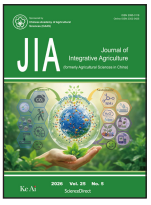
Journal of Integrative Agriculture 2026, 25(5): 2041–2050

Available online at ScienceDirect

Journal of Integrative Agriculture



RESEARCH ARTICLE

A rescued virus from the infectious clone of a PRRSV NADC34-like strain exhibits high pathogenicity for nursery pigs

Zhenbang Zhu[1*], Zhengqin Ye[1*], Wenqiang Wang[1], Yanhua Li[1], Zhe Sun[2], Xiuling Yu[2], Kegong Tian[2, 3#], Xiangdong Li[1, 4#]

[1] *Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, College of Veterinary Medicine, Yangzhou University, Yangzhou 225009, China*
[2] *National Research Center for Veterinary Medicine, Luoyang 471003, China*
[3] *College of Veterinary Medicine, Henan Agricultural University, Zhengzhou 450002, China*
[4] *Joint International Research Laboratory of Agriculture and Agri-Product Safety, Ministry of Education/Yangzhou University, Yangzhou 225009, China*

Highlights

- A NADC34-like porcine reproductive and respiratory syndrome virus (PRRSV) was successionally rescued by reverse genetics technique.
- The rescued virus proliferated well in porcine alveolar macrophages.
- The rescued virus showed high pathogenicity on pigs.

Abstract

NADC34-like porcine reproductive and respiratory syndrome virus (PRRSV) has been circulating in China for several years and became the dominant field strain in some provinces. Current commercial vaccines could not provide complete cross-protection to NADC34-like PRRSV infection, which led to huge economic losses on pig farms. Co-infections of NADC34-like PRRSV with some other PRRSV strains are commonly found in many clinical cases, and successful isolation of NADC34-like PRRSV strain from the clinical samples has been a challenge to study its biological characters and perform animal experiments to evaluate its pathogenicity. In this study, we constructed a NADC34-like PRRSV infectious clone derived from the isolated JS2021NADC34 PRRSV strain using the reverse genetics technique and investigated its virulence and pathogenicity for nursery pigs. The rescued (rNADC34) strain could proliferate well in porcine alveolar macrophages (PAMs), and the viral copy number and titers were comparable to parental strain. For pathogenicity, the rNADC34 strain-infected pigs showed high body temperature and body weight loss. The histopathological results presented interstitial pneumonia and severe hemorrhage, infiltration of neutrophils and lymphocyte in lungs, lymph nodes, and tonsils. The viral proteins were also detectable in rNADC34 strain-infected pigs using immunohistochemistry staining. Moreover, the trends of PRRSV-specific antibody and viremia in PRRSV rNADC34-infected pigs were similar with the parental strain-infected pigs. These data indicated that rNADC34 strain manifested strong virulence and high pathogenicity for nursery pigs.

Keywords: PRRSV, NADC34-like strain, infectious clone, pathogenicity

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is one of the most important diseases in swine industry, which is characterized by reproductive disorders in sows and severe respiratory symptom in piglets (Cho and Dee 2006). PRRS is caused by porcine reproductive and respiratory syndrome virus (PRRSV), which is an enveloped virus belonging to the genus *Porartevirus* of the family *Arteriviridae* in the order *Nidovirales* (King *et al.* 2018). PRRSV is about 15 kb at length of genome, encoding at least 11 open reading frames

Received 2 March 2024; Received in revised form 28 June 2024; Accepted 21 July 2024; Available online 22 August 2024
Zhenbang Zhu, E-mail: 007583@yzu.edu.cn; Zhengqin Ye, E-mail: yezhengqin@126.com; #Correspondence Xiangdong Li, E-mail: 007352@yzu.edu.cn; Kegong Tian, E-mail: tiankg@263.net
* These authors contributed equally to this study.

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doi: 10.1016/j.jia.2024.08.015

(ORF). ORF1a and ORF1b occupy most of the full-length genome, and code viral non-structural proteins (nsps). These nsps are responsible for PRRSV replication, which mainly act as RNA replicase and RNA polymerase. Some of nsps are associated with viral pathogenicity (Fang and Snijder 2010). The rest of ORFs encode viral glycoprotein GP2-GP5, matrix protein M, and the nucleocapsid protein N, which are relevant to viral entry and immunogenicity (Montaner-Tarbes *et al.* 2019).

High genetic diversity is a significant characteristic of PPRSV. Shi *et al.* (2010) constructed the global classification system of PRRSV based on the comprehensive analysis of the complete ORF5 gene sequence. Two genotypes of PRRSV have been described, including genotype 1 (also known as the European type), and genotype 2 (the North American type). According to the classification system, the PRRSV-1 was classified as three subtypes (subtype 1–3) and the PRRSV-2 was divided into 9 lineages with several sublineages in each lineage (Guo *et al.* 2018). PRRSV-2 are the major epidemic genotype in China, PRRSV-1 also emerged sporadically in some provinces/regions.

PRRS was firstly reported in America in 1987, since then it was described in Europe and Asia. In 1995, PRRSV was firstly described and isolated in China, but PRRSV circulated in China seemed to be earlier than 1995 according to serological studies (Zhou and Yang 2010). In 2006, a variant PRRSV, which was now known as highly pathogenic PRRSV (HP-PRRSV), caused hyperpyrexia and 20% mortality for nursery pigs, especially in southern China (Zhou *et al.* 2011; Han *et al.* 2017). Several years later, the NADC30-like PRRSV emerged and spread quickly in China. The NADC30-like PRRSV strains are homologous to the NADC30 strains circulating in North America in 2008, but with extensive variation (Zhou *et al.* 2015). Since then, HP-PRRSV and NADC30-like PRRSV are predominantly epidemic strains in the fields, which cause severe and complicated situation in swine industry of China. In 2017, two new PRRSV isolates were first identified and reported in Liaoning Province, China (Zhang *et al.* 2018). These isolates show similar genetic characteristics with IA/2014/NADC34 strain, which belongs to ORF5 1-7-4 lineage, so it was designated as NADC34-like strains (van Geelen *et al.* 2018). NADC34-like strains were subsequently reported in more than 10 provinces, suggesting that NADC34-like PRRSV could be the next dominant PRRSV strain in China (Liu *et al.* 2019; Song *et al.* 2020; Xu *et al.* 2020). NADC30-like and NADC34-like strains were probably imported by breeding pig trade from foreign countries and went through mutation and recombination in China (Liu *et al.* 2022).

Nsp2 is a varied and diverse gene in different PRRSV lineages, presenting length differences and nucleotide deletions, mutations and insertions. Compared to the standard strain VR-2332, HB-2(sh)/2002 strain was the first reported PRRSV deletion strain by Gao with a 12-amino acid (aa) deletion in nsp2 (Gao *et al.* 2004). HP-PRRSV was identified a discontinuous deletion of 30-aa (481 aa, 532-560 aa) in nsp2 (Tian *et al.* 2007). NADC30 strains also show a 131-aa discontinuous deletion in nsp2, while NADC34 strains shows a unique continuous 100-aa deletion at positions 328–427 in nsp2 (Xu *et al.* 2022). Nsp2 is involved in viral replication, proliferation, and variation. Different strains with specific nsp2 gene present distinct cytotropism and virulence, which make it different in pathogenicity for nursery pigs *in vivo*. Even PRRSV strains in the same lineage exert different pathogenicity and different clinical symptoms. HP-PRRSV could affect pigs of all ages with rubefaction and obvious respiratory distress, it is characterized by high body temperature (41°C–42°C) and high morbidity (50–100%) and mortality (20–100%) (Han *et al.* 2017). NADC30-like strains are mainly characterized by reproductive disorders in sows, abortion rate in infected sows is up to 30–40%. Infected nursery pigs display respiratory symptom and easily cause secondary bacterial infection (Yu *et al.* 2021). For different NADC34-like strains, there is a difference of pathogenicity in piglets. The origin strain IA/2014/NADC34 could cause dramatic abortion in sows and serious clinical symptoms in piglets (van Geelen *et al.* 2018). NADC34-like PRRSV HLJDZD32-1901 identified in China was recognized as a mildly pathogenic strain in piglets (Song *et al.* 2020). However, JS2021NADC34 PRRSV, another Chinese NADC34-like PRRSV isolate, was highly pathogenic for nursery pigs (Yuan *et al.* 2022).

The reverse genetics technique is a powerful molecular tool, which contributes to the study of viral genome function, virulence, pathogenesis, immune responses, and vaccine development. PRRSV infectious clones are also responsible for the research on cell tropism, virulence immunogenicity, and the design for PRRS vaccines (Han and Yoo 2014). PRRSV infectious clones can serve as a gene delivery vector for foreign gene expression or construct chimeric viruses. Clone pABV437 was the first PRRSV infectious clone derived from PRRSV Lelystad virus (Meulenberg *et al.* 1998). Subsequently, numerous infectious clones for VR-2332, HP-PRRSV and NADC30 strains had been developed and widely used in many researches. In this study, we rescued a virus from the infectious clone of a PRRSV NADC34-like strain to study its virulence and pathogenicity on nursery pigs.

2. Materials and methods

2.1. Virus and cells

Porcine alveolar macrophages (PAMs), the target cells for PRRSV strains, were isolated from 4-wk-old specific-pathogen free pigs, and were cultured in RPMI 1640 medium (Gibco, Waltham, MA, USA) with 10% fetal bovine serum (FBS) at 37°C in 5% CO_2. BHK-21 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Waltham, MA, USA) with 10% FBS at 37°C in 5% CO_2, providing a high efficiency of transfection for plasmids of infectious clones. JS2021NADC34 PRRSV, a NADC34-like PRRSV strain, was isolated and identified in our laboratory. The virus was purified by plaque assay for three rounds and titrated to 50% tissue culture infective dose ($TCID_{50}$). JS2021NADC34 PRRSV was designated as PRRSV NADC34 in this study due to its high genomic similarity (96.1%) with IA/2014/NADC34 strain (Yuan *et al.* 2022).

2.2. PRRSV infectious clones construction

pCMV-vector plasmid containing a human cytomegalovirus immediate-early promoter (CMV) and the unique restriction enzyme sites was provided by Prof. Yanhua Li from Yangzhou University, China. The complete genomic cDNA of JS2021NADC34 strain was divided into four overlapping fragments (F1–F4). These four fragments and a hepatitis D virus ribozyme (HDV Rbz) were cloned into the pCMV-vector *via* homologous recombination technology. The whole strategy was divided into two steps. Initially, viral RNA was extracted using TIANamp Virus DNA/RNA Kit (TIANGEN, Beijing, China) and inverse transcription was carried out using Superscript IV first-strand synthesis system (ThermoFisher Scientific, Waltham, MA, USA). The cDNA was used as a template and amplified using the Q5 high-fidelity DNA polymerase (NEB, Ipswich, MA, USA). The primers for four viral amplified fragments were listed in Table 1. Additionally, the primers for HDV were also designed and shared 20 nucleotide acids with the fourth fragment primer. For the first step homologous recombination, F1 and F2 were cloned into the pCMV-vector which was linearized with restriction enzymes (*Mlu*I and *Spe*I) using the NEBuilder HiFi DNA Assembly Master Mix (NEB, Ipswich, MA, USA). For the second step, F3 and F4 (containing HDV) were inserted into the first-step constructed plasmid which was linearized with restriction enzymes (*Not*I and *Spe*I). The four fragments were assembled into a full-length cDNA clone and designated as pCMV-JS2021NADC34. To distinguish between parent virus and infectious cloned virus, a single-base mutation at 15,852 bp in *Bsr*GI restriction site (A>C) was introduced. The plasmids were transfected in to BHK-21 cells for 48 h using Lipofectamine 3000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and then virus supernatants were harvested and passaged to PAMs for three generations. Finally, the rescued virus, thereafter named PRRSV rNADC34, was determined by Western blot, immunofluorescence, and RT-qPCR.

2.3. Animal trials

Fifteen pigs aged 2 mon were selected for the viral infection study *in vivo*. These pigs were free of PRRSV vaccination or infection tested by RT-qPCR and Herdcheck PRRSV X3 antibody test (IDEXX Laboratories, Westbrook, ME). These pigs were randomly divided into three groups (five pigs per group) and kept in the same temperature and humidity. After one week of acclimation, the 1st group of pigs was challenged with JS2021NADC34 PRRSV at 3×10^6 $TCID_{50}$ per pig *via* intranasal (0.5 mL per nasal) and intramuscular (2 mL) routes simultaneously. The 2nd group of pigs was challenged with PRRSV rNADC34 at 3×10^6 $TCID_{50}$ per pig *via* intranasal (0.5 mL per nasal) and intramuscular (2 mL) routes simultaneously. The 3rd group of pigs received the same volume of DMEM and served as a control. Rectal temperature and body weight were measured daily. In addition, the condition of animal survival was recorded and the mortality was calculated. All animals were euthanized with propofol (2.5 mg kg^{-1}, Peifen, China) and potassium chloride (0.1 g kg^{-1}, Huamu, Jilin, China) *via* intravenous injection at the end of this study (14 days post-infection, dpi). Serum samples were collected for viremia and antibody detection, and different tissues were isolated for H&E staining and immunohistochemistry.

2.4. Viral copy number quantification

Serum samples were collected on 0, 3, 7, 9, and 14 dpi. To detect the PRRSV RNA in serum, quantitative real-time reverse-transcription polymerase chain reaction (RT-qPCR) was used. Viral RNA was extracted using TIANamp Virus DNA/RNA Kit (TIANGEN, Beijing, China) and reverse transcripted using HiScript III 1st Strand cDNA Synthesis Kit (Vazyme, China) according to the manufacturer's instructions. RT-qPCR was performed using a Premix Taq (TaKaRa, Japan) on ABI QuantStudio3 (Applied Biosystems, Waltham, MA, USA). Primers and probes used for viral quantification are listed below: PRRSV2-UF: TTGTGCTTGCTAGGCCGC; PRRSV2-UR: ACGACAAATGCGTGGTTATCA; PRRSV-UP: FAM-TCTGGCCCCTGCCCA-MGB (Chen *et al.* 2019). Ct values were calculated for absolute PRRSV RNA quantity (copy number) according to the standard curve produced by the different dilutions of the positive PRRSV plasmids.

2.5. Immunofluorescence assay

PAMs were seeded in twelve-well plate and were mock infected or infected with PRRSV NADC34 or rNADC34 for 36 h. Cells were fixed with 4% paraformaldehyde (Biosharp, China) for 10 min and then permeabilized by 0.5% Triton X-100 diluted in PBS for 15 min. After blocking with 1% BSA for 30 min, cells were incubated with an anti-PRRSV N antibody (MEDIAN, Republic of Korea) overnight at 4°C. Rinsing with PBS for three times, cells were incubated with an Alexa fluor 555-conjugated anti-mouse IgG (Cell

Table 1 List of primers for the construction of rNADC34

Primer	Sequence (5′→3′)
JS2021-F1-F	GGTCTATATAAGCAGAGCTACGCGTTAATAGCATGACGTATAGGTGTTGGC
JS2021-F1-R	CACCTACCTTGCGAAAGTAC
JS2021-F2-F	CTTATGTACTTTCGCAAGGT
JS2021-F2-R	CCAGCCGGAAGGGCTTAAAACTAGTGCGGCCGCGTGTACTGG
JS2021-F3-F	TGGGAGTCCAGTACACGC
JS2021-F3-R	ACTGGGTCTTCTAGCGAG
JS2021-F4-F	AGAGTTCTCGCTAGAAGACC
JS2021-F4-R1	AGCGAGGAGGCTGGGACCATGCCGGCCTTTTTTTTTTTTTTTTTTTTAATTTCGGCCGC
JS2021-F4-R2	CCAGCCGGAAGGGCTTAAAACTAGTGTCCCATTCGCCATTACCGAGGGGACGGTCCCCTCGGAATGTTGCCCAGCCGGC GCCAGCGAGGAGGCTGGGAC

Signaling Technology, Danvers, MA, USA) for 1 h. Finally, 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Beyotime, Shanghai, China) was used to stain nucleus for another 5 min. Images were acquired with an inverted fluorescence microscope (UHGLGPS; OLYMPUS, Tokyo, Japan).

2.6. Western blot

PAMs were seeded in six-well plate (2×10^6cells per well). When grew to 60–70% confluence, cells were mock infected or infected with PRRSV NADC34 or rNADC34 for 36 h. Cells were lysed in cell lysis buffer (Beyotime, Shanghai, China) on ice and lysates were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was electric-transferred onto a polyvinylidene fluoride (PVDF) membrane (Merck Millipore, Billerica, MA, USA). The membranes were blocked with 5% nonfat-dried milk for 1 h at room temperature. Then membranes were incubated with an anti-PRRSV N antibody (MEDIAN, Republic of Korea) and anti-GAPDH antibody (Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. After washing the membranes with TBST (20 mmol L^{-1} Tris-HCl pH=8.0, 150 mmol L^{-1} NaCl, 0.05% Tween 20) for three times, the corresponding secondary antibodies were used for additional 1 h at room temperature followed by washing with TBST for 3 times. The proteins were visualized using an enhanced chemiluminescence (ECL) reagent (NCM Biotech, Suzhou, China) on Tanon 5200 system (Tanon, Shanghai, China).

2.7. Histopathology and immunohistochemistry staining

Healthy pigs or pigs with PRRSV NADC34/rNADC34 challenge were euthanized and necropsied on the indicated days. Lung, lymph node, and tonsil samples were collected and fixed in 4% paraformaldehyde (Biosharp, Hefei, China) for 18 h. Then the samples were dehydrated, paraffin embedded, and cut into 5 μm thick sections. The sections were mounted onto microscope slides and stained automatically by Leica fully automatic dyeing machine for histopathology staining. Immunohistochemistry was performed with the Cell and Tissue Staining Kit (R&D, Emeryville, CA, USA) according to the manufacturer's instructions. The anti-PRRSV N antibody (MEDIAN, Republic of Korea) was used as the primary antibody. The images of sections were acquired using 200× microscope.

2.8. Viremia and serological test

Serum samples of pigs were collected at 0, 3, 7, 9, and 14 dpi. For detection of viremia, viral RNA was extracted from serum samples using TIANamp Virus DNA/RNA Kit (TIANGEN, Beijing, China). The quantitative PCR (qPCR) was performed as described above. For the measure of PRRSV-specific antibody, Herdcheck PRRSV X3 antibody test (IDEXX Laboratories, Westbrook, ME) was used according to the manufacturer's instructions. Sample-to-positive (S/P) ratios reflected PRRSV-specific antibody level. Results were reported as negative (ELISA S/P ratio of <0.4) or positive (ELISA S/P ratio of ≥0.4).

2.9. Statistical analysis

Statistical analysis was performed using SPSS 17.0 and GraphPad Prism 6.0. Data are mean±SE. The Student's *t*-test, one-way or two-way ANOVA were used to analyze the data. *P*-value less than 0.05 was considered to be significant.

3. Results

3.1. The construction and identification of PRRSV rNADC34 strain

Firstly, we amplified two cDNA clones from JS2021NADC34 strain, a NADC34-like strain isolated in our laboratory. These two cDNA clones and skeleton vector were overlapped through homologous recombination *in vitro*. Secondly, another two fragments of NADC34-like strains were amplified into cDNA, and cloned to the first constructed plasmid through homologous recombination. Ultimately, a full-length cDNA clone of JS2021NADC34 strain was constructed (Fig. 1-A). The viruses were rescued by plasmids transfection in BHK-21 cells. At 48 hours post-transfection (hpt), PAMs were incubated and passaged with the harvested virus supernatants for three generations. As shown in Fig. 1-B, the PRRSV rNADC34 could replicate in PAM with typical cytopathic effect (CPE), which was similar with parental strain-infected PAMs. Meanwhile, PRRSV N protein expression was observed in cells infected with parental strain and PRRSV rNADC34 strain, but not in mock-infected cells (Fig. 1-C and D). These data suggested that PRRSV rNADC34 strain was successfully rescued and had cytotropism to PAMs.

3.2. The replication of PRRSV rNADC34 in PAMs

In order to compare the level of replication in PAMs between NADC34 strain and rNADC34 strain, the viral titers and copy numbers of the third passaged viruses were measured. As shown in Fig. 2-A, the viral titers of rNADC34 strain were comparable with NADC34 strain, and the trend of the one-step growth curves were consistent with each other. Simultaneously, the copy number of rescued virus showed slightly lower than that of parental strain at some time points, but showed no significant difference (Fig. 2-B). The results indicated that the rescued strains had the same potential to replicate in PAMs.

3.3. Clinical symptoms of pigs upon PRRSV rNADC34 challenge

In order to evaluate the pathogenicity of PRRSV rNADC34, fifteen pigs were divided into three groups, including a mock group, NADC34 group, and rNADC34 group. Pigs in the mock group were received DMEM as placebo, in NADC34 group were challenged with JS2021NADC34 PRRSV using a nasal drip and intramuscular injection, in rNADC34 group were infected with PRRSV rNADC34 using the same routes. Body temperature and body weight of the pigs were measured every day for the next 14 d. As shown in Fig. 3-A, the body temperature of pigs in mock group remained in the normal range and varied between 38.5°C and 39.5°C, while the body temperatures of NADC34- and rNADC34- infected pigs were 1–2 degree higher than the mock-infected pigs. Pigs infected

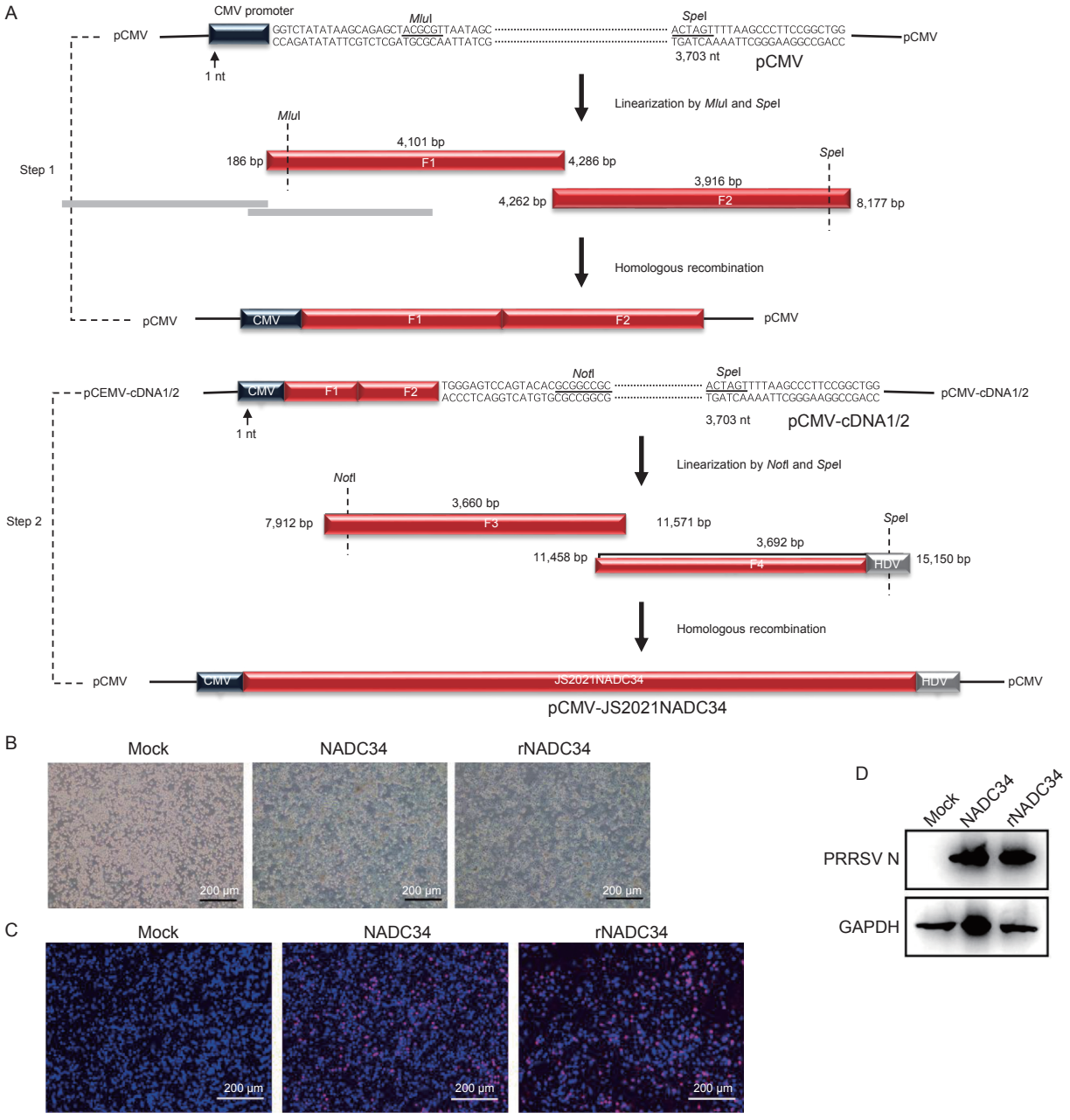


Fig. 1 Construction and rescue of an infectious cDNA clone of JS2021NADC34 porcine reproductive and respiratory syndrome virus (PRRSV). A, two-step assemblage of rNADC34 strain through homologous recombination *in vitro*. HDV, hepatitis D virus ribozyme sequence. *Mlu*I, *Spe*I, and *Not*I were restriction enzymes for plasmid linearization. B, porcine alveolar macrophage (PAM) were mock-infected or infected with NADC34 and rNADC34 strains (P3) at an MOI of 0.5 for 36 h. The CPE was shown using 200× microscope. C and D, PAMs were mock-infected or infected with NADC34 and rNADC34 strains (P3) at an MOI of 0.5 for 36 h. Then cells were fixed with 4% paraformaldehyde and incubated with an anti-PRRSV N (4A5) antibody, followed by an Alexa fluor 555-conjugated anti-mouse IgG (red) (C). Cells were harvest for Western blot (D). The protein levels of PRRSV N and GAPDH were detected.

with PRRSV rNADC34 had the similar variation tendency with PRRSV NADC34 in body temperature. The temperature climbed sharply on the first day post-infection in both NADC34- and rNADC34-infected pigs. The highest temperature of NADC34- and rNADC34-infected pigs reached up to 41.5°C on 8 and 6 dpi, respectively. Moreover, the mock-infected pigs had more than 0.25 kg of body weight gain per day, while PRRSV NADC34-infected pigs lost more than 0.3 kg of body weight and PRRSV rNADC34-infected pigs lost almost 0.4 kg of body weight in average (Fig. 3-B). When infected pigs were dead and survival pigs were euthanized at the end of the study, pigs were necropsied and the pathology of their lungs were scored. The lungs in mock-infected pigs looked normal and no obvious lesions were observed. By contrast, NADC34- and rNADC34-infected pigs showed severe hemorrhage, congestion, pulmonary consolidation, and even necrosis in the lungs. As shown in Fig. 3-C, the scores in rNADC34-infected pigs were comparable with the scores in NADC34-infected pigs, but much higher than the mock-infected pigs. In addition, two of NADC34-challenged pigs died at 9 and 11 dpi, with the

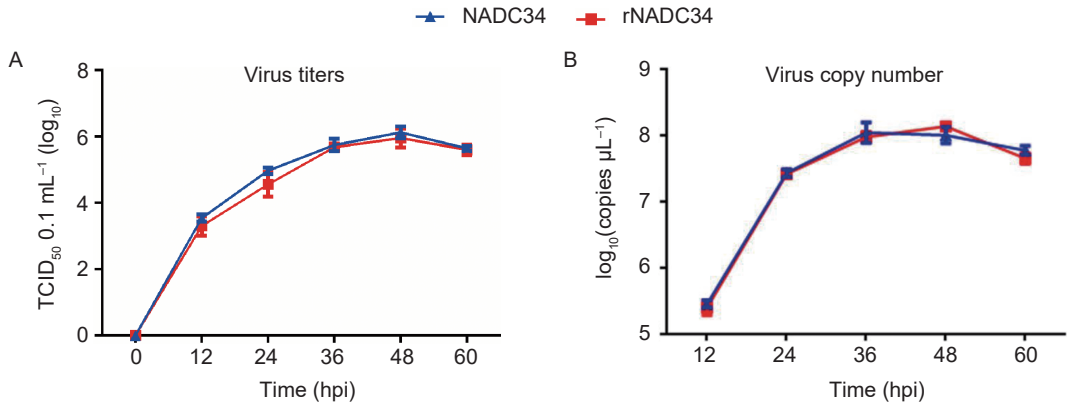


Fig. 2 rNADC34 strain could replicate in porcine alveolar macrophages (PAMs). A and B, PAMs in a 12-well plate were infected with rNADC34 strain of P3 and NADC34 strain at an MOI of 0.5, respectively. To detect multi-step virus growth curves, virus supernatants were harvested at 0, 12, 24, 36, 48, and 60 hours post-infection (hpi) and titrated by $TCID_{50}$ assay (A). At the time points 0, 12, 24, 36, 48, and 60 hpi, cells were harvested for RT-qPCR (B). The copy numbers of rNADC34 and NADC34 strains were calculated according to standard plasmid's amplification curve. Data are mean±SE (*n*=3).

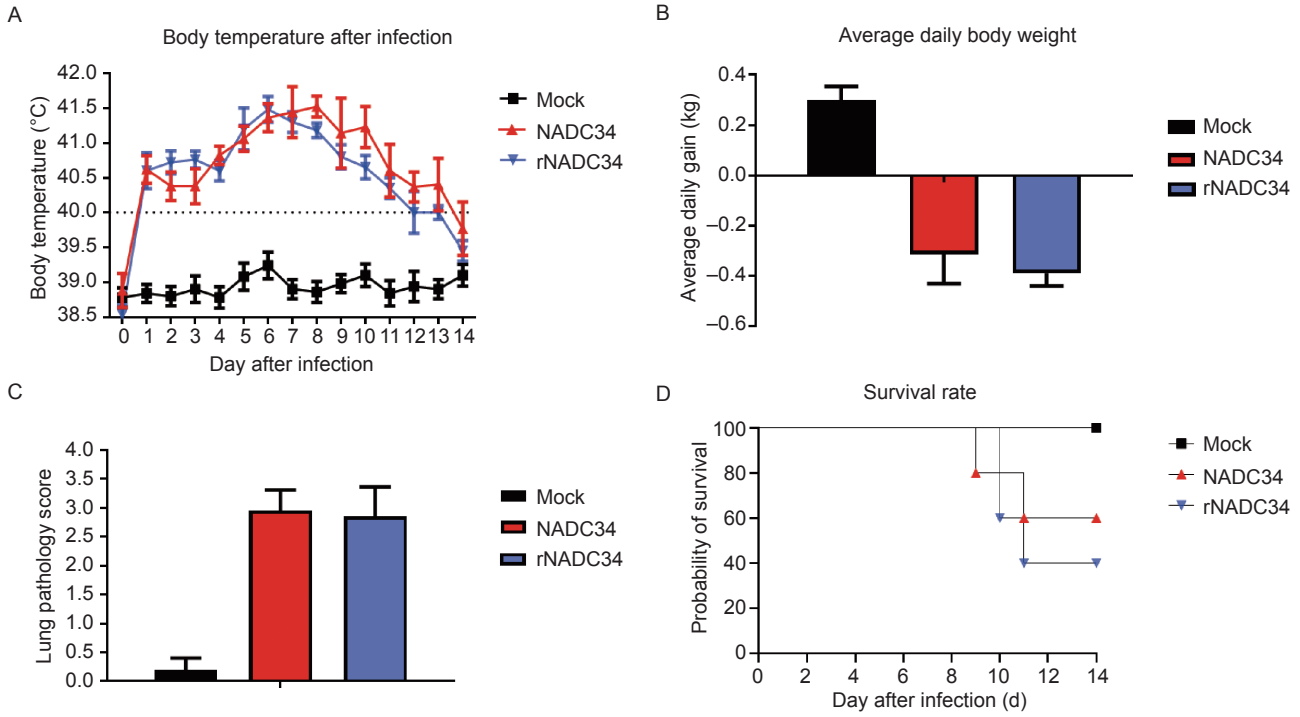


Fig. 3 Clinical feature of pigs after PRRSV rNADC34 infection. A, body temperature was measured every day when pigs were challenged with NADC34 and rNADC34 strains. B, averaged body weight gain was calculated. C, pathology of lungs was scored according to the pathological changes of lung lesions visually. D, mortality and survival curve of pigs during NADC34 and rNADC34 challenge. Data are mean±SE (*n*=5).

mortality rate of 40%. One of rNADC34-infected pigs died at 10 dpi, and another two rNADC34-challenged pigs were died at 11 dpi, with the mortality rate of 60%. All mock-infected pigs survived at the end of study (Fig. 3-D). The above data indicated that rNADC34 strain showed high pathogenicity for nursery pigs.

3.4. Histopathological examinations after PRRSV rNADC34 infection

Tonsil, lung, and lymph node samples were collected for histopathological examination. As shown in Fig. 4, NADC34 and rNADC34 strains caused severe tissue damage in lung, lymph node, and tonsil samples according to the results of H&E staining. The lungs infected with NADC34 and rNADC34 strains manifesting hemorrhage, thickening of the alveolar walls and infiltration of a large number of inflammatory cells in the pulmonary interstitium. Moreover, the lymph nodes and tonsils showed acute hemorrhage, lymphocyte depletion, and infiltration of neutrophils in both NADC34- and rNADC34-infected pigs. The damage to the

tissues was comparable between NADC34-infected pigs and rNADC34-infected pigs. By contrast, no pathological lesions were observed in the pigs of control group.

3.5. Immunohistochemistry examinations after PRRSV rNADC34 infection

In order to detect the PRRSV antigen in these tissue samples, a monoclonal antibody specific to PRRSV N protein was used for immunohistochemistry staining. As shown in Fig. 5, there was no positive signal in the sections of mock-infected pigs. On the contrary, the virus antigens (brown staining dots) were detected in the lung, tonsil, and lymphoid of NADC34-infected pigs. Meanwhile, positive staining signals were also detected in these samples of pigs infected with rNADC34 and the amount of PRRSV antigen was similar to NADC34 strain. These data indicated rNADC34 also showed high pathogenicity for nursery pigs *in vivo*.

3.6. Viremia and anti-PRRSV antibody response after PRRSV rNADC34 challenge

In order to investigate the specific antibodies and viremia caused by rNADC34 infection, pig serum samples were collected at 0, 3, 7, 9, and 14 dpi. As expected, the antibody levels of mock-infected pigs remained negative, while PRRSV-specific antibodies after NADC34 and rNADC34 infection were gradually increased until 14 dpi. The level of antibodies in rNADC34 infection was mildly lower than NADC34 infection at 14 dpi (Fig. 6-A). In addition, viral nucleic acid was detected at 3 dpi in NADC34- and rNADC34-infected pigs. The levels of viral nucleic acids rapidly increased and reached to the maximum nearly 4×10^8 copies PRRSV RNA mL^{-1} at 7 dpi, and the viremia in the survived pigs declined at the end of the study. The viral copy number in rNADC34-infected pigs was slightly higher than that in NADC34-infected pigs at 3 and 9 dpi (Fig. 6-B). These data demonstrated that PRRSV rNADC34 could induce PRRSV-specific antibodies and cause viremia.

4. Discussion

PRRSV is one of the most important pathogens in global swine industry. Among them, NADC34-like strains are detected frequently in the field in recent years. Remarkably, NADC34-like strains have potential to become one of the main epidemic strains in some areas of China (Zhao *et al.* 2022). In our previous study, we found that an isolated JS2021NADC34 strain, showed high pathogenicity for nursery pigs, manifesting lasting fever, reduced body weight, and high mortality, accompanied with histopathological interstitial pneumonia, lymphocyte depletion, and acute hemorrhage. The pathogenicity of this isolate is quite different from another Chinese NADC34-like strain isolated by Song *et al.* (2020). Moreover, we demonstrated that common commercial PRRSV vaccine could not provide complete protection to this NADC34-like PRRSV infection (Yuan *et al.* 2023). In this study, we successfully constructed a full-length infectious clone of the JS2021NADC34 strain, named rNADC34 strain. Consistent with parental strain, rNADC34 only proliferated in PAMs, but not Marc-145 cells, a cell line permitted for most PRRSV strain replication (Fig. 1-B and D). Moreover, rescued virus copy numbers and virus titers were generally

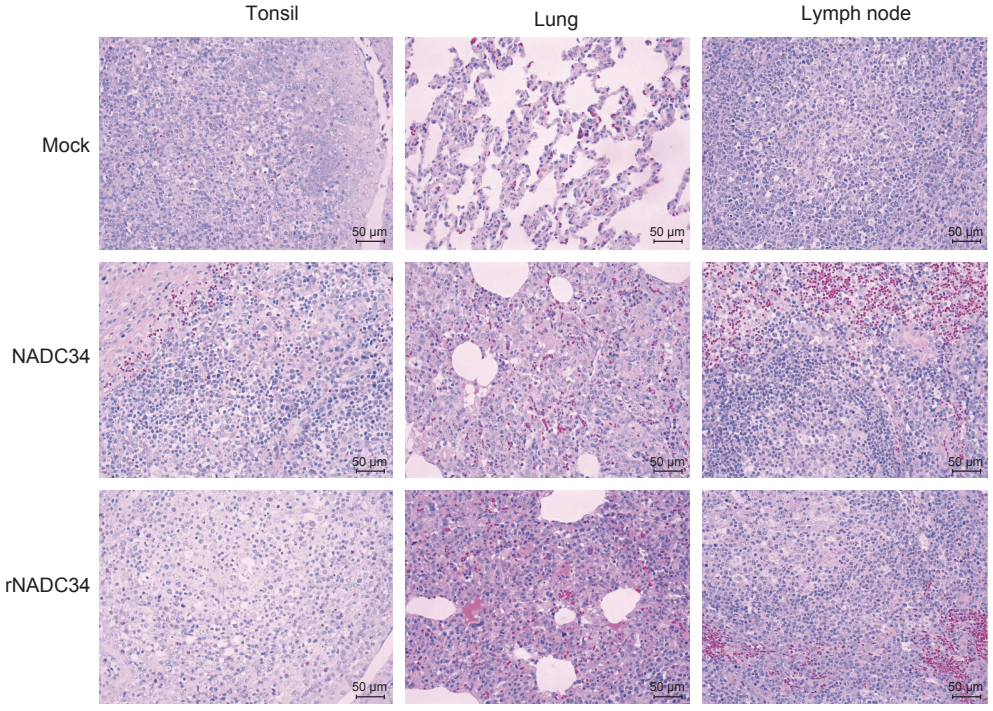


Fig. 4 Histopathological examination in mock-infected pigs (first row), NADC34 -infected pigs (second row), and rNADC34 -infected pigs (third row). Tonsil, lung, and lymph node samples from three groups were collected and embedded in paraffin, making pathological section. Then paraffin sections were stained with H&E. Typical histopathological manifestations were visualized using 200× microscope.

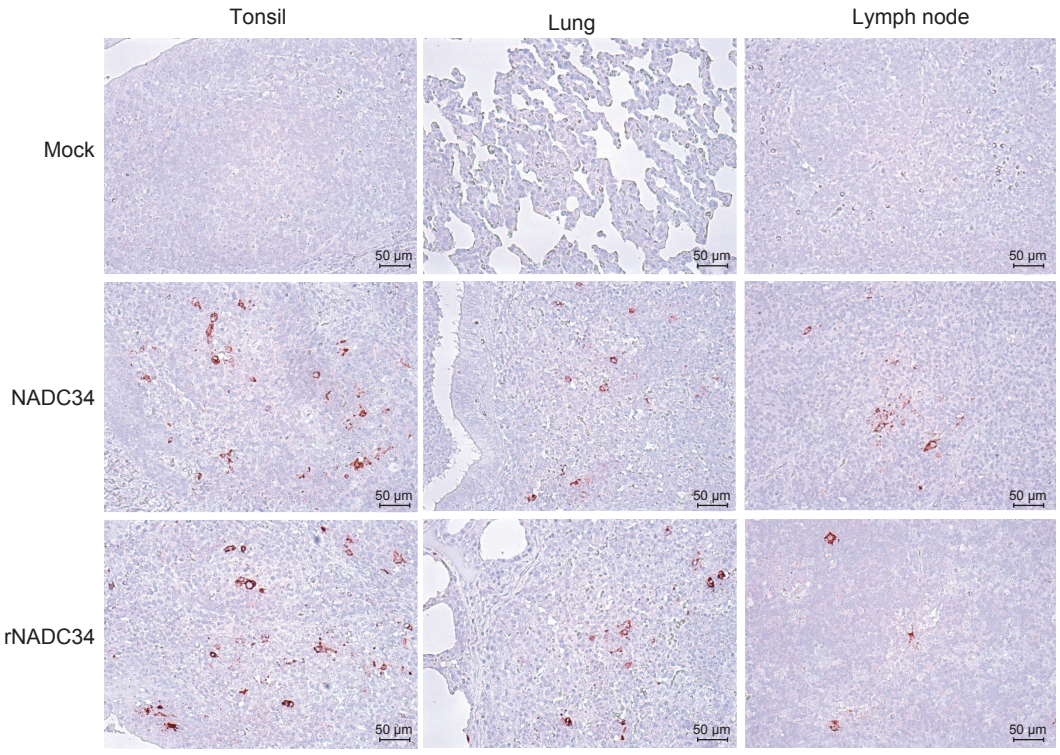


Fig. 5 Immunohistochemistry examination in the mock-infected pigs (first row), porcine reproductive and respiratory syndrome virus (PRRSV) NADC34-infected pigs (second row), and PRRSV rNADC34-infected pigs (third row). Tonsil, lung, and lymph node paraffin sections were stained with PRRSV N protein antibody. The dark brown staining represented PRRSV antigen.

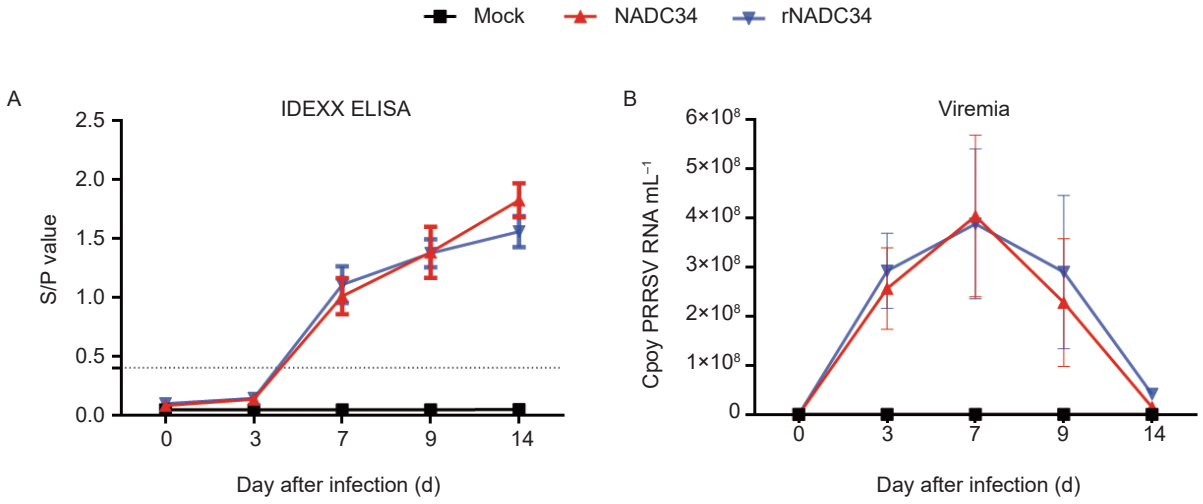


Fig. 6 Viremia and PRRSV-specific antibodies present in rNADC34-infected pigs. A, blood samples were collected to detect anti-porcine reproductive and respiratory syndrome virus (PRRSV) antibody titers using IDEXX HerdCheck ELISA at 0, 3, 7, 9, and 14 days post-infection (dpi). Sample-to-positive (S/P) ratios reflected PRRSV-specific antibody level. B, PRRSV viral RNA in serum was measured by RT-qPCR at 0, 3, 7, 9, and 14 dpi. Data are mean±SE (n=5).

the same with parental strain at different time points in PAMs (Fig. 2). However, the replication level of rNADC34 at some time points seemed slightly lower than parental strain, the possible reason may be that the virus was not fully adapted to PAMs for only three-generation passages.

In terms of pathogenicity, the rescued virus was also highly pathogenic for nursery pigs, which was consistent with parental strain. The rNADC34 strain caused lasting fever and weight loss for nursery pigs, the highest temperature almost reached 41.5°C, and the average bodyweight was decreased nearly 0.4 kg daily. The mortality of rNADC34 infected pigs (3/5) was higher than NADC34 infected pigs (2/5, Fig. 3). Moreover, the pathological change in tonsil, lung, and lymph node of rNADC34 infected pigs was obvious,

with hemorrhage and severe infiltration of neutrophils (Fig. 4). In addition, the ability to induce neutralizing antibody and viremia was comparable between rNADC34 and NADC34 strains (Fig. 6). These results indicated rNADC34 strain we constructed showed high pathogenicity for nursery pigs. The reason for severe pathogenicity of rNADC34 strain was probably its parental strain JS2021NADC34, which showed high homology with IA/2014/NADC34 strain in America without recombination (Zhu *et al.* 2022). IA/2014/NADC34 strain caused more severe disease and was characterized by high levels of pyrexia (van Geelen *et al.* 2018). JS2021NADC34 strain also caused similar clinical symptom and high mortality with IA/2014/NADC34 strain, so that rNADC34 strain triggered similarly severe disease for nursery pigs. In addition, PRRSV NADC34 caused notable abortion in sows in the USA, whether JS2021NADC34 PRRSV or its clone strain is highly pathogenic to sows needs further investigation.

The reverse genetics technique is rapidly developed and commonly used in the study of virology and vaccinology. It was a powerful tool to investigate virulence, pathogenesis, immune responses, and vaccine development (Han and Yoo 2014). There are three possible applied fields as follows: firstly, infectious clones are usually used for viral tropism identification *via* substitution genes among arteriviruses. JS2021NADC34 strain and its rescued virus could only proliferate in PAMs, but not in Marc-145 cells. To further identify the reasons for the viral cytotropism and better develop the corresponding vaccine, the substitution of structural genes with PRRSV strains which are susceptible to Marc-145 cells can take into consideration. It was reported that PRRSV minor proteins were responsible for cell tropism (Dokland 2010). Due to the cell restriction of rNADC34 strain, we can use rNADC34 infectious clone as a backbone substituting structural and even only minor envelope of other PRRSV strains, thus allowing the chimeric PRRSV to acquire a broad cell tropism. Secondly, rNADC34 strain can be used as a vector to investigate the virulence and pathogenic gene of NADC34-like PRRSV. Engineering the PRRSV NADC34 genome *via* amino acid substitution, mutation and depletion make it possible to manipulate the viral genome and create genetically mutant viruses. We can utilize this genetic tool to identify the precise amino acid sites associated with virulence and pathogenesis of NADC34 strain. In addition, NADC34 strain induced low neutralizing antibodies, but its infectious clones can be used as a backbone for chimeric viruses, which could substitute with some envelope proteins (GP3, GP4, GP5, and M) of strains that susceptible to serum neutralization. Thirdly, rNADC34 infectious clone could be used as a tool for vaccine development. In our previous study, we identified some commercial vaccines could not provide complete protection against PRRSV NADC34 infection. NADC34 strains were frequently detectable in the field, therefore, it is necessary to study the protective vaccines for NADC34 strain. The infectious clone rNADC34 we constructed may play an important role as a vector or the basis skeleton for reconstruction.

cDNA infectious clone construction, virus rescue, and animal experiments targeting different PRRSV strains have been reported in many reports. Unlike the direct homologous recombination of four PRRSV overlapping fragments, we divided the constructed strategy into two steps of homologous recombination. During plasmid construction, enzyme digestion and sequencing analysis could be performed at each step to ensure the integrity and accuracy of the rescued viruses. Although there are more steps in our method, the efficiency of homologous recombination is higher, back mutation is more convenient, and the chance of positive rescued viruses are higher. For improvement of construction methods, the homologous recombination conditions can be optimized, and fluorescent protein or luciferase can be inserted for labeling to improve the screening efficiency.

5. Conclusion

In summary, we successfully rescued a PRRSV NADC34 strain using infectious clone technique and evaluated its pathogenicity for nursery pigs. We found rNADC34 strain could replicate in PAMs, and the virulence of rNADC34 strain was comparable to parental strain. Moreover, rNADC34 strain showed high pathogenicity for nursery pigs with severe fever and histopathological lesion, which was consistent with PRRSV NADC34 strain. The reverse genetics of PRRSV NADC34 provides a basic understanding of the biology of emerging NADC34 strain and promotes the development of corresponding live modified vaccines.

Acknowledgements

This work was supported by the National Natural Science Foundation of China-CGIAR (32361143511), the National Key Research and Development Program of China (2022YFD1800301), the Natural Science Foundation of Jiangsu Province, China (BK20210804), the Jiangsu Innovative and Entrepreneurial Talent Team Project, China (JSSCTD202224), the 111 Project D18007, and the Priority Academic Program Development of Jiangsu Higher Education Institutions, China (PAPD). Zhenbang Zhu is supported by the “LvYangJinfeng Program” of Yangzhou City, China.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscript.

Ethical approval

Pigs used in our study were kept in housing with suitable temperature and humidity where water and food were available ad libitum. All animal experiments were approved by the Institutional Animal Care and Use Committee of Yangzhou University, China under the Laboratory Animals-Guideline of welfare and ethics. The protocol license number was 202204002.

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