene, for DNA shuffling of the GP5 genes. The resulting 468-nt GP5 genes from seven PRRSV strains, each representing a distinct genetic lineage or sublineage (16) (Fig. 1), were shuffled with DNase I digestion, followed by PCR without primers for reassembly. A PCR product consisting of reassembled shuffled products with an expected size of 468 bp was obtained after a second PCR with specific primers flanking the shuffled region. To generate chimeras containing segments derived from all seven parental viral strains, the shuffling process was iterated by using the shuffled DNA pool from the first-round shuffling as the parents (2, 4).

Sequence analyses of 30 representative clones that were randomly selected from the shuffled library revealed that they all contained chimeric GP5 sequences, but only two clones contained sequences from all 7 parental viruses. The numbers of crossovers ranged from 8 to 12 in the shuffled GP5 gene products. The GP5 clone DS722, which contains segments derived from all seven parental viral sequences with 12 crossovers, was selected from the shuffled library (Fig. 2) and cloned into the backbone of a DNA-launched PRRSV infectious clone (39). The resulting chimeric virus, DS722, containing the shuffled GP5 genes from 7 different strains of PRRSV was successfully rescued from transfected cells (Fig. 4B).

Similar approaches were used to shuffle the region spanning the GP5-M genes of 6 distinct strains of PRRSV. Sequence analyses of 10 representative clones that were randomly selected from the shuffled library revealed that all contained the chimeric GP5 sequences, and 6 of the 10 clones contained sequences derived from all 6 parental viruses. The numbers of crossovers ranged from 12 to 22 in the shuffled GP5 gene products. The chimeric GP5-M clone DS5M3, containing segments derived from all 6 parental viral sequences with 18 crossovers (Fig. 2), was selected and cloned into the backbone of the DNA-launched PRRSV infectious clone. The GP5-M chimeric virus DS5M3 was successfully rescued from transfected cells (Fig. 4C). The amino acid differences in GP5 among the parental and shuffled viruses are indicated in Fig. 3.

The two chimeric viruses replicated at a lower level, both in MARC-145 cells and in PAMs, and formed smaller plaques in MARC-145 cells than the parental virus. To characterize and compare the growth characteristics between the two chimeric viruses (DS722 and DS5M3) and the parental virus (VR2385), the growth kinetics of the three viruses were analyzed by infection of MARC-145 cells or PAMs with the respective virus at the same MOI of 0.1. The infectious-virus titers were determined at different times p.i. The results showed that, in MARC-145 cells, the chimeric virus DS722 replicated to significantly lower levels than the parental virus, VR2385, at all time points except 6 and 12 h p.i. The other chimeric virus, DS5M3, displayed a significantly lower level of replication at all time points than VR2385 (Fig. 5B).

Both chimeric viruses and the parental virus developed plaques within 4 days p.i. in MARC-145 cells. However, the diameters of the plaques formed by the two chimeric viruses were significantly smaller than that formed by the parental virus (P < 0.001) (Fig. 5C), further indicating that the chimeric viruses had a lower growth capacity in vitro than the parental virus.

Both chimeric viruses (DS722 and DS5M3) were attenuated in pigs. The pathogenicity study revealed that pigs infected with the chimeras DS5M3 had significantly lower serum viral-RNA loads than pigs infected with the parental virus, VR2385, at both 7 (P = 0.02) (Fig. 6A) and 14 (Fig. 6B) days p.i. (P = 0.0009). Similarly, the serum samples from pigs infected with chimera DS722 also had lower viral-RNA loads than pigs infected with the parental virus at both 7 and 14 days p.i., and the difference was significant at 7 days p.i. (P = 0.03) (Fig. 6A), but not at 14 days p.i., although most sera from the DS722 group displayed viral-RNA loads lower than those from the VR2385 group at 14 days p.i. (Fig. 6B).

Similarly, pigs infected with the chimera DS5M3 had significantly lower viral-RNA loads (P < 0.0001) in the lung tissues than pigs infected with VR2385. The pigs infected with the chimera DS722 also had a lower viral load in the lung than pigs infected with the parental virus, although the difference was not significant (Fig. 6C).

Macroscopic lung lesions were generally absent or mild in pigs inoculated with DMEM and with the two chimeras, DS5M3 and DS722 (Fig. 7A). In pigs infected with the parental VR2385 virus, visible gross lung lesions were more pronounced and affected an average of 42% of the lung surfaces (Fig. 7A). The mean scores of
the gross lung lesions in pigs inoculated with the two chimeric viruses, DS722 (P = 0.005) and DS5M3 (P < 0.0001), were significantly lower than that of the pigs inoculated with the parental virus, VR2385 (Fig. 7A). The mean scores of the histological lung lesions in pigs infected with the chimeric viruses DS722 (P = 0.0002) and DS5M3 (P < 0.0001) were significantly lower than that in pigs infected by the parental VR2385 virus (Fig. 7B).

Chimera DS722, but not chimera DS5M3, elicited protection in pigs against PRRSV challenge at a level similar to that in the parental virus. To investigate whether the two attenuated chimeras could still elicit protection against PRRSV, pigs were first vaccinated with the parental virus, VR2385; the chimera DS722 or DS5M3; or DMEM (Table 2). Eight or nine vaccinated pigs each in groups 1, 3, 5, and 7 were then challenged at 35 days p.v. with the parental VR2385 virus (lineage 5). Nine vaccinated pigs each in groups 2, 4, 6, and 8 were challenged at 35 days p.v. with a heterologous NADC20 virus (lineage 9) (Table 2). At the time of challenge at 35 days p.v., viremia was not detected in any of the pigs by RT-PCR. All pigs were necropsied at 14 days p.c.

For the pigs challenged with the parental strain VR2385, at 7 days p.c., the serum viral-RNA loads significantly decreased in pigs vaccinated with VR2385 (P = 0.005) or with the chimera DS722 (P = 0.003), but not in pigs vaccinated with the chimera DS5M3, compared to group 7 control pigs (Fig. 8A). Similarly, at 14 days p.c., the serum viral-RNA loads significantly decreased in pigs that were vaccinated with VR2385 (P = 0.01) or with the chimera DS722 (P = 0.01), but not in pigs vaccinated with the chimera DS5M3, compared to group 7 control pigs (Fig. 8B). The reduction of the serum viral-RNA loads against challenges with parental VR2385 and heterologous NADC20 in pigs vaccinated with the chimera DS722 was similar to that in pigs vaccinated with the parental virus, VR2385, at both 7 and 14 days p.c. (Fig. 8A and B). The viral-RNA loads in the lung tissues at 14 days p.c. were significantly decreased in pigs vaccinated with the parental virus, VR2385 (P = 0.002), or with the two chimeras DS722 (P < 0.0001), but not in pigs vaccinated with the chimera DS5M3, compared to group 7 control pigs (Fig. 8C). The pigs vaccinated with the chimera DS722 displayed significantly lower viral-RNA loads in the lung tissues than the pigs vaccinated with VR2385 (Fig. 8C).

For pigs challenged with a heterologous NADC20 strain, at 7 days p.c., the serum viral-RNA loads were significantly lower in pigs vaccinated with the parental virus (P = 0.0002) or with the two chimeras DS722 (P = 0.0002) and DS5M3 (P = 0.02) than in group 8 controls (Fig. 8A). Similarly, at 14 days p.c., there were significant reductions in the serum viral-RNA loads in pigs vaccinated with the parental virus, VR2385 (P = 0.01), or with two
chimeras, DS722 (P = 0.03) and DS5M3 (P = 0.02), compared to group 8 control pigs (Fig. 8B). Also, the viral-RNA loads in the lung tissues at 14 days p.c. were significantly reduced in pigs vaccinated with the parental virus, VR2385 (P = 0.002), and with the chimera DS722 (P = 0.01), but not in pigs vaccinated with the chimera DS5M3, compared to group 8 control pigs (Fig. 8C). The reduction of viral-RNA loads in the lung tissues of pigs vaccinated with the chimera DS722 was similar to that in pigs vaccinated with the parental virus, VR2385, at both 7 and 14 days p.c. (Fig. 8C).

At necropsy, the average scores of both macroscopic and microscopic lung lesions in pigs vaccinated with two chimeras (groups 3, 4, 5, and 6) were significantly lower than those in the control pigs in groups 7 and 8 (Fig. 9A and B). The protection, based on the macroscopic and microscopic lung lesions, was much more effective in the DS722-vaccinated pigs than in DS5M3-vaccinated pigs. The average scores of gross and microscopic lung lesions in DS722-vaccinated pigs were mostly similar to those in VR2385-vaccinated pigs, although the scores in DS5M3-vaccinated pigs were significantly higher than those in VR2385-vaccinated pigs (Fig. 9).

Both chimeras DS722 and DS5M3 were stable in vivo. The shuffled genes of chimeric viruses DS722 and DS5M3 recovered from the sera of pigs in the respective groups at 14 days p.i. were amplified by RT-PCR and sequenced. Sequence analyses revealed that the sequences of the recovered viruses were the same as those of the original virus inocula, indicating the genetic stability of these two chimeric viruses in animals.

**DISCUSSION**

Molecular breeding through DNA shuffling can direct the evolution of viruses in vitro and select new strains with desired traits. To determine if molecular breeding of virus envelope genes that are important virulence determinants can produce an attenuated virus that retains its protective ability against challenge, we bred the GP5 genes of 7 and the GP5-M genes of 6 genetically distinct strains of PRRSV by DNA shuffling and iteration of the shuffling process. The application of iteration of the DNA-shuffling process increased the chances to incorporate all parental viral genes into the small GP5 region (2, 4). Two representative chimeric viruses, a GP5 chimera, DS722, and a GP5-M chimera, DS5M3, were rescued and selected for further studies. Although both chimeras were infectious in vitro, they both displayed a lower level of virus replication in both MARC-145 cells and PAMs. In addition, both chimeric viruses formed smaller plaques in MARC-145 cells than the parental virus, indicating that the two shuffled chimeric viruses exhibited an attenuated phenotype in vitro.

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**FIG 7** Macroscopic and microscopic lesions in the lung tissues from pigs experimentally infected with the parental virus, VR2385, and the two chimeric viruses DS722 and DS5M3 during necropsy at 14 days p.i. (A) Macroscopic-lesion scores of lung tissues at 14 days p.i. from the 6 pigs infected with the parental virus, VR2385, and the chimeric viruses DS722 and DS5M3 or inoculated with DMEM (negative control). (B) Microscopic-lesion scores of lung tissues at 14 days p.i. Significant differences are indicated with asterisks (**, P < 0.01; ***, P < 0.001).
To further determine whether DNA shuffling of the GP5 or GP5-M gene altered virus virulence in vivo, we conducted a pathogenicity study (Table 1) and showed that there was a significant reduction in both the macroscopic- and microscopic-lung-lesion scores in pigs infected with the two chimeras compared to those infected with the parental virus. Significant reductions in viral-RNA loads in sera and lung tissues were also found in pigs infected with the chimera DS5M3. The in vitro growth and the in vivo pathogenicity studies indicated that both chimeras were attenuated. Therefore, rapid attenuation of PRRSV was achieved in this study by shuffling of the virulence determinant GP5 genes from multiple genetically divergent virus strains. It is important to note that GP5 is not the sole gene responsible for PRRSV virulence (37), and thus, DNA shuffling of other PRRSV genes also involved in virulence in the future may further improve virus attenuation. Nevertheless, this unique DNA-shuffling approach to attenuate a virus is more advantageous than many other traditional reverse-genetics system approaches in that DNA shuffling mimics the natural evolution of viruses and does not require an understanding of the functionality of the shuffling regions; rather, the approach relies on functional screening for the desired traits of the shuffled viruses, such as the attenuation phenotype in this study.

Since the two chimeric viruses displayed an attenuated phenotype in vivo, we next evaluated whether the chimeric viruses could still induce protection against PRRSV challenge. Eight groups of pigs were first vaccinated with the parental virus, VR2385; the two chimeras; or DMEM (Table 2). At 35 days p.c., pigs in each group were challenged with a homologous (lineage 5) or a heterologous (lineage 9) PRRSV. The results revealed that the chimera DS722 still elicited solid protection against challenges by both homologous and heterologous PRRSV strains. However, the GP5-M-shuffled chimeric virus DS5M3 did not induce a sufficient level of protection, even though there was a significant reduction in macroscopic- and microscopic-lung-lesion scores. Thus, the GP5-shuffled chimeric virus DS722 still retains its ability to elicit protection against PRRSV, suggesting that the DNA shuffling of the virulence determinant gene attenuated the virus but did not impair the ability of the shuffled virus to elicit protection.

We had initially thought that the GP5-M chimera DS3M3 would also retain its ability to elicit protection, since GP5 and M form heterodimers (45). The observed poor protection of the chimera DS5M3 was likely due to the low replication fitness of the chimera in vivo, since only low levels of chimera DS5M3 viral RNA were detected in both sera and lung tissues in the pathogenicity study. The GP5-M DNA shuffling included some critical regions for virus replication, such as the GP5 signal peptide sequence and the overlapping region, and thus, shuffling of these critical regions likely affected the viral replication efficiency in vivo, leading to overattenuation of the chimera DS5M3 and thus poor protection compared to the GP5 chimera, DS722. In addition, glycosylation of the major envelope protein GP5 is known to play an important role in PRRSV virulence (46). For the chimera DS5M3, it appears that DNA shuffling resulted in the loss of an important glycosylation site at amino acid position 34 of the chimera compared to its parental virus, VR2385 (Fig. 3), and this might contribute to the virus attenuation phenotype of the chimera (46). Although the exact mechanism of attenuation by DNA shuffling remains unknown, the attenuation phenotype of the shuffled viruses may be attributed to the altered growth efficiency of the chimeric viruses. In addition, potential conformational changes of the shuffled GP5 in the chimeras may alter its interactions with other viral proteins or host cells, leading to attenuation (6).

In conclusion, attenuation of a virus by DNA shuffling of its envelope genes was demonstrated for the first time. We successfully produced two chimeric viruses that displayed attenuated phenotypes both in vitro and in vivo by shuffling the GP5 gene containing major virulence determinants and the GP5-M genes. The attenuated shuffled virus DS722 still induced protection similar to that of its parental virus against PRRSV infection. Although development of an improved PRRSV vaccine with better protection was not within the scope of the present study, it is logical to speculate that further shuffling of other structural genes, such as GP3 and GP4, which are relevant for neutralizing activities, in the future may lead to the generation of a more broadly protective PRRSV modified live-attenuated vaccine. Therefore, attenuation of a positive-strand RNA virus by DNA shuffling, as demonstrated in this study, has important implications for potential future vaccine development and thus is of broad general interest to the scientific community, as this approach of rapid virus attenuation can be easily applied to other important human and veterinary viruses.

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