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RESEARCH ARTICLE

Development of Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) mRNA Vaccine against Highly Pathogenic PRRSV Challenge

Mirwaise Khan[1#], Xinqi Shi[1#], Ziyi Wei[1], Fandan Meng[1], Peiyu Xiao[1], Tao Wang[1], Lingzhi Luo[1], Dasong Xia[1], Tongqing An[1], Haiwei Wang[1,2*] and Xuehui Cai[1,3*]

[1]State Key Laboratory for Animal Disease Control and Prevention, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin, 150069, China
[2]Heilongjiang Provincial Key Laboratory of Veterinary Immunology, Harbin 150069, China
[3]Heilongjiang Veterinary Biopharmaceutical Engineering Technology Research Center, Harbin 150069, China
#These authors contributed equally to this work
*Corresponding authors: wanghaiwei@caas.cn (HW); caixuehui@caas.cn (XC)

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ABSTRACT

Infection with the Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) results in a chronic and occasionally severe illness that affects pregnant sows and is characterized by respiratory issues, weight loss, poor growth performance, and reproductive failure. The emerged messenger RNA (mRNA) is a promising approach to preventing various diseases due to its favorable safety profile, ease of design, and scalable production. In this study, we developed a messenger RNA (mRNA) vaccine against a highly pathogenic PRRSV strain HuN4. Recombined multiple antigenic proteins, including GP5-M, GP3-NSP9, and GP2-GP4, were designed and codon-optimized. Indirect immunofluorescence assay (IFA) and Western blot detected the expression levels of different mRNA-LNPs. The outcomes of IFA demonstrated that GP3-NSP9 and GP2-GP4 had stronger fluorescence in their mRNA-LNP expressions, GP3-NSP9 expressing themselves better than GP2-GP4. Conversely, GP5-M exhibited hardly little fluorescence. The GP2-GP4 and GP3-NSP9 fusion proteins were expressed in the cells, according to the Western blot data. However, GP5-M was not. The GP3-NSP9 and GP2-GP4 were used to immunize pigs alone or in combination. The challenge of PRRSV HuN4 after immunization revealed that N protein antibody titers and viral load in the blood and lungs were much lower than those of mock-challenged pigs. All piglets were euthanized, and their lungs were examined macroscopically and histopathologically. In addition, the GP2-GP4 and GP3-NSP9 combined mRNA immunization showed effective and protective immune response than GP3-NSP9 mRNA individual immunization.

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INTRODUCTION

Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) is one of the most economically significant pathogens globally and causes substantial economic losses. Porcine reproductive and respiratory syndrome (PRRS) originated in 1980s from North America and expanded to Europe (Lugo Mesa *et al.*, 2024). Estimated annual losses caused from PRRSV alone exceeding $600 million in the USA and €1.5 billion in Europe. Clinical signs of respiratory disease associated with pneumonia in pigs of all ages and problems with sow reproduction, including premature farrowing or late-term abortion, as well as a higher rate of stillborn piglets, are the hallmarks of PRRS.

Along with Coronaviridae and Roniviridae, members of the Arteviridae family within the Nidovirales order, Betaarterivirus suid 1 (PRRSV-1) and Betaarterivirus suid 2 (PRRSV-2) are responsible for PRRS (Brinton *et al.*, 2021). PRRSV is a single-stranded positive-strand enveloped RNA virus. The genes that code for structural proteins are located at the 3′ end of the approximately 15 kb PRRSV genome, while the genes that code for replication are located at the 5′ end. The genome of

PRRSV has more than 10 open reading frames (ORFs). While ORFs 2–7 encode eight structural proteins, including glycoprotein (GP) 2, envelope (E), GP3, GP4, GP5, ORF5a, membrane (M), and nucleocapsid (N) protein, more than two-thirds of the viral genome is encoded by ORF1a and ORF1b, which also encode fourteen non-structural proteins that are critical for viral replication, including NSP1a, NSP1b, NSP2-6, NSP7a, NSP7b, and NSP8-12.

According to the most recent classification, PRRSV is classified into two species, PRRSV-1 and PRRSV-2, under the Porartevirus genus (Zhao *et al.*, 2022). Although strains of PRRSV-1 and PRRSV-2 have different serotypes, they share only 60% nucleic acid sequence identity and cause similar disease phenotypes, clinical symptoms, genomic structure, and incubation period (Martín-Valls *et al.*, 2023).

The major envelope proteins GP5 and M, coded by open reading frames 5 and 6, form a heterodimer connected by a disulfide bond. The GP5 has a variety of glycosylation sites, depending on the heterologous PRRSV strains. The non-glycosylated M protein is a 19 kDa protein and is believed to have three hydrophobic connected regions that together form several membrane-spanning domains. Furthermore, for both proteins to be transported from the endoplasmic reticulum to the Golgi apparatus efficiently, only a small portion of M, the surface of the virion displays the first 16 amino acids at the amino terminus.

PRRSV all viral protein that interacts with the host during PRRSV infection. The RNA-dependent RNA polymerase structure of the NSP9, which is around 72–95 kDa in PRRSV-infected MARC-145 cells, formed key for PRRSV replication (Zhang *et al.*, 2022). The T cell epitopes discovered in the NSP9 sequence are highly advantageous for next-generation vaccines. More GP3 is secreted by PRRSV-1 strains than PRRSV-2 strains, the GP3 is 45–50 kDa. One of the most important factors in pig PRRSV infection is the released GP3. PRSSV-1 and strains of PRSSV-2, GP3 proteins have been found to include many linear antigenic regions; nevertheless, antibodies targeting most of these epitopes cannot neutralize the virus. PRRSV evades the antibody response in pigs inoculated experimentally and reported that it alters GP3 and GP5 primary glycoproteins (Zhang et al., 2018). GP2 and GP4 were two of the minor envelope glycoproteins usually 29 kDa and 31 kDa, in size respectively. Many studies reported that all the additional GPs were discovered to interact with GP2 and GP4 proteins, creating a complex protein structure. While it has been proposed that GP4, which is recognized as the target of a monoclonal antibody that neutralizes PRRSV, might also play a part (Liu *et al.*, 2024). In addition to acting as the viral attachment protein that mediates contacts with CD163 for virus entrance into susceptible host cells, the GP4 protein is essential for mediating interglycoprotein connections. It also functions in tandem with GP2a.

PRRSV has specific tropisms for the monocyte-macrophage lineage cells. These cells are primarily immune cells that are susceptible to PRRSV infection (Xu *et al.*, 2022). PRRSV vaccines primarily include attenuated and inactivated vaccines, with the ongoing development of subunit, DNA, and virus-vectored vaccines (Madapong *et al.*, 2020b), the protective effect of inactivated vaccines is unsatisfactory. Although the immune effect of attenuated vaccines is good, their potential safety issues preclude them from being an ideal PRRSV vaccine. Currently available vaccines only protect against homologous strains of PRRSV; they are unable to effectively cross-protect against heterologous strains. Thus, it is imperative to create new vaccines that are safe and efficient against both homologous and heterologous strains of PRRSV (Nan *et al.*, 2017a).

Recently, mRNA vaccines have gained much attention for their potential to accelerate vaccine development and offer protection against virus challenges. mRNA vaccines have considerable advantages, including better safety profiles, non-infectiousness, rapid production, enhanced efficacy, and immune response. Despite the antigens encoded by the mRNA, consistent techniques in vaccine production simplify the process, ensuring timely and scalable vaccine development. The major antigenic viral proteins involved in protection are envelope proteins GP5 and M. The GP5 has a variety of glycosylation sites, depending on the heterologous PRRSV strains. The non-glycosylated M protein is believed to have three hydrophobic connected regions that together form several membrane-spanning domains (Luo *et al.*, 2023). Additionally, the NSP9 encoding the RNA-dependent RNA polymerase structure, which is crucial for PRRSV pathogenicity (Zhang *et al.*, 2022). The T cell epitopes discovered in the NSP9 sequence are highly advantageous for next-generation vaccines. Moreover, the GP3 plays a crucial role in PRRSV infection (Lv *et al.*, 2024). PRRSV evades the antibody response in pigs inoculated experimentally and reported that it alters GP3 and GP5 primary glycoproteins (Zhang *et al.*, 2023a). Besides the minor envelope glycoproteins GP2 and GP4 were discovered to interact with GP3, creating a complex protein structure. It has been proposed that GP4 is recognized as the target of a monoclonal antibody that neutralizes PRRSV (Perez-Duran *et al.*, 2024), suggesting that GP4 could induce neutralizing antibodies. In addition to acting as the viral attachment protein that mediates contacts with CD163 for virus entrance into susceptible host cells, the GP4 protein is essential for mediating inter glycoprotein connections.

Several highly pathogenic PRRSV virulent strains of PRRSV-2, including JXAI and HuN4 are the etiological agents of many PRRS outbreaks (Ruedas-Torres *et al.*, 2021). This study aimed to develop an mRNA vaccine against PRRSV, in which GP2, GP3, GP4, GP5, NSP9 and M proteins of PRRSV HuN4 were selected as antigens. To increase protein expression, the trans-membrane domains and signal peptides of the GP2, GP3, GP4, and GP5 proteins were eliminated. To create mRNA vaccines, the GP5-M, GP3-NSP9, and GP2-GP4 proteins were constructed. To assess the effectiveness following PRRS mRNA vaccine vaccination, the pigs were challenged with PRRSV HuN4. The results showed that N protein antibody titers and the virus load in the blood were much lower than those of the mock challenge indicating that the mixed immunization of GP3-NSP9 and GP2-GP4 provides better immune protection than GP3-NSP9 alone.

MATERIALS AND METHODS

Cell culture and virus: High glucose Dulbecco's modified Eagle's medium (DMEM) Gibco Grand Island, NY, USA, supplemented with 10% fetal bovine serum (FBS, Gibco), 100 IU/mL penicillin, and 100 IU/streptomycin (1% PS solution, Corning) was used to maintain and subculture HEK293T and Marc-145 cells that were acquired from the American Type Culture Collection (ATCC). The highly pathogenic PRRSV HuN4 (GenBank accession no. EF635006), used in this study, isolated and preserved in our laboratory, previously described by Tong *et al.* (2007) and Zhou *et al.* (2008).

PRRSV mRNA sequence optimization synthesis and lipid nanoparticles (LNPs) encapsulation: The sequences of GP2, GP3, GP4, GP5, M and NSP9 referenced the prototype PRRSV-2 strain HuN4 (Genbank ID: EF635006). We designed and synthesized three fusion proteins, named GP2-GP4, GP3-NSP9 and GP5-M. The trans-membrane domain and signal peptides of GP2, GP3, GP4 and GP5 were predicted by TMHMM. In brief, the amino acid sequences of GP2, GP3, GP4, and GP5 were inputted to predict trans-membrane domains and signal peptides on https://dtu.biolib.com/DeepTMHMM, GP2 and GP4 were fused by GS-linker (GGGGSGGGGSGGGGS). A tPA-secreting peptide (MDAMKRGLCCVLLLCGAVFVSPS) was added to the 5' end of GP2-GP4, and 3×Flag (DYKDHDGDYKDHDIDYKDDDDK) tags were added to the 3' end of GP2-GP4. GP3 and NSP9 were fused in the same way as the GP2-GP4 fusion protein. GP5 and M were fused by GS-linker (GGGGSGGGGSGGGGS). GP5 sequence was modified, and NetNGlyc was used to predict GP5 glycosylation sites by inputting the amino acid sequence of GP5 on https://services.healthtech.dtu.dk/services/NetMHCpan-4.1/. and GP5 glycosylation sites were mutated to N30T, N34S, N35S, N44T and N51R. We added an additional median epitope (SHLQLIYTL) to the 5' end of GP5. tPA-secreting peptide was added before the modified GP5 protein and 3×Flag was added after M protein. DNAs codon-optimized, mRNAs synthesized and LNP encapsulated were completed by a commercial company (CSPC Pharmaceutical Group Limited China).

PRRSV mRNA-LNPs transfection into HEK293 T cells: The HEK293T cells were seeded in 12-well plates with 1×10^5 cells per well. When the cell monolayer reached an 80% confluency, HEK293T cells were transfected with mRNA-LNP of GP5-M, GP3-NSP9 or GP2-GP4 with the mixture of 2 μg of each mRNA-LNP and 200μL DMEM. After 6 h of incubation at 37 °C, the cells were transferred to a new DMEM containing 10% FBS for a further 24h. LNP-only transfected cells served as a negative control.

Indirect immunofluorescence assay (IFA): After being fixed with 4% paraformaldehyde for 30min at room temperature, the HEK293T cells were triple washed with PBS. 500μL of permeabilization solution (0.2% Triton-X100) per well was added, and the wells were left at room temperature for 15min. Following three PBS washes, a blocking solution containing 1% BSA was applied for 1 h. Following three additional washes with PBS, anti-Flag mouse monoclonal antibody (F1804, Sigma Adrich, USA) diluted 1:1000, was added to the wells as a primary antibody and incubated at room temperature for 1h at 37°C. After three PBS washes, a goat anti-mouse secondary antibody labeled with fluorescein isothiocyanate (FITC) (Sigma, Burlington, MA, USA) was added at a dilution of 1:200 and incubated for 1h at 37°C. Following three PBS washes, the samples were viewed using the EVOS FL Auto 2 Cell Image System.

Western blot: The cells were lysed with 200μL RIPA high-efficiency lysis buffer containing 1% PMSF (Solarbio, China) for 20 min on ice. After centrifuging the cell lysates for 10min at 12,000 rpm/min, the supernatant was gathered and put through SDS-PAGE. The blot was washed three times with PBS-T after the protein was moved to a polyvinylidene difluoride membrane (PVDF) (ISEQ00010, Millipore, Germany) and blocked with 5% skim milk in PBS (BD USA) for one h at room temperature. Anti-Flag mouse monoclonal antibody diluted 1:10000 was used as a primary antibody, while anti-β-actin loading control mouse monoclonal antibody (66009-1-Ig proteintech, Rosemont, USA) diluted 1:10000 was used as internal control, incubated at room temperature for 1h, and washed with PBS-T three times. The secondary antibody, Alexa Fluor 680-Goat anti-mouse IgG (H+L) (Biodragon, China, 1:10000), was first incubated for one h at room temperature before being rinsed three times with PBS-T. Finally, an infrared fluorescence scanning imaging equipment (Licor Odyssey, Lincoln, NE, USA) was used to scan the blot.

Animal experiment: Four-week-old Duroc, male piglets were kept in Harbin Veterinary Research Institute based animal center in Harbin, and all piglets tested negative for PRRSV and the classical swine fever virus by commercial IDEAXX ELISA kits and RT-qPCR. The following 4 groups of pigs were designed with 3 pigs in each group, including the HuN4-F112 attenuated vaccine control group, combined mRNAs immunization group (GP2-GP4 and GP3-NSP9), mRNA individual immunization group (GP3-NSP9), and negative control group (mock). On day 0, pigs were immunized with commercial HP-PRRSV vaccine (HuN4-F112 Strain, Weike China) with 2mL per pig as the HuN4-F112 attenuated vaccine control group. The pigs were intramuscularly administered with l00 μg/pig of the PRRSV mRNA-LNP of GP2-GP4 and GP3-NSP9 as a combined mRNAs vaccine group and administered with mRNA-LNP of GP3-NSP9 as an mRNA individual group with the same dose. The same dosage of DMEM was administered to the piglets as negative control. A similar protocol was repeated on day 21 as a second immunization. Two weeks after the second immunization, each piglet was challenged with 3mL of PRRSV HuN4 strain at $10^{5.5}$ $TCID_{50}$/mL, and 1mL and 2 mL were injected intramuscularly and through the nostrils. The clinical symptoms in the test animals were observed daily, and rectal temperatures were tested daily after the challenge. The blood was collected at 0, 7, 14, and 21 days post-challenge (dpc), and the viral load in the blood was measured. A commercial ELISA (IDEXX,

America) analyzed the antibody level against the PRRSV N protein. At 21 dpc, all piglets were euthanized.

Analyses of antibodies against PRRSV N protein: Blood was collected on days 0, 7, 14, and 21dpc. The antibody to PRRSV N protein in the serum was found using an ELISA kit (IDEXX, America) in accordance with the manufacturer's instructions. Briefly, 100µL diluted samples were added into appropriate wells. Incubated for 30min at 18-26°C. Following the washing of each well with wash solution 3-5 times. Then, dispense 100µL of conjugate into each well. Incubate it again for 30 min at 18-26°C. Remove the solution again after washing each well three times with 300µL of wash solution. After the last wash, tap each plate onto absorbent material to ensure that any remaining wash liquid is removed. Distribute 100µL of a chromogenic substrate solution (TMB) into each well. Incubate it for 15min at 18-26°C by dispensing 100µL of stop solution in each well. Following and recording the A (650) for samples and controls. The S/P ratio was calculated according to the formula, S/P ratio = 100 x Corrected optical density (COD) sample /COD positive reference serum (Walker and Crowther, 2009).

Flow cytometry: After being separated at 28dpc, the piglets' peripheral blood lymphocytes were put into a 1.5 mL centrifuge tube with 1 × 106 cells per tube and given a single PBS wash. Following staining with fluorescent antibodies, the pellet was resuspended in 100µL of PBS which included Mouse Anti-Porcine CD3ε-SPRD (SouthernBiotech USA), Mouse Anti-Porcine CD4-FITC (SouthernBiotech USA) and Mouse Anti-Porcine CD8α-PE (SouthernBiotech USA). The mixture was incubated on ice in the dark for 30 min. After centrifuging the tubes for 5min at 500rpm and discarding the supernatant, the pellet was rinsed three times with PBS. 500µL of a cell stain buffer (FBS) (554656, BD, USA) was used to resuspend the cell pellet. The number of CD4+ and CD8+ T lymphocytes per 1 × 105 cells was then calculated using flow cytometry on an Accuri C6 Plus device (BD Biosciences). To analyze the data, FlowJo v10.8.1 was used.

Histopathology: Necropsies of euthanized pigs were performed to assess potential gross lung lesions. Additionally, lung tissue samples were obtained for histological analyses and preserved for 48 to 72h in 10% buffered formalin. Subsequently, they underwent a series of alcohol solution immersions to dehydrate and were regularly imbedded in paraffin (Paraplast Plus®, Leica Biosystems, Germany). Hematoxylin and eosin (H&E) was used to stain dewaxed slices that were 2-5µm thick for histopathological analysis.

Quantitative PCR analysis: On day 21 after the challenge, lung samples were taken from each pig to determine the virus copy number. The PRRSV RNA was then isolated using a virus extraction kit (TIANGEN, China), and the mRNA was transcribed using a One Step PrimeScriptTM RT-PCR kit (TaKaRa Dalian, China) for quantitative PCR analysis (Chen *et al.*, 2019a).

Statistical analysis: The mean ± standard deviation (SD) was used to express the results. The GraphPad Prism version 9.00 (GraphPad, USA) was used to analyze the data utilizing the t-test. P-values below 0.05 were considered significant.

RESULTS

PRRSV mRNA vaccine sequence design and construction: After removing the signal peptides and trans-membrane domains of GP2, GP3, GP4, and GP5, glycosylation sites were modified to replace the decoy epitope of GP5 with a second neutralizing epitope. It has been suggested that mutate on the glycosylation site of the second neutralizing epitope, facilitating the production of neutralizing antibodies. The amino acid sequences of G2 to G5 are shown in Table 1 and the signal peptides, trans-membrane domains and glycosylation site were indicated in different colors. Besides, the construction of mRNA vaccine is shown in (Fig. 1), GP2 and GP4 were fused by GS-linker (GGGGSGGGGSGGGGS). A tPA-secreting peptide (MDAMKRGLCCVLLLCGAVFVSPS) was added to the 5' end of GP2-GP4, which improved the expression and helped secrete proteins outside of cells, and 3×Flag (DYKDHDGDYKDHDIDYKDDDDK) tags were added to the 3' end of GP2-GP4. Both the M and NSP9 sequences use the full-length sequence, and GP3 fusion with NSP9 and GP5 fusion with M use the same strategy (Fig. 1).

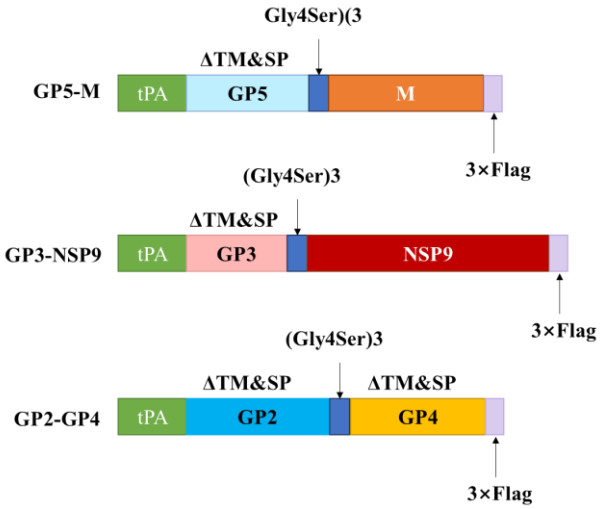

Fig. I: Schematic diagram of PRRSV HuN4 mRNA design. GP5-M fusion protein includes tPA-secreting peptide, modified GP5 protein, GS-linker, M protein and 3×Flag from 5' end to 3' end. GP3-NSP9 includes tPA-secreting peptide, modified GP3 protein, GS-linker, NSP9 protein and 3×Flag from 5' end to 3' end, while GP2-GP4 included tPA-secreting peptide, modified GP2 protein, GS-linker, modified GP4 protein and 3×Flag from 5' end to 3' end. In ΔTM&SP the transmembrane domain and single peptide were deleted. (Gly4Ser)3 is followed by GS-linker, GGGGSGGGGSGGGGS.

Expression of mRNA-LNPs *in vitro*: The HEK293T cells were transfected by 2µg each mRNA-LNP of GP2-GP4, GP3-NSP9 and GP5-M, which were collected 24 h after transfection, and the expression of mRNA-LNP was analyzed by IFA (Fig. 2) and Western blot (Fig. 3). The results confirmed that the expressions of positive control

Table I: Amino acid sequence for PRRSV antigens.

Proteins	Amino acid sequence before modification X: Signal peptide X: Trans membrane region X: Glycosylation site	Modified Amino acid sequence
GP2	MKWGLCKASLTKLANFLWMLSRNFWCPLLISSYFWPFCLASQSPV GWWSYASDWFAPRYSVRALPFTLSNYRRSYEAFLSQCQVDIPTW GVKHPLGVLWHHKVSTLIDEMVSRRMYRIMEKAGQAAWKQVVSE ATLSRISGLDVVAHFQHLAAIEAETCKYLASRLPMLHNLRLTGSNVT IVYNSTLDQVFAIFPTPGSRPKLHDFQQWLIAVHSSIFSSVAASCTLF VVLWLRIPMLRSVFGFRWLGATFLLNSW	PFCLASQSPVGWWSYASDWFAPRYSVRALPFTLSNY RRSYEAFLSQCQVDIPTWGVKHPLGVLWHHKVSTLI DEMVSRRMYRIMEKAGQAAWKQVVSEATLSRISGL DVVAHFQHLAAIEAETCKYLASRLPMLHNLRLTGSN VTIVYNSTLDQVFAIFPTPGSRPKLHDFQQRIPMLRSV FGFRWLGATFLLNSW
GP3	MANSCTFLHIFLRCSFLYSFCCAVVANSNATFCFWFPLVRGNFSFEL MVNYTVCPLCPTRQAAAEILEPGKSLWCRIGHDRCSENDHDELGF MVPPGLSSEGHLTSVYAWLAFLSFSYTAQFHPEIFGIGNVSQVYVDIK HQFICAVHDGDNATLPRHDNISAVFQTYYQHQVDGGNWFHLE WLRPFFSSWLVLNVSWFLRRSPANHVSVRVFRTSKPTPPQHQTSLSS RTSAALGMATRPLRRFAKVLSAARR	TFCFWFPLVRGNFSFELMVNYTVCPLCPTRQAAAEIL EPGKSLWCRIGHDRCSENDHDELGFMVPPGLSSEGH LTSVYAWLAFLSFSYTAQFHPEIFGIGNVSQVYVDIK HQFICAVHDGDNATLPRHDNISAVFQTYYQHQVD GGN
GP4	MAASFLFLLVGFKCFVVSQAFACKPCFSSSLSDIKTNTTAASDFVVL QDISCLRHGDSSSPTIRKSSQCRTAIGTPVYITITANVTDENYLHSSD LLMLSSCLFYASEMSEKGFKVVFGNVSGIVAVCVNFTSYVQHVKEFT QRSLVVDHVRLLHFMTPETMRWATVLACLFAILLAI	CKPCFSSSLSDIKTNTTAASDFVVLQDISCLRHGDSSS PTIRKSSQCRTAIGTPVYITITANVTDENYLHSSDLLM LSSCLFYASEMSEKGFKVVFGNVSGIVAVCVNFTSYV QHVKEFTQRSLVVDHVRLLHFMTPE
GP5	MLGKCLTACCCSRLLFLWCIVPFYLAVLVNASNNNSSHIQLIYNLT LCELNGTDWLAQKFDWAVETFVIFPVLTHIVSYGALTTSHFLDTV GLATVSTAGYYHGRYVLSSIYAVCALAALICFVIRLAKNCMSWRYS CTRYTNFLLDTKGRLYRWRSPVIVEKGGKVEVKGHLIDLKRVVLD GSAATPLTRVSAEQWGRL	SHLQLIYTLAVLVTAKYSSSSHIQLIYTLTLCELRGTD WLAQKFDWAVEAKNCMSWRYSCTRYTNFLLDTK GRLYRWRSPVIVEKGGKVEVEGHLIDLKRVVLDGSA ATPLTRVSAEQWGRL

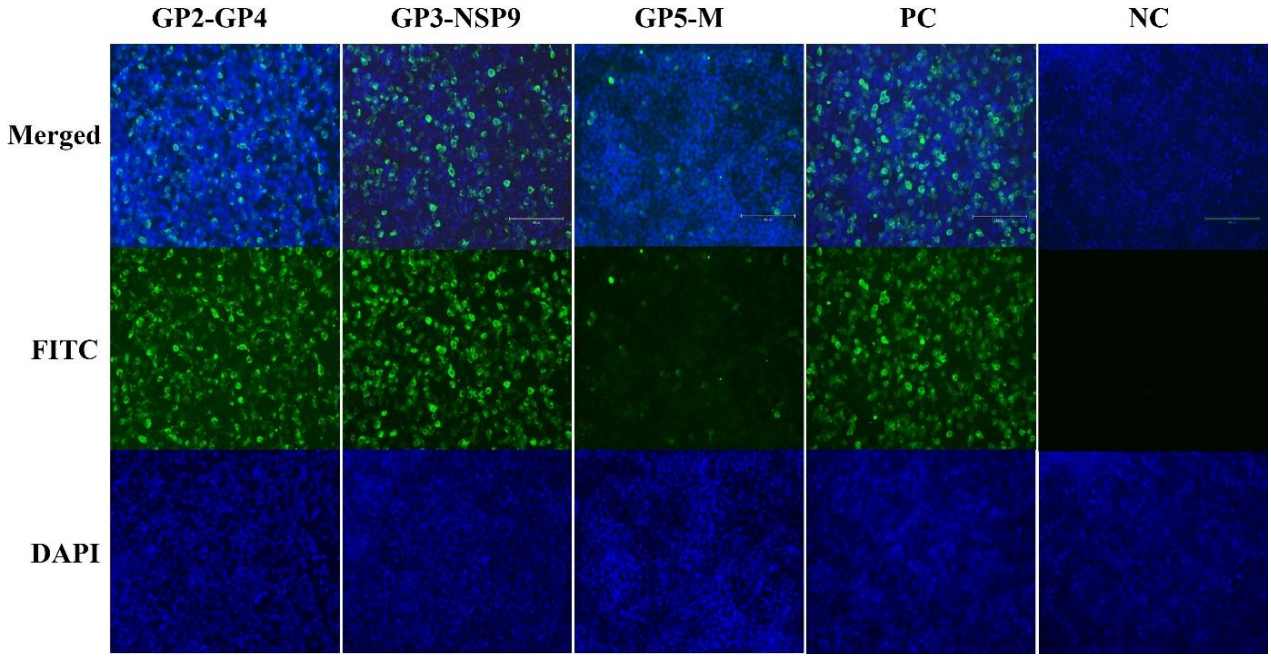

Fig. 2: Response of different antigenic proteins through Immunofluorescence assay including GP2-GP4, GP3-NSP9 and GP5-M. PC positive control transfected with 3× Control of Flag-tagged plasmid and NC indicated negative control untransfected blank control.

and mRNA-LNPs of GP2-GP4 and GP3-NSP9 showed strong fluorescence, and the expression of GP3-NSP9 was better than GP2-GP4. In contrast, GP5-M showed very weak fluorescence. In the negative control group, there was no fluorescence signal found (Fig. 2). The findings imply that GP2-GP4 and GP3-NSP9 are well expressed in HEK-293T cells, while the expression of GP5-M was deficient. Since it is challenging to express M protein *in vitro*. Furthermore, the results of Western blot showed that the GP2-GP4 and GP3-NSP9 fusion protein as well as the positive control expressed within the cells, while the GP5-M was not expressed. β-actin was identified as an internal control. No band was detected in the negative control (Fig. 3). The observed protein sizes were approximately 55 kDa for GP2-GP4 (expected size 45 kDa) and 110kDa for GP3-NSP9 (expected size 94.7 kDa), which were significantly larger than predicted. The The results obtained from IFA and Western blot expression of GP5-M agreed, suggesting that the fusion protein was not strongly expressed in cells. Consequently, the mRNA-LNP of GP5-M was not chosen for use in any further animal experiment.

Combination of GP2-GP4 and GP3-NSP9 mRNA-LNP decreases viral load in pigs after PRRSV challenge: The mRNA-LNP with higher expression levels were selected to immunize the pigs with GP2-GP4 and GP3-NSP9 combined mRNAs immunization groups and GP3-NSP9 mRNA individual immunization group. After completing the immunization program, the pigs were

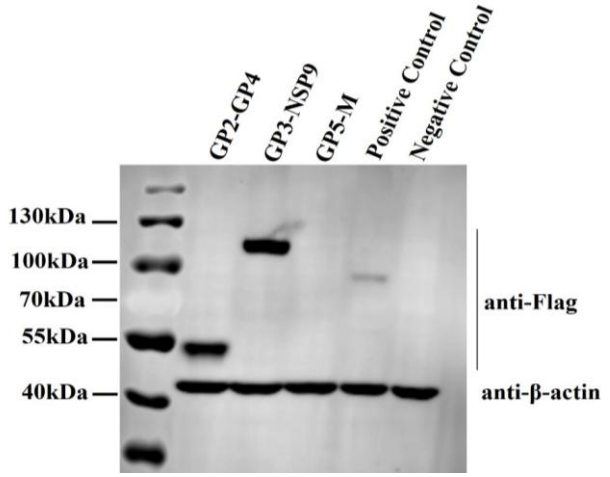

Fig. 3: Western blot for detection of GP2-GP4, GP3-NSP9 and GP5-M protein expression with a mouse anti-Flag monoclonal antibody, after mRNA-LNP transfection into HEK293T cells. An anti-β-actin monoclonal antibody from mice was used to detect β-actin as an internal control. The positive control group is a recombinant expression plasmid with 3×Flag tag. The negative control is the HEK293T cell lysis.

challenged with HP-PRRSV, and the rectal temperature was recorded every day up to the first 21 consecutive days, as shown in (Fig. 4A). A total of nine pigs were noted with fever, with an average duration of eight days. In the combined mRNAs immunization group, two pigs experienced fever, averaging five days. In the mRNA individual immunization group, fever was noted for three pigs with an average duration of four days. One pig in the HuN4-F112 attenuated vaccine control group developed a fever, with an average duration of only two days, while three pigs, with an average duration of six days, developed a fever in the negative control group (mock), indicating the need for further optimization of the antigen.

On day 0, 7, 14, and 21 following the challenge, N protein antibody level in mRNA-LNP-immunized pigs was detected. According to the findings, the combined mRNA immunization group's level of N protein antibody significantly below than mock-infected group (Fig. 4B), indicating that mRNA-LNP mixed immunization can effectively inhibit PRRSV replication in piglets. Additionally, following the PRRSV challenge, the virus load in the serum was found. The viral load in the serum of the mRNA-LNP combined mRNAs vaccinated group showed a much lower level than that of the mock-infected group (Fig. 4C), suggesting the PRRSV replication was inhibited in the LNP combined mRNAs vaccinated group. Seven days after virus infection, the number of virus copies in the single immunization group was reduced by about 1 Log compared to the mock group, while nearly 4 Logs decreased the number of virus copies in the combined immunization group. Thus, the mixed mRNA immunization group induced antibodies that could significantly inhibit the replication of PRRSV in pigs at the early stage of infection (Fig. 4C). On the 21st day following the challenge, there was no discernible change in the number of viral copies in the lungs of the single immunization group, combined immunization group, and the HuN4-F112 attenuated vaccine compared to the mock control group, not significant (NS) difference was observed (Fig. 4D). These results suggest that the HuN4-F112 attenuated vaccine and mRNA-LNPs combined immunization group can promote the clearance of PRRSV in PRRSV-infected piglets. The design of mRNA-LNP needs to be further optimized to achieve a better immune protection effect.

Evaluation of PRRSV specific T cell responses in piglets: Pigs' cellular immunological response to the vaccine was determined by isolating peripheral blood lymphocytes at 28dpc and using flow cytometry to analyzed them, paying particular focus to T lymphocyte subsets. The percentage of CD3+CD4+ T cells in the combined mRNAs group was not statistically significant when compared to mock (Fig. 5A). Nonetheless, the combined mRNAs group had a greater proportion of CD3+CD8+ T cells than mock (Fig. 5B). The above results show that the two groups of vaccines cannot significantly stimulate the body to produce $CD3^+CD4^+$ T cells, but $CD3^+CD8^+$ T cells increase slightly. This may also be related to the number of samples and the individual differences between samples.

Macroscopic gross and histopathological examination of the lung: Lung lesions were examined after the pigs in each group were dissected. The lung of the HuN4-F112 attenuated vaccine pigs' group (Fig. 6A) exhibited normal texture with gross features consistently and effectively maintained the specifications of the lung as evidenced by macroscopic gross pathological investigation, including all visible parameters. In the combined mRNAs immunization group, pig's lung appeared healthy, with smooth surfaces. No such atrophy or other pathological demarcation (Fig. 6B), the results showed that pigs in the (mixed) combined mRNAs immunization group have the least lung lesions but effectively protect pigs from PRRSV infection. The virus causes lung tissue damage, and nodules have been noticed, either caseous or noncancerous, appeared haphazardly internally in the lung of mRNA individual immunized pigs where the lung manifested multifocal whitish discoloration with some white appearance of suppuration and anemia without significant gross lesions (Fig. 6C). Meanwhile, the mock exhibited severe gross lesions characterized by different patches covering the uneven and indistinct borders. They were in the cranial, middle, and accessory lobes and the medial half of the caudal lobes with consolidation and hemorrhages. The infected lung appeared firmer, shrinker, and anemic. The lungs were congested with dark black discoloration that is multi-focally mottled tan to black with gross internal hemorrhages, indicating lung parenchymal anemia, and the surface displayed slightly watery mucus (Fig. 6D). A histological investigation was done on each group of lung. Infiltration of inflammatory cells appeared in the lung of the HuN4-F112 attenuated vaccination pig group (Fig. 7A). Pig lung in the combined mRNA immunization group show little thickening of the infiltration of inflammatory cells (Fig. 7B). According to the findings, the pig in the mRNA individual immunization group had diffuse thickening of the alveolar septa, interstitial edema, and hyperemia (Fig. 7C). The mock exhibited simulated alveolar gaps which showed proteinaceous fluid, neutrophils, and epithelial hyperplasia in multifocal bronchi (Fig. 7D).

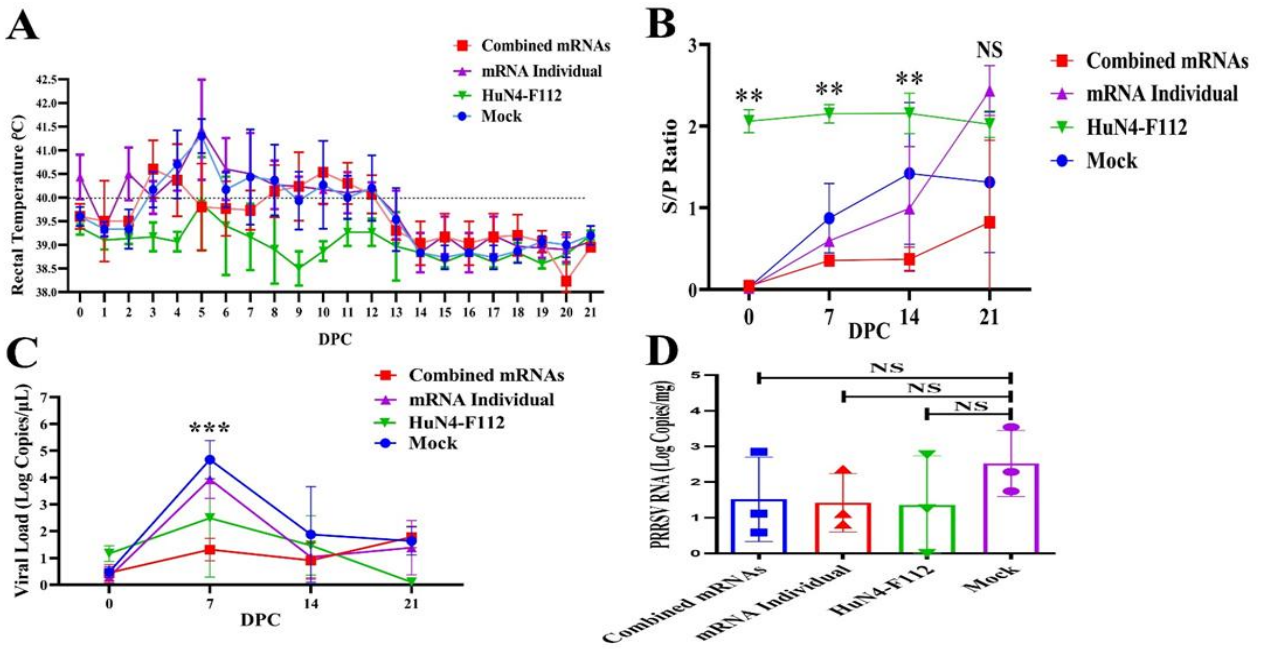

Fig. 4: Evaluation of the immune efficacy of PRRSV mRNA-LNP in pigs after challenge. (A) Changes in body temperature of PRRSV mRNA-LNP immunized pigs. (B) N protein antibody levels in PRRSV mRNA-LNP immunized pigs. (C) Virus load in serum of PRRSV mRNA-LNP immunized pigs. (D) Viral load in the lungs of PRRSV mRNA-LNP immunized pigs. The mean ± SD of three pigs per group is used to show the data. Significant differences are indicated by asterisks in the figures (*p ≤0.05; **p ≤0.01; ***p ≤0.001; NS, not significant).

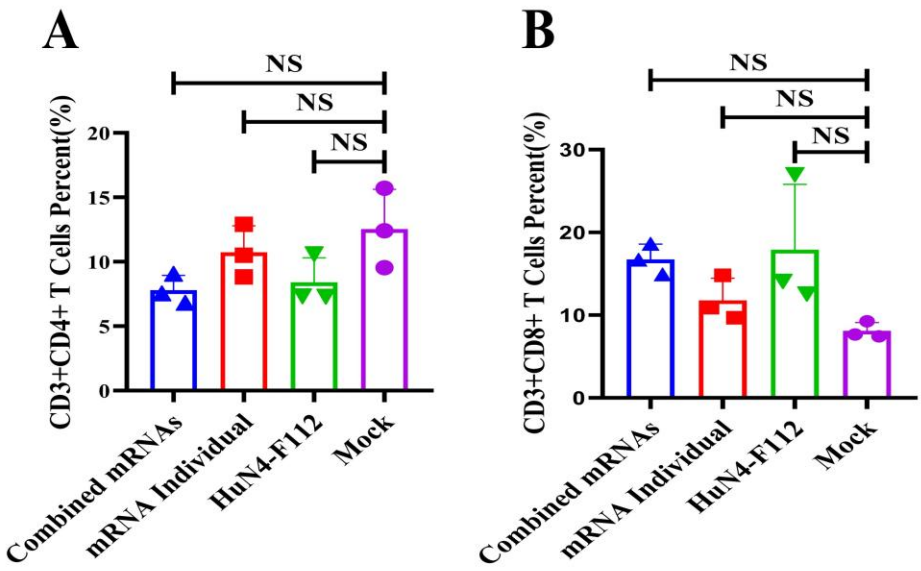

Fig. 5: Evaluation of PRRSV specific T cell responses in different groups of piglets. Peripheral blood lymphocytes from piglets were separated at 28dpc and sent for assessment of cellular immune responses. (A, B) CD3+CD4+ and CD3+CD8+ T cell frequencies in piglets following immunization. After being stained with CD3, CD4, and CD8 fluorescent antibodies, the piglets' extracted lymphocytes were analyzed using flow cytometry to determine the number of CD4+ and CD8+ T cells per 1 × 105 cells obtained. Data are presented as the mean ± SD of three piglets per group. (*P≤0.05; **P≤0.01; ***P≤0.001; NS, not significant).

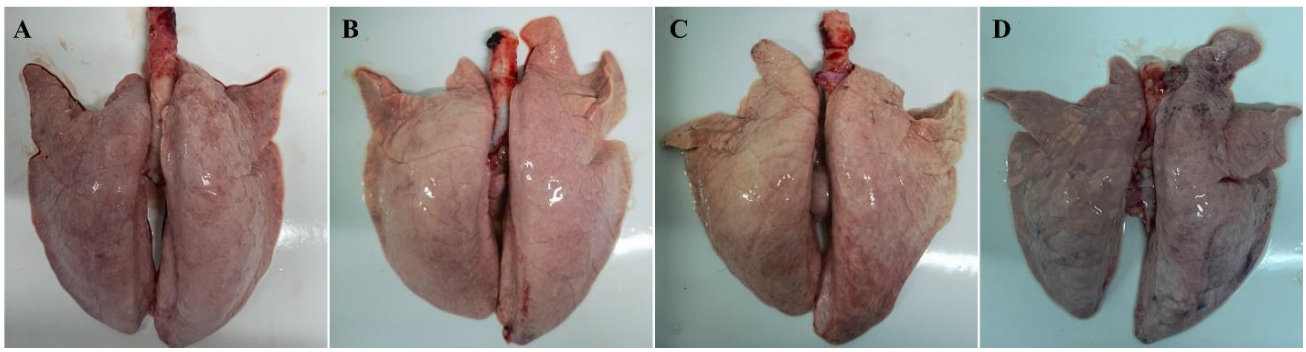

Fig. 6: Animals in the study were examined for macroscopic (gross) pathology. (A) The lung of HuN4-F112 attenuated vaccine as positive control at necropsy. (B) The lung of the combined mRNAs group appeared normal. (C) The mRNA individual group lung appeared anemic, with white internal spots and consolidated areas. (D) The mock showed lung atelectasis, dark black charcoal discoloration of the bronchial mucosa, and the appearance of respiratory lesions that were fully dispersed throughout the lung tissue.

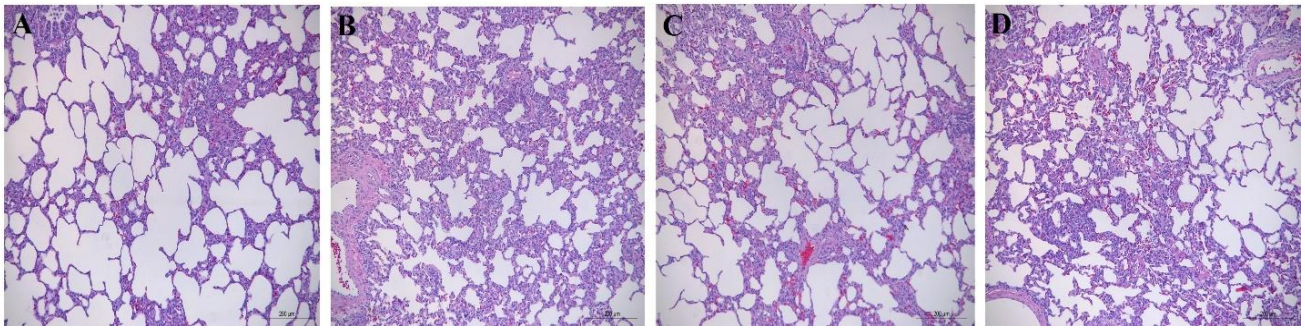

Fig. 7: The lungs histological architecture. (A) The HuN4-F112 lung show inflammatory cell infiltration. H and E staining (40X). (B) The combined mRNAs, show thickening of small amount of inflammatory cell infiltration. H and E staining (40X). (C) The mRNA individual lung shows alveolar septa thickening, extensive inflammatory cell infiltration and mild alveolar epithelial cell hyperplasia. H and E staining (40X). (D) The mock appeared extensive inflammatory cell infiltration by lymphocytes and plasma cells, proliferation and alveolar epithelial cell hyperplasia. H and E staining (40X).

DISCUSSION

PRRSV is a worldwide severe illness that costs the global pig industry a lot of money (Boeters *et al.*, 2023). The mRNA vaccines gained momentum after the COVID-19 pandemic, provide a new strategy since they have faster preparation cycles, and exceptionally high safety compared to commercial PRRS inactivated and attenuated vaccinations. Thus, the development of PRRSV mRNA vaccine can potentially overcome the drawbacks of traditional vaccines. Here, we showed the immunization of GP2-GP4 and GP3-NSP9 combined mRNA-LNPs provided a more protective immune response than GP3-NSP9 mRNA-LNP alone, which showed N protein antibody titers and the virus load in the blood were much lower.

GP5-M heterodimers appear to be involved in virus assembly (Veit *et al.*, 2022). According to reports, GP5 and M are somewhat effective against heterologous challenges, demonstrating the potential of GP5 and M in developing a universal vaccine against PRRSV (Zhang *et al.*, 2023b; Liu *et al.*, 2024). Here barely any expression of GP5-M was observed in indirect immunofluorescence and Western blot. We speculated that the full length of M protein may interfere with the expression of mRNA-LNP of GP5-M *in vitro*.

Multiple transmembrane areas make M protein expression difficult and result in a small amount of product being produced *in vitro*, despite suggestions that M protein could trigger protective antibodies (Frölichová *et al.*, 2017; Nan *et al.*, 2017b). Several groups attempt to express the M protein through the baculovirus system and Pichia pastoris cells (Qian *et al.*, 2003), but the yield of M protein is relatively poor. The primary explanation is its extreme hydrophobicity, which makes recombinant expression challenging. Since the M protein is not glycosylated, expression in the E. coli system provides a straightforward and reasonably priced technique to produce the optimal recombinant protein (Frölichová *et al.*, 2017; Luo *et al.*, 2023).

Relying solely on GP5-M protein as an antigen may not be an optimal strategy. The immune effect of individual mRNA using GP2, GP3, GP4, NSP9, GP5, and M as mRNA vaccines in pigs remains to be explored. Neutralizing epitopes have been identified in the proteins, GP2, GP3, and GP4 which can be used as antigens to increase the protective antibody titers (Loving *et al.*, 2015; Kim *et al.*, 2017; Rahe and Murtaugh, 2017; Montaner-Tarbes *et al.*, 2019). NSP9 encodes RNA-dependent RNA polymerase (RdRp) is involved in the virulence of HP-PRRSV (Li *et al.*, 2014). Multiple strains and inactivated vaccines are also distinguished by antigens targeting the RdRp fusion protein (Zhao *et al.*, 2013). Thus, NSP9 was selected as an antigen for the PRRSV mRNA vaccine.

The combined mRNAs immunization group maintains a low replication load in peripheral blood, suggesting that combined mRNAs immunization may promote the clearance of PRRSV. Antibodies against the N protein are commonly used to assess the level of PRRSV infection (Chen *et al.*, 2019b). We confirmed that N protein antibody level in the mixed immunized group remained low compared to the HuN4-F112 vaccine immunized group, indicating the mixed mRNA immunized inhibited PRRSV replication. The pigs immunized with MLV vaccines or infected with virulent PRRSV strains frequently experience weak and sluggish neutralizing antibody responses (Zuckermann *et al.*, 2007; Butler *et al.*, 2014). Numerous investigations on vaccines have shown that pig protected against challenge infection in the absence of neutralizing antibodies (Trus *et al.*, 2014) including the study where the acceptable level of neutralizing antibodies wasn't achieved (NAbs titer < 2 log2) (Gu *et al.*, 2015; Vu *et al.*, 2015). Our findings show that PRRSV combined mRNA group did not significantly stimulate CD4+ T cells, CD8+ T cells increased marginally. Gross pathological alterations revealed that pigs in the combined mRNA immunization group had the fewest lung lesions and were healthy, with no signs of atrophy, more pathological changes were observed in the lung of mRNA individual group. Our report's observations of lung pathological changes are in line with those of earlier research (Guo *et al.*, 2013). Histopathological changes in the lung of combined mRNAs show small amount of inflammatory infiltrate which consist of atypical lymphocytes while in mRNA individual group, the primary changes were lymphocyte and plasma-cell infiltrates in the periarteriolar as well as the interlobular septa.

Nevertheless, more research is necessary to fully characterize the cellular immunity brought on by the mRNA vaccination. Currently, PRRS vaccines include inactivated, live, and subunit vaccines, but the protective effects of inactivated and subunit vaccines are suboptimal

(Zhang *et al.*, 2023a). Live vaccines control some PRRSV outbreaks, while significant safety concerns arise due to high mutation rates of virulence reversion or recombination between the vaccine and wild strains. Still, most vaccines lack reliable indicators. The response of lymphocytic proliferative cells to homologous stimulation was comparatively moderate in magnitude and delayed (Madapong *et al.*, 2020a). There have been attempts to employ DNA, subunit, and virus-vectored vaccines that incorporate additional viruses. But the majority of vaccinations created with these methods seem to be less effective. All of the previously approved PRRS vaccines for genotypes 1 and 2 cause rather moderate humoral and cell-mediated immune responses, according to the evidence of available data, similar to what is seen following infection with highly pathogenic PRRSV strains (Nan *et al.*, 2017a). Differentiating the vaccines from the wild-type virus is also challenging. The problem has been made worse by the appearance of recombinant viruses (Liu *et al.*, 2017). The research was done to create Sa-mRNA vaccines against PRRSV to facilitate RNA self-amplification. These vaccines would encode the replication machinery of alphaviruses, such as the Sindbis or Venezuelan equine encephalitis viruses (Démoulins *et al.*, 2017; Rehman *et al.*, 2022).

Now it's time to use this nanotechnology LNPs in the animal division section (Khan *et al.*, 2023; Lam *et al.*, 2023). The scientific community is currently greatly interested in the first clinical efficacy results. It's obvious that a safer and more effective PRRSV vaccine is desperately needed, and that creating the means to produce one will be difficult. Numerous mRNA vaccine applications are currently being tested to combat many other infectious pathogens in animals, including rabies and influenza. However, PRRSV mRNA needs special attention to cope with that specified virus and prevent the pig's industry from losing millions of dollars annually.

Conclusions: The immunization of GP2-GP4 and GP3-NSP9 combined mRNA-LNPs showed a more protective immune response than GP3-NSP9 mRNA-LNP alone. Our results will aid in design novel PRRSV mRNA vaccine. These findings demonstrated the potential protective effect of the proposed PRRSV mRNA vaccination against PRRSV challenge. The PRRSV mRNA vaccine's development in pigs may be able to overcome the shortcomings of the pig industry.

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Authors contribution: MK, XS and ZW conceived, designed and conducted the research. FM, PX, TW and LL collected the data. DX, TA and HW analysed the data. XC was actively involved in the supervision of research.

Ethics approval and consent to participate: This study was carried out in accordance with the legislation and the Ministry of Science and Technology of the People's Republic of China's guidelines for the care and use of laboratory animals. The Animal Care and Ethics Committee of the Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, approved all animals research under protocol number 202207-02.

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