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The mRNA Vaccine Expressing Fused Structural Protein of PRRSV protects piglets against PRRSV challenge

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Highlights

- The mRNA vaccines targeting the structural proteins of HP-PRRSV were engineered.
- The GP5-M mRNA vaccine induced PRRSV-specific humoral and cellular immune responses.
- The GP5-M mRNA vaccine protected piglets from HP-PRRSV challenge.

Abstract

The swine industry experiences substantial economic losses annually due to the porcine reproductive and respiratory syndrome virus (PRRSV). The limited protective efficacy of existing commercial vaccines against epidemic PRRSV underscores the urgent need for innovative solutions. The mRNA vaccines, which elicit robust immune responses, have emerged as a promising avenue in vaccine development. In this study, two distinct mRNA vaccines were engineered: one encoding the full-length GP5 and M proteins (GP5-M), and the other encoding the full-length N protein along with epitope peptide segments of the M and E proteins (NMEpep). Our findings indicate that, compared with NMEpep, piglets immunized with the GP5-M mRNA vaccine produced specific antibodies, exhibited elevated levels of PRRSV-specific IFN-γ, and demonstrated effective activation of $CD4^+$ and $CD8^+$ T cells as well as $CD21^+$ B cells. Furthermore, the GP5-M vaccine conferred protective efficacy against HP-PRRSV challenge, evidenced by the mitigation of clinical symptoms, reduction in viral loads, and alleviation of tissue damage. In conclusion, this study presents a promising candidate vaccine for addressing epidemic PRRSV and establishes the GP5-M mRNA vaccine as a viable platform for the development of next-generation PRRSV vaccines.

Keywords

PRRSV; mRNA vaccine; piglets; protective immunity; structural proteins.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is a widespread disease that exerts a substantial impact on swine development (VanderWaal and Deen, 2018). The primary clinical manifestations of PRRS include reproductive and respiratory disorders affecting pigs at various growth stages. These manifestations encompass abortion in sows, fetal or premature death in piglets, and symptoms such as fever, hemorrhage, and respiratory distress (Montaner-Tarbes et al., 2019). Owing to its high transmissibility and severity, PRRS has been designated as a Class B infectious disease by the World Organization for Animal Health (WOAH) and as a second-class infectious disease in China. As a result, PRRS represents a significant challenge to the advancement of the global pig industry (Whitworth et al., 2016).

Current commercial vaccines for porcine reproductive and respiratory syndrome virus (PRRSV) are

classified into two categories: inactivated and attenuated live vaccines (Lunney et al., 2016). Despite extensive efforts by vaccine developers to enhance their efficacy, inactivated vaccines are often criticized for their insufficient ability to elicit robust cell-mediated immune responses and neutralizing antibody production (Toman et al., 2019). Similarly, attenuated live vaccines have raised concerns due to the potential risks associated with viral recombination and reversion to virulence (Chae, 2021). Unfortunately, these commercial vaccines have shown limited or no efficacy in conferring protection against the emergent NADC30-like strain, which has become prevalent in China in recent years (Li et al., 2023). Consequently, there is an urgent need for the development of next-generation PRRSV vaccines to effectively mitigate the impact of PRRSV on swine husbandry.

PRRSV is a single-stranded, positive-sense RNA virus belonging to the *Arteriviridae* family within the *Nidovirales* order. The PRRSV genome is approximately 15.4 kb in length and comprises at least ten open reading frames (ORFs), which encode a minimum of 14 non-structural proteins and eight structural proteins, namely GP2a, GP2b, GP3, GP4, GP5, 5a, M, and N (Chen et al., 2010). PRRSV is characterized by a high rate of mutation and recombination during genome replication. These genetic variations frequently modify the virus's virulence and antigenicity, thereby presenting substantial challenges for vaccine development (Chen et al., 2017). In addition, the virus exhibits multiple small transmembrane structural proteins on its envelope surface, which assemble into heterodimers or trimers essential for viral infection processes (Dokland, 2010). This distinctive viral architecture furtherly complicates the identification of neutralizing antibody targets and hinders the selection of protective antigens (Zhang et al., 2021b). Consequently, innovative strategies are imperative for the design of effective vaccines against PRRSV.

Messenger RNA (mRNA)-based vaccines exhibit robust immunogenicity and high efficacy, demonstrating significant potential in combating viral diseases such as influenza, Zika, Dengue, Hepatitis C, and HIV (Bollman et al., 2023; Fang et al., 2022; McMahon et al., 2022; Patra et al., 2023; Wollner et al., 2021; Zhang et al., 2021a). The unexpected emergence of COVID-19 notably accelerated the research and development of mRNA vaccines, facilitating advancements in the prevention and control of other highly mutable pathogens. The mRNA platform enables the rapid production of vaccines targeting multiple antigens while inherently functioning as a self-adjuvant

to activate the innate immune system (Pardi et al., 2020). The swift progress in mRNA vaccine technology offers substantial promise for the comprehensive prevention and management of the PRRS epidemic.

In this study, we developed two mRNA vaccines targeting the structural proteins of HP-PRRSV. One vaccine encoded the full-length GP5 and M proteins (GP5-M), while the other encoded the full-length N protein along with epitope peptide segments of the M and E proteins (NMEpep). These mRNA constructs were subsequently encapsulated in lipid nanoparticles (LNPs). Furthermore, we assessed the capacity of these mRNA vaccines to elicit PRRSV-specific antibodies and cellular immune responses prior to evaluating their protective efficacy in piglets. Our findings indicated that the GP5-M mRNA vaccine successfully induced an effective immune response and conferred protection to the immunized piglets against HP-PRRSV.

2. Materials and Methods

2.1. Cells and virus

Both the HEK293T and Marc-145 cell lines were grown and subcultured in high glucose Dulbecco's Minimum Essential Medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Solabrio, China). The HP-PRRSV strain JXWN06 was cultured in Marc-145 cells.

2.2. Reagents

The commercial PRRSV live vaccine (JXA1-R) was purchased from SG-Biotech. HRP-Goat Anti-Mouse IgG H&L (#AS003) were purchased from Abclonal. Carboxyfluorescein diacetate succinimidyl ester (CFSE) was purchased from Selleck. PerCP-Cy™5.5 Mouse Anti-Pig CD3ε (#561478), PE-Cy™7 Mouse Anti-Pig CD4a (#561473), and Purified Mouse Anti-Pig CD8b (#552769) were purchased from BD Pharmingen. Mouse Anti-Porcine CD21-FITC (BB6-11C9.6) (#4530-02) was purchased from SouthernBiotech.

2.3. Ethical statement

The use of animals was approved by the Jiangsu Province Administrative Committee for Laboratory Animals (SYXK-SU-2021-0026) and followed the Jiangsu Province Laboratory Animal Welfare and Ethics guidelines of the Jiangsu Province Administrative Committee of Laboratory Animals.

2.4. The mRNA vaccine design and production

The structural proteins of the JXWN06 strain of PRRSV were employed as the reference amino acid sequence. Two antigens, the GP5-M and NMEpep proteins, were selected for mRNA vaccine design. The GP5-M protein was composed of the secretory signal peptide derived from Sus scrofa (Pig) CD5, followed by the full-length GP5 protein, IRES, and full-length M protein. The NMEpep protein was composed of the full-length N protein, 4 epitope peptide segments of M protein, and 2 epitope peptide segments of E protein (NMEpep). To efficiently express the target protein, we designed a modified mRNA encoding a type 1 (N7mGpppAm) cap, 5' and 3' untranslated sequences, and an optimized target antigen GP5-M and NMEpep sequence (Fig. 1A). For plasmid construction, the pUC57 vector (sourced from GenScript) was utilized to facilitate in-vitro mRNA transcription.

The plasmid DNA is enzymatically linearized to obtain a linearized plasmid for in vitro transcription. The in vitro co-transcription capping reaction is performed to the linearized plasmid, the 7-methylated guanylate cap structure is added to the 5' terminus of the transcribed mRNA, and the template DNA is degraded. The resulting mRNA pellet was dissolved in nuclease- and endotoxin-free water, followed by quantification and quality control using a Nanodrop spectrophotometer and capillary electrophoresis, respectively. Finally, the purified mRNA was aliquoted and stored at -80°C.

Lipid nanoparticles encapsulating mRNA (mRNA-LNPs) were produced using microfluidic technology. Lipids were dissolved at a molar ratio of 50:10:38.5:1.5 (SM102: DSPC: cholesterol: DMG-PEG2000). mRNA was prepared at a concentration of 0.1 mg/mL in 20 mM sodium acetate buffer (pH 5.5). The lipid and mRNA solutions were then mixed using a microfluidic cartridge at a total flow rate of 6 mL/min, with an aqueous to organic phase flow rate ratio of 3:1, resulting in an N/P ratio of 6. The obtained mRNA-LNPs were dialyzed three times against 20 mM Tris-HCl buffer (pH 7.4) using a Slide-A-Lyzer dialysis cassette with a 3.5 kD molecular weight cut-off. The final mRNA-LNP solution was adjusted to a concentration of 100 μg/mL in 10% sucrose (w/v) and could be stably stored at 2-8°C for six months. During utilization at swine farms, the mRNA-LNP could be placed in containers filled with ice packs. It is crucial to emphasize that freezing of the mRNA-LNP was strictly prohibited throughout storage and transportation.

To detect the expression of LNP-delivered mRNA, HEK293T cells were seeded into 24-well plates. After 18 h, the cells were treated with LNPs containing NMEpep or GP5-M mRNA (1 μg per well). Followed by another 24 h incubation, the expression of mRNA was detected by western blotting assays. The untreated cells served as the control group.

2.5. Western blotting

HEK293T cells treated with LNPs containing NMEpep mRNA were lysed using RIPA buffer (Thermo Scientific) following the manufacturer's protocol. In a parallel experiment, HEK293T cells treated with LNPs containing GP5-M mRNA had their supernatant concentrated through ultracentrifugation. The cells underwent two distinct treatments: one involved lysing the entire cell population with RIPA buffer, while the other involved extracting the cell membranes using the Membrane and Cytosol Protein Extraction Kit (Beyotime, China). All the protein samples were separated on SDS-PAGE gel and transferred to polyvinylidene difluoride membrane. The membrane was blocked for 1 h in PBS containing 0.2% Tween 20 (PBST) with 5% skim milk. The membrane was washed 3 times in PBST and incubated with anti-PRRSV-M monoclonal antibody (kept in our laboratory) overnight at 4°C. After 3 washes in PBST, the membrane was incubated with HRP-Goat Anti-Mouse IgG antibody for 2 h at room temperature. After 3 washes in PBST, the membrane were visualized using an enhanced chemiluminescence system (Tanon, China).

2.6. The pig vaccination experiments and viral challenge

In the active immunization experiments (Fig. 2A), twenty-five healthy piglets, aged 28 days and free from PRRSV, ASFV, CSFV, and PCV2 infections, were procured from a farm in Jiangsu Province, China. These piglets were allocated into five groups and housed in separate rooms. The vaccine group received intramuscular injections in the neck with either the NMEpep mRNA vaccine (45 μg per piglet), the GP5-M mRNA vaccine (45 μg per piglet), or the commercial PRRSV live vaccine (JXA1-R) as a positive control. The piglets in the control group and the PRRSV challenge group were treated with PBS. A booster dose was administered to the piglets 21 days later. Serum samples were collected 7 days after boosting and stored at −80°C for subsequent analysis of PRRSV-specific antibodies and neutralizing antibodies. Additionally, 7 days after boosting the peripheral blood mononuclear cells (PBMCs) were also collected to assess PRRSV-specific T cell proliferation,

IFN-γ secretion, and lymphocyte subsets.

At 35 days post-primary vaccination, each piglet was exposed to 4 mL of the HP-PRRSV (JXWN06 strain) at a concentration of 10^5 $TCID_{50}$/mL. The administration was conducted via both intramuscular and intranasal routes, with 2 mL allocated to each route. The control group consisting of five piglets remained unchallenged. Following the viral challenge, clinical symptoms were systematically monitored and scored on a daily basis. Rectal body temperature was recorded daily for a duration of 14 days post-challenge (dpc). The weight was measured every two days. The serum and nasal swab samples were also collected every two days and stored at −80°C for subsequent viral load analysis. On the 14 dpc, the piglets were euthanized and subjected to necropsy. Lung, spleen, and cervical lymph node tissues were harvested and preserved in paraformaldehyde for histopathological examination.

2.7. ELISA

PRRSV-specific IgG antibody titers in piglet serum were determined by ELISA kit (PP23104, BHBT, China). Briefly, diluted serum samples were added to the plates and incubated at 37°C for 1 h. After washing the plates with PBST, HRP-conjugated goat anti- Pig IgG (H+L) (1:10000) was added. Plates were incubated at 37°C for 1 h, washed, and TMB solution was added to the plates for 10 min at room temperature. The reaction was stopped using 2 M H_2SO_4. Absorbance was measured at OD_{450} nm with a microplate reader.

2.8. Serum neutralization assay

The neutralizing antibody (NAb) titers piglet serum were determined on Marc-145 cells. Briefly, the viruses were diluted to a concentration of 100 $TCID_{50}$ per 50 μL in DMEM supplemented with 2% FBS. Serial dilutions of serum, starting at a 1:4 dilution, were mixed with the diluted viruses and incubated at 37°C for 1 h. The positive serum was kept in our laboratory. Subsequently, 100 μL of each mixture was transferred to Marc-145 monolayers in 96-well plates and incubated for an additional 2-3 days at 37°C with 5% CO_2. Cytopathic effects were then detected using an immunofluorescence assay. A reduction of more than 90% in infected cells was considered indicative of neutralization at that dilution; otherwise, the sample was deemed negative for neutralization. Each serum dilution was tested in quadruplicate wells. NAb titers were calculated

using the Reed-Muench method.

2.9. Lymphocyte proliferation assay

The peripheral blood lymphocytes (PBMCs) were isolated using a commercial kit according to the manufacturer's instructions (Solabrio, China). The isolated cells were labeled with Carboxyfluorescein diacetate succinimidyl ester (CFSE) according to manufacturer's instructions. Responder T cells (5×10^5/well) were co-cultured with inactivated PRRSV JXWN06 strain (MOI=10) in 24-wells plates for 5 days in 5% CO_2 incubator at 37°C and then subjected to flow cytometry analysis. The untreated T cells were served as a negative control.

2.10. Flow cytometry

The PBMCs (2×10^6 per tube) were stained with antibodies (anti-Pig CD3, CD4, CD8, or CD21) of interest for 40 min at 4℃, followed by centrifugation at 1600 rpm for 5 min. After washing twice with PBS with 0.5% BSA and centrifugation at 1600 rpm for 5 min, the cells were resuspended in 1 mL of PBS with 0.5% BSA for FACS by FACS LSRFortessa (BD Biosciences, NJ, USA). Data analysis was subjected to FlowJo software 10.8.1 version.

2.11. ELISpot assay

Harvested PBMCs were dissociated using gentle MACS Dissociator (Miltenyi Biotec) and filtered through 70 μm cell strainer. Cells were treated with 1× Red Blood Cell Lysis Solution (Solabrio, China). After RBC removal, cells were washed and resuspended in RPMI with 10% FBS with a concentration of 1.0×10^7 cells/mL. 2.0×10^5 cells per well was added and incubated overnight in a cell incubator for cell resting. After the resting, cells were stimulated by the inactivated PRRSV JXWN06 strain (MOI=0.1) for 24 h, and IFN-γ secretion were detected by FluoroSpot Plus (Mabtech) following the manufacturer's instructions. The spot-forming cells (SFCs) were manually counted using inverted microscope (IX71, Olympus).

2.12. Quantification of PRRSV RNA by quantitative real-time PCR

The number of viral RNA copies in serum and nasal swab samples was determined by quantitative real-time PCR (RT-qPCR). Viral RNA was extracted from serum and nasal swab samples the TIANamp Virus RNA Kit (TIANGEN, China). The viral RNA was reverse transcribed into cDNA

using the Reverse Transcription Kit (Vazyme, China). The cDNA amplification was performed using the Taq Pro HS Probe Master Mix (Vazyme, China) according to the manufacturer's instructions. The following PRRSV-N gene primer and probe sequences were used: sense, 5'-AATGGCCAGCCAGTCAATCA-3';

antisense, 5'-CAAACACAATTGCCGCTCAC-3'.

2.13. Statistical analysis

GraphPad Prism 8 software was used for statistical analysis and figure generation. All data were expressed as the mean ± SD. Differences between groups were examined for statistical significance using a one-way analysis of variance (ANOVA) with Tukey's multiple comparison post-test. The asterisks in the figures indicate significant differences, with $P < 0.05$ (*$P < 0.05$; **$P < 0.01$; ns, not significant).

3. Results

3.1. The expression of mRNA encoding the fused structural proteins

Two mRNA vaccines based on PRRSV structural proteins were developed, each encoding distinct fused structural proteins. The first vaccine encoded a construct comprising the full-length GP5 and M proteins (GP5-M). The second vaccine encoded the NME protein, which included the full-length N protein, four epitope peptide segments of the M protein, and two epitope peptide segments of the E protein (NMEpep) (Fig. 1A). Both the GP5-M and NMEpep mRNAs were encapsulated within lipid nanoparticles (LNPs). The expression of these immunogens in vitro was confirmed by detecting the GP5-M and NMEpep proteins in HEK293T cells transfected with the mRNA. Western blotting analysis demonstrated that the incubation of HEK293T cells with mRNA-LNPs led to efficient expression of the GP5-M and NMEpep proteins, and the GP5-M protein was predominantly found in the cell supernatant (Fig. 1B).

3.2. The mRNA vaccine elicits a PRRSV-specific immune response

To evaluate the immunogenicity of these mRNA vaccines, the piglets were allocated into five groups: Control, NMEpep, GP5-M, JXA1-R, and the PRRSV challenge groups. The commercial PRRSV live vaccine (JXA1-R) was served as a positive control. Each group of piglets received an initial

immunization, followed by a booster dose after 21 days (Fig. 2A). Following two rounds of immunization, both the mRNA vaccine GP5-M and JXA1-R elicited a specific immune response in the piglets, as indicated by elevated levels of PRRSV-specific IgG antibodies (Fig. 2B) and enhanced PRRSV-specific lymphocyte proliferation (Fig. 2C). However, neutralizing antibodies were not detected in the serum of the immunized piglets (Fig. 2D)

IFN-γ has been recognized as a critical cellular factor in the infection and clearance of PRRSV in pigs (Li et al., 2023). To evaluate PRRSV-specific IFN-γ production post-vaccination, PBMCs were collected from immunized piglets at one week post-boosting and stimulated in vitro with inactivated PRRSV to assess the production of specific IFN-γ. ELISpots analysis revealed that immunization of piglets with GP5-M and JXA1-R effectively stimulated the PRRSV-specific IFN-γ secretion (Fig. 2E).

To analyze the various lymphocyte subsets elicited by these mRNA vaccines, we subsequently assessed the vaccine's ability to stimulate $CD4^+$ T and $CD8^+$ T cells within PBMCs using flow cytometry. In comparison to piglets immunized with PBS and the NMEpep mRNA vaccine, the proportion of $CD3^+CD8^+$ T cells was significantly elevated in piglets immunized with the GP5-M mRNA vaccine, which is critical for viral clearance (Fig. 3A and 3B). However, there was no significant increase in $CD3^+CD4^+$ cell numbers (Fig. 3A and 3B), indicating that our mRNA vaccines have a limited ability to induce a balanced Th1 and Th2 immune response. Humoral immunity, mediated by B cells, was assessed by CD21 expression in mature B lymphocytes. Figures 3C and 3D illustrate a marked increase in $CD21^+$ B lymphocytes subsequent to immunization with GP5-M and JXA1-R. Conversely, the NMEpep mRNA vaccine did not elicit a significant stimulation of $CD21^+$ B lymphocytes in piglets. Collectively, the GP5-M mRNA vaccine elicits a PRRSV-specific immune response and cell immunity in piglets, underscoring its potential as a promising candidate for combating PRRSV.

3.3. The mRNA vaccine mitigates PRRSV-induced clinical symptoms and viremia.

After two weeks post-boosting, the piglets were exposed to HP-PRRSV via intramuscular and intranasal routes. Daily monitoring of rectal temperature and clinical symptoms was conducted. Additionally, weight, serum, and nasal swab samples from each piglet were collected every two

days (Fig. 2A). As depicted in Figure 4A-C, piglets in the PRRSV group consistently displayed characteristic clinical symptoms, including elevated fever, coughing, and anorexia, throughout the challenge period. The peak rectal temperature recorded was 42.8°C, and all piglets succumbed by 10 dpc. The NMEpep mRNA vaccine failed to significantly stimulate immune responses in the piglets, resulting in diminished weight gain, persistent high fever, and a 100% mortality rate by 12 dpc. Notably, while the GP5-M mRNA vaccine did not completely mitigate the clinical symptoms induced by PRRSV, the piglets only experienced elevated rectal temperatures between 6 and 11 dpc. Their daily weight gain returned to levels comparable to the non-challenged group after 10 dpc, with a survival rate of 60%. In contrast, piglets immunized with JXA1-R maintained a relatively healthy clinical status, with a mortality rate of zero (Fig. 4A-C).

Subsequently, we evaluated viremia levels among the groups following the PRRSV challenge. Viremia was sustained in the piglets of the PRRSV and NMEpep+PRRSV groups until the point of mortality. In contrast, the administration of the GP5-M mRNA vaccine resulted in a significant reduction of viremia after 7 dpc, with the JXA1-R treatment facilitating a more rapid clearance of viremia compared to the GP5-M mRNA vaccine (Fig. 4D). Furthermore, viral load was assessed in all piglets using nasal swab samples. The findings further indicated that the GP5-M mRNA vaccine effectively immunizes piglets against PRRSV infection (Fig. 4E). Collectively, these suggest that the GP5-M mRNA vaccine can mitigate the clinical symptoms and viremia associated with PRRSV infection in piglets.

3.4. The mRNA vaccine reduces histopathological lesions induced by PRRSV

To evaluate the efficacy of the vaccine in mitigating organ damage, the piglets were euthanized and dissected at 14 dpc, and samples from the lung, spleen, and cervical lymph node were collected. The autopsy findings indicated that all piglets in the PRRSV and NMEpep+PRRSV groups exhibited distinct PRRSV-associated lesions, including pulmonary consolidation and significant hemorrhaging in the spleen and cervical lymph nodes (Fig. 5A). In contrast, only 2/5 piglets in the GP5-M+PRRSV group and 1/5 piglets in the JXA1-R+PRRSV group exhibited mild tissues lesions (Fig. 5A). The gross pathology scoring of lungs also revealed that piglets in GP5-M+PRRSV group and JXA1-R+PRRSV group have significantly slighter pathological lesions than piglets in the PRRSV and NMEpep+PRRSV groups (Fig. 5B). Subsequently, we assessed the histopathological

lesions. The PRRSV challenge induced severe interstitial pneumonia characterized by expanded alveolar septa and a reduced number of alveoli in the lungs; indistinct medullary areas with cell necrosis in the spleen; a significant hyperaemia and lymphocyte necrosis in the cervical lymph nodes (Fig. 5C). However, immunization with the GP5-M mRNA vaccine and JXA1-R was able to reduce the hemorrhagic necrotic patches and tissue damage induced by PRRSV (Fig. 5C). These results illustrate the protective efficacy of the GP5-M mRNA vaccine against PRRSV in piglets

4. Discussion

In 1994, the United States pioneered the introduction of the first commercial vaccine for PRRS, employing a live attenuated virus. Following this, in 2007, China approved the registration of an attenuated vaccine developed from the CH-1a isolate. The overarching clinical aim has consistently been the prevention of PRRSV through the mitigation of symptoms via immunization (Zhou et al., 2021). However, due to the inherent limitations associated with inactivated and attenuated PRRSV vaccines, particularly regarding efficacy and biosafety concerns, current research efforts are increasingly focused on the development of novel vaccine modalities (Zhou et al., 2024a). The messenger RNA-lipid nanoparticle (mRNA-LNP) platform represents an optimal framework for evaluating the protective potential of novel vaccine targets against infectious diseases (Alameh et al., 2022). In contrast to conventional vaccines presently employed for the control of PRRS, mRNA vaccines can offer advantages such as reduced preparation time, enhanced safety profiles, and prolonged efficacy, suggesting significant potential for the comprehensive prevention of PRRS. Nonetheless, the mRNA vaccines have not yet been applied in the development of PRRS vaccines, rendering the research and development of PRRS mRNA vaccines an emerging and innovative area of study (Zhou et al., 2024b). In this study, we developed two mRNA vaccines expressing fused structural proteins from the HP-PRRSV: GP5-M and NMEpep. We observed that piglets vaccinated with the GP5-M mRNA vaccine exhibited significant immune responses against HP-PRRSV, in contrast to those receiving the NMEpep vaccine. The GP5-M mRNA vaccine was capable of inducing PRRSV-specific antibodies, promoting T cell proliferation, and enhancing IFN-γ secretion. Additionally, it increased the number of $CD8^+$ T cells and $CD21^+$ B cells. Notably, piglets immunized with GP5-M mRNA vaccine showed reduced clinical symptoms, viremia, mortality, and organ damage induced by HP-PRRSV.

Despite GP5 is recognized as the most significant immunogenic protein and the primary inducer of neutralizing antibodies among the viral proteins encoded by PRRSV (Cui et al., 2020), studies have demonstrated that the co-expression of GP5 with GP3, M, or N as fusion proteins, in conjunction with appropriate adjuvants, elicits enhanced immunogenicity compared to the expression of GP5 alone (Cho et al., 2020; Du et al., 2012; Jiang et al., 2006). GP5 and M proteins are the main structural proteins of PRRSV virus, and the GP5-M heterodimeric complex plays an important role in virus budding and invasion of host cells. Therefore, GP5-M protein is often used as the main target antigen for the development of genetically engineered vaccines. The recombinant Pseudorabies virus (PRV) that stably co-expresses the GP5 and M proteins of the NADC30-like PRRSV has been shown to induce both humoral and cellular immune responses specific to this PRRSV variant (Zhao et al., 2022). In addition, in the realm of novel vaccine formulations, multi-epitope vaccines represent a distinctive design paradigm predicated on the incorporation of multiple overlapping antigenic epitopes (Wang and Feng, 2024). The presentation of viral immunogenic proteins in complex formations is essential for the efficient induction of specific and protective B- and T-cell responses, which are crucial for eliciting robust immunity against PRRSV (Wang and Feng, 2024). GP5, along with M, N, E and certain non-structural proteins, harbors virus-neutralizing B cell epitopes and potential T cell epitopes (Wang and Feng, 2024). In our study, considering the high glycosylation of PRRSV membrane proteins and the expression efficiency of mRNA, we have opted to utilize a limited selection of full-length antigen proteins or truncated antigen peptides of PRRSV to developed mRNA vaccines: one encoding the full-length GP5 and M proteins (GP5-M), and the other encoding the full-length N protein along with epitope peptide segments of the M and E proteins (NMEpep). Our objective was to identify PRRSV antigens that confer protection against PRRSV challenge. The findings demonstrated that the GP5-M mRNA vaccine elicited more robust immune responses and conferred greater protection against PRRSV challenge compared to the NMEpep. These results underscore the GP5-M mRNA vaccine's potential as a promising candidate for PRRSV mitigation.

The effectiveness of mRNA vaccines can be attributed to their capacity to elicit both humoral and cellular immune responses (McMahon et al., 2022). The mRNA vaccine expressing the PEDV spike protein likely achieves its efficacy through a synergistic effect of neutralizing antibodies and the

cellular immune response (Zhao et al., 2024). Cui et al. developed an mRNA vaccine targeting an HP-PRRSV strain, which was shown to stimulate both cellular and humoral immune responses in mice (Zhou et al., 2024b). Nevertheless, due to the ability of PRRSV to establish persistent infections and its strategies to evade neutralizing antibodies, the humoral immune response alone may not suffice for complete protection against PRRSV infection in pigs. Conversely, the cellular immune response is considered to play a more critical role than humoral immune response (Guo et al., 2016; Sun et al., 2025). This is why vaccines can confer partial cross-protection and homologous protection even in the absence of neutralizing antibodies (Park et al., 2015; Park et al., 2014). In our study, although the GP5-M mRNA vaccine induced PRRSV-specific IgG antibodies, it lacked neutralizing activity against PRRSV. Comparing the mRNA vaccine with a commercial PRRSV vaccine, the commercial live vaccine (JXA1-R) outperformed the GP5-M mRNA vaccine in survival (100% vs. 60%) and viremia reduction. This suggest that it is important to optimize the epitope combinations in mRNA vaccine, which will enable the commercial application of mRNA vaccine against PRRSV.

T cells demonstrate a significantly greater complexity and diversity in their functions compared to B cells. Various subsets of porcine T cells, including γδ T cells, $CD4^+$ T cells, and $CD8^+$ T cells, have been shown to respond to PRRSV infection by proliferating vigorously, secreting cytokines, and exhibiting cytotoxicity upon ex vivo restimulation with PRRSV (Cao et al., 2019). Notably, Li et al. discovered that among the different T-cell subsets examined, only $CD8^+$ T cells mounted a robust response following PRRSV vaccination (Li et al., 2023). The TJM-F92 vaccine boosts $CD8^+$ T cell memory response but not the humoral immune response, indicating these T cells may provide partial protection against NADC30-like PRRSV strains (Li et al., 2023). In our study, the GP5-M mRNA vaccine elicited both the proliferation of PRRSV-specific T cells and a marked expansion of $CD8^+$ T cells. These researches highlights the role of PRRSV-specific cytotoxic T lymphocytes (CTLs) in viral clearance, indicating that the antigen-specific cellular immunity extends beyond humoral immunity and contributes to protective immunity against PRRSV (Sun et al., 2025). The activated $CD8^+$ T cells are not only essential for immune defense and viral elimination but also play a crucial role in secreting IFN-γ (Liu et al., 2021). Research has demonstrated a significant expansion of $CD8^+$ T cells in PRRSV-infected pigs, identifying them as the predominant IFN-γ-

secreting cells upon antigen re-stimulation (Charerntantanakul et al., 2006; Li et al., 2023). Piglets immunized with nanoparticles containing epitopes targeting the viral GP3, GP4, and GP5 proteins were capable of generating specific antibodies, PRRSV-specific IFN-γ production, and demonstrated protective efficacy against PRRSV challenge (Sun et al., 2025). Consequently, we hypothesize that the observed expansion of $CD8^+$ T cells and the elevated level of PRRSV-specific IFN-γ secretion in our study may be the primary contributors to the protection of piglets against the HP-PRRSV challenge, and further investigation into these mechanisms could yield valuable insights into vaccine efficacy.

In summary, our preliminary investigation into the HP-PRRS mRNA vaccine reveals that the GP5-M mRNA vaccine significantly induces a PRRSV-specific immune response and a T-cell-mediated immune response in piglets. Furthermore, this mRNA vaccine effectively reduces high fever, mortality, viremia, and organ damage caused by the HP-PRRSV challenge, thereby alleviating clinical symptoms and disease progression. However, we did not evaluate long-term immunity or protection against heterologous PRRSV strains. In future studies, we aim to address the challenges posed by the NADC30-like and NADC34-like PRRSV strains and optimize antigen combinations to enhance PRRSV-specific cellular and humoral immune responses.

Declarations

We are greatly indebted to our colleagues for providing technical support and valuable suggestions.

Institutional review board statement

Not applicable.

Informed consent statement

Not applicable.

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Declaration of Competing Interest

The authors have no conflict of interest to declare.

Data availability

The datasets generated and analyzed in the current study are available from the corresponding author upon reasonable request.

Author Contributions

CM, XZ, TH, and SX designed, performed, and analyzed the experiments and data. CZ, QH, MX, and KS developed reagents and helped with the experiments. CM and ZC planned the experiments and secured the funding. CM wrote the manuscript.

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Figure Legends

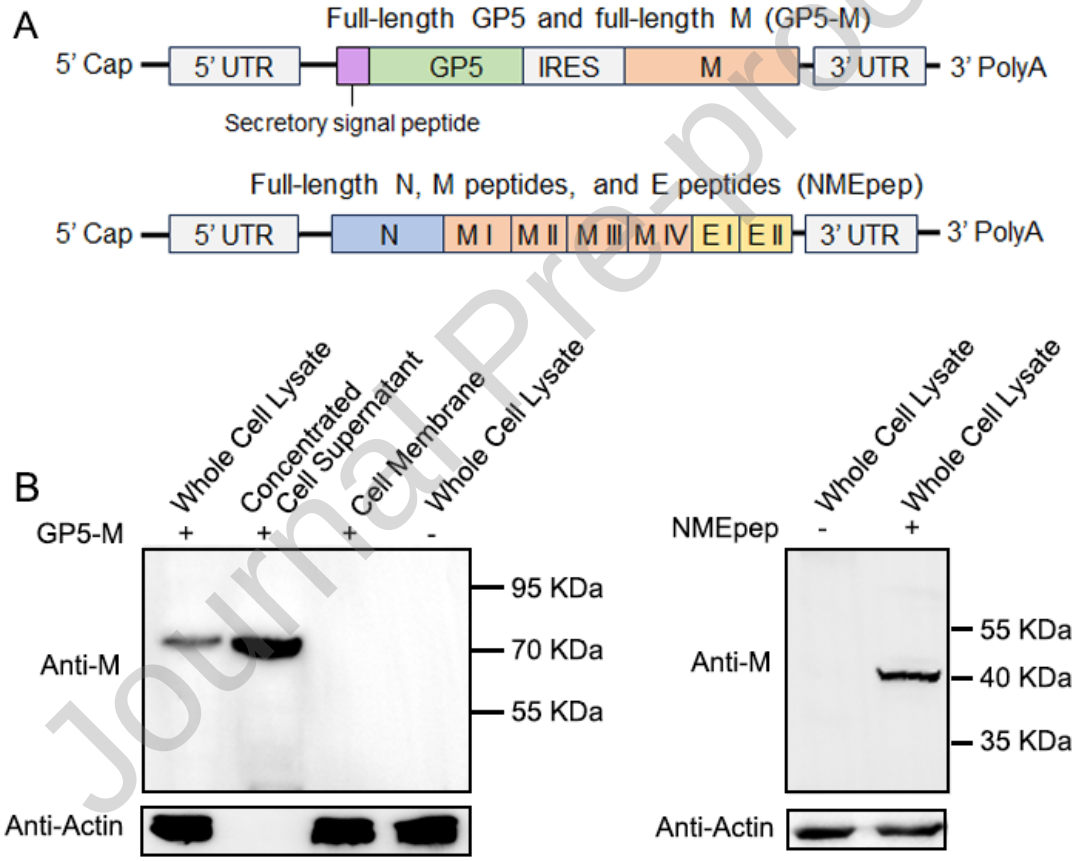

Figure 1 The production and characterization of PRRSV-based mRNA vaccines.

(A) The illustration presents mRNA constructs that encompass the full-length GP5 and M proteins (GP5-M), another construct encoding the full-length N protein, epitope peptide segments of the M and E proteins M and E proteins (NMEpep). (B) The in vitro expression of mRNA-LNPs is demonstrated. HEK293T cells were incubated with mRNA-containing

LNPs for 24 h, followed by cell lysis for analysis via western blotting.

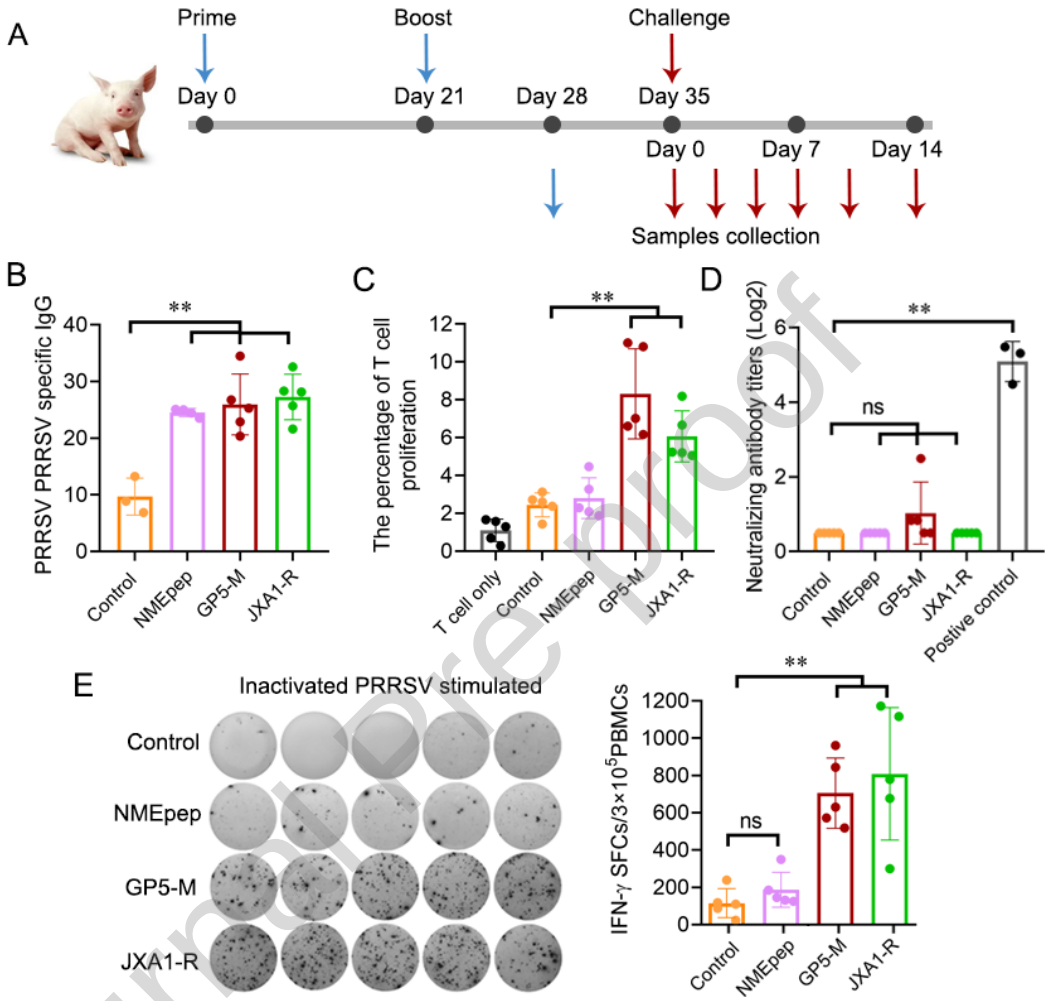

Figure 2 Immunogenicity of mRNA vaccines in piglets.

(A) Experimental design for piglet vaccination: piglets were immunized on days 0 and 21 with mRNA vaccines, PBS, or the commercial PRRSV vaccine (JXA1-R). (B) Serum levels of PRRSV-specific IgG antibodies were measured 28 days following the initial immunization. (C) Neutralizing antibodies against PRRSV JXWN06 strains were assessed, with neutralizing antibody (NAb) titers expressed as the highest serum dilution capable of protecting more than 50% of cells from cytopathic effects (CPE). (D) PRRSV-specific lymphocyte proliferation assay. PBMCs were isolated 28 days post-immunization and stimulated with inactivated PRRSV for five days. PRRSV-specific lymphocyte proliferation was characterized using carboxyfluorescein succinimidyl ester (CFSE) and

quantified via flow cytometry, acquiring data from 1 × 10^5 cells. Data analysis was performed using FlowJo software version 10.8.1. (E) Levels of PRRSV-specific IFN-γ secretion were determined using an enzyme-linked immunospot (ELISpot) assay. Representative images of ELISpot wells and PBMC IFN-γ spot-forming cells (SFCs) were recorded following stimulation with inactivated PRRSV JXWN06. Results are expressed as the mean ± standard deviation (SD) for five piglets per group. Asterisks in the figures denote statistically significant differences, with $P < 0.05$ indicating significance (*$P < 0.05$; **$P < 0.01$; ns, not significant).

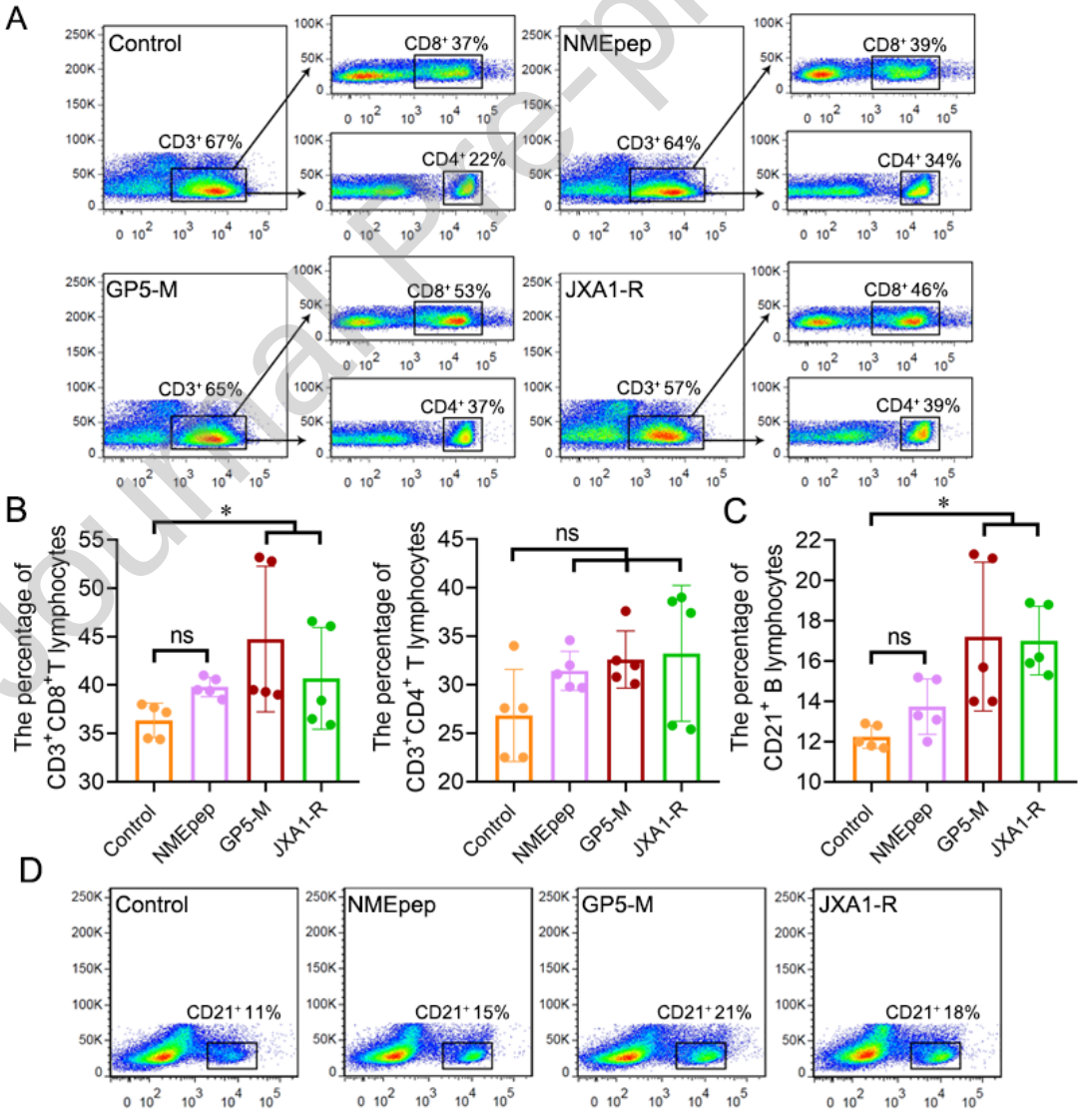

Figure 3 The evaluation of cellular responses in piglets immunized with mRNA vaccines.

Peripheral blood lymphocytes were isolated from the piglets 28 days post-primary immunization to assess cellular responses. (A and B) the frequencies of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells, respectively, in the immunized piglets, as determined by flow cytometry with acquisition of 1×10^5 cells. (C and D) the frequencies of $CD21^+$ B cells, also characterized by flow cytometry with acquisition of 1×10^5 cells. Data were analyzed using FlowJo software version 10.8.1. Results are expressed as the mean ± SD for five piglets per group. Asterisks in the figures denote statistically significant differences, with $P < 0.05$ indicating significance (*$P < 0.05$; **$P < 0.01$; ns, not significant).

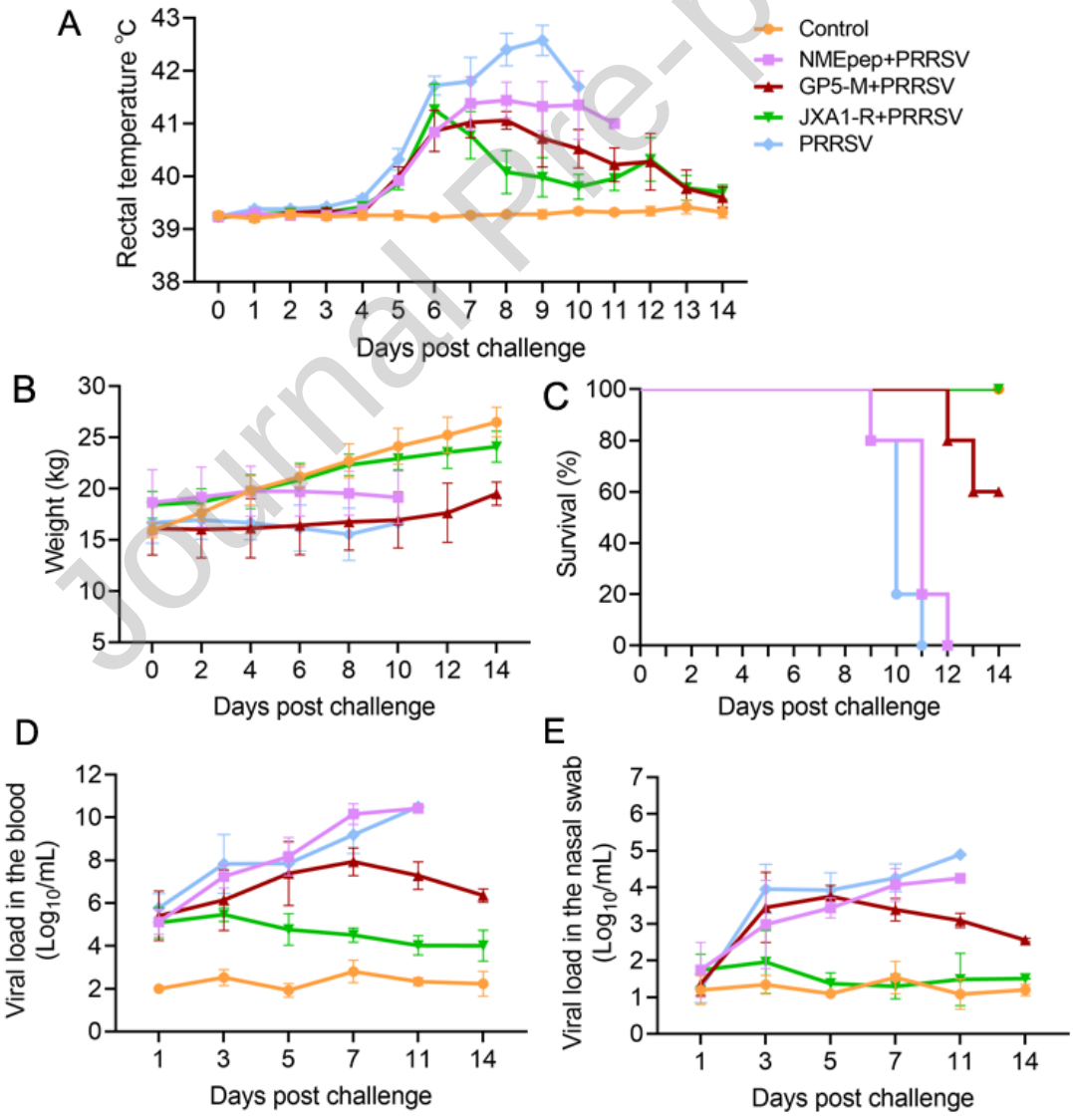

Figure 4 The mRNA vaccine confers protection against PRRSV in immunized piglets.

Piglets receiving two doses of mRNA vaccine were exposed to PRRSV on day 35 and subsequently euthanized 14 dpc, as illustrated in Figure 2A. (A) The rectal temperatures of piglets following PRRSV JXWN06 challenge are presented as mean ± SD for five piglets per group, with rectal temperatures ≥ 40.0°C classified as febrile. (B) The daily weight of piglets post-challenge is depicted. (C) Mortality rates in piglets subjected to PRRSV challenge are shown. (D-E) Viral loads in serum and nasal swab samples from various groups of piglets were measured at 1, 3, 5, 7, 11, and 14 dpc, with data expressed as mean ± SD.

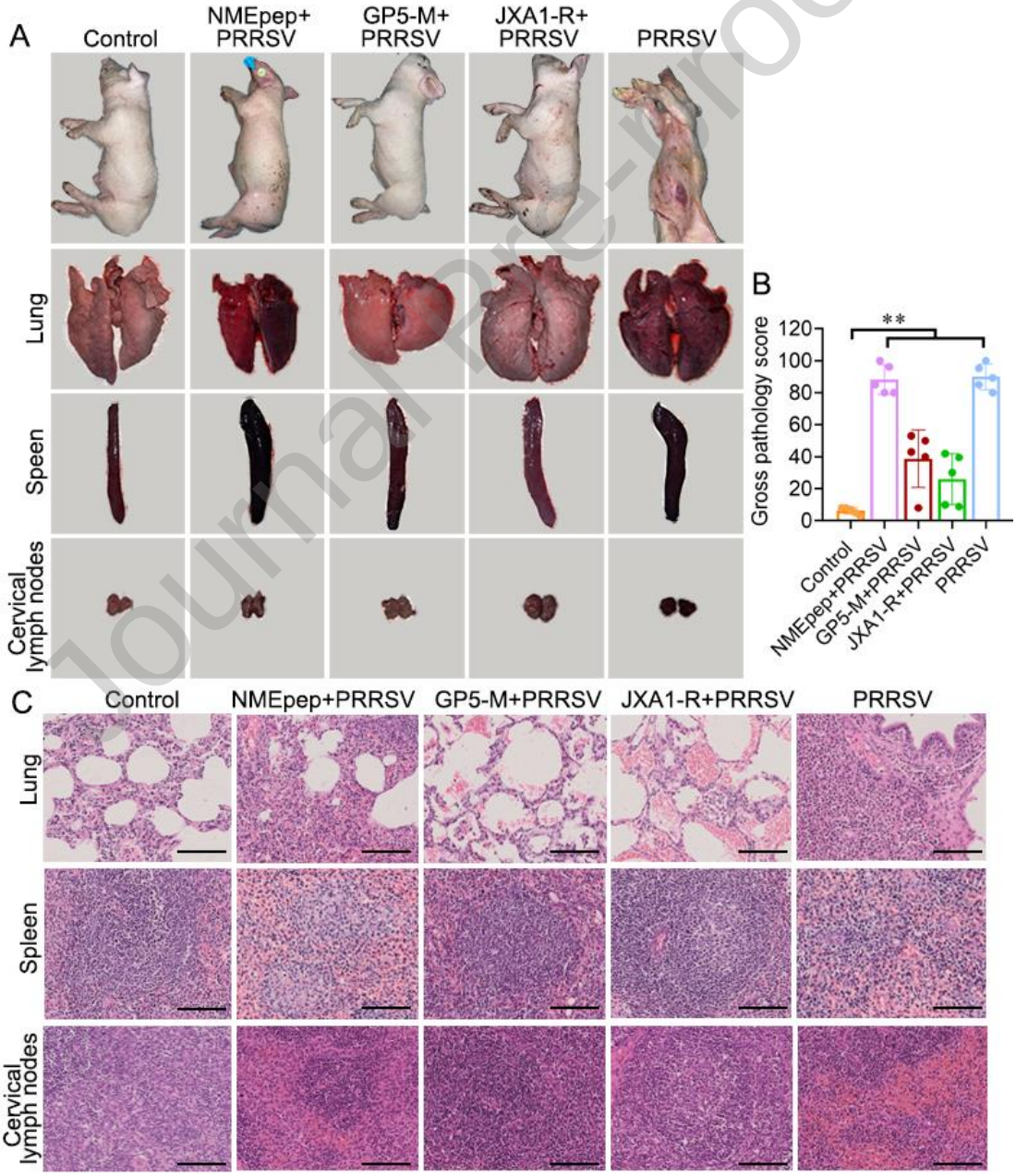

Figure 5 The histopathological lesions observed in piglets subjected to mRNA vaccination.

(A) The clinical and histological lesions in the lungs, spleens, and cervical lymph nodes of piglets euthanized at 14 dpc are depicted. (B) The gross pathology of the lungs was scored. (C) Representative images of the lungs, spleens, and cervical lymph nodes are presented, as visualized through hematoxylin and eosin (H&E) staining. Scale bar represents 50 μm.

Declarations

We are greatly indebted to our colleagues for providing technical support and valuable suggestions.

Informed consent statement

Not applicable.

Declaration of Competing Interest

The authors have no conflict of interest to declare.

Highlights

• The mRNA vaccines targeting the structural proteins of HP-PRRSV were engineered.

• The GP5-M mRNA vaccine induced PRRSV-specific humoral and cellular immune responses.

• The GP5-M mRNA vaccine protected piglets from HP-PRRSV challenge.