



A strain of highly pathogenic porcine reproductive and respiratory syndrome virus: genomic characterization, pathogenicity, and construction of an infectious full-length cDNA clone

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Abstract

Porcine reproductive and respiratory syndrome (PRRS) is a highly contagious infectious disease caused by porcine reproductive and respiratory syndrome virus (PRRSV), which inflicts major economic losses on the global pig farming industry. Based on its similarity to highly pathogenic strains, the GDzj strain isolated in this study was predicted to be highly pathogenic. We therefore analyzed the pathogenicity of this strain experimentally in piglets. All piglets challenged with this virus experienced fever or high fever, loss of appetite, decreased food intake, daily weight loss, shortness of breath, and listlessness, and the necropsy results showed that they had experienced severe interstitial pneumonia. We then used the BAC system to construct a full-length cDNA infectious clone of GDzj, and the rescued virus displayed *in vitro* proliferation characteristics similar to those of the parental PRRSV strain. In summary, we successfully isolated a highly pathogenic PRRSV strain and constructed a full-length infectious cDNA clone from it, thereby providing an effective reverse genetics platform for further study of viral pathogenesis.

Abbreviations

HP-PRRSV Highly pathogenic porcine reproductive and respiratory syndrome virus
PRRS Porcine reproductive and respiratory syndrome

ORFs Amino acid, aa; open reading frame
NSP Non-structural protein
BAC Bacterial artificial chromosome
RT-PCR Reverse transcription polymerase chain reaction
MOI Multiplicity of infection
MCS Multiple cloning site
FBS Fetal bovine serum
IFA Immunofluorescence assay

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Introduction

Porcine reproductive and respiratory syndrome (PRRS) is a highly contagious infectious disease caused by porcine reproductive and respiratory syndrome virus (PRRSV), a member of the family *Arteriviridae* [4]. It is an enveloped, positive-sense, single-stranded RNA virus. The genome of this virus is approximately 15 kb long, with a methylated 'cap' structure at the 5' end, a polyadenylic acid (poly A) tail at the 3' end, and at least 10 open reading frames (ORFs) [26, 30]. Most of the adjacent ORF gene structures in PRRSV overlap. ORF1, which accounts for 80% of the total genome length, encodes proteins involved in viral replication. This largest segment of the genome includes ORF1a and ORF1b, which encode two polyprotein types, pp1a and pp1ab. The pp1a polyprotein is processed to form nine non-structural proteins (NSPs)—NSP1 α , NSP1 β , and NSP2–NSP8—whereas pp1ab is processed to form NSP9–NSP12 [32]. ORFs 2–7, at the 3' end of the genome, mainly encode glycoprotein (GP) 2, the envelope protein, GP3, GP4, GP5, ORF5a, the membrane matrix protein, and the nucleocapsid protein (N) [13]. Studies in China and abroad have shown that the variation seen in different strains of PRRSV is distributed across the genome, with a difference of up to 40% among them, but two genes, NSP2 and GP5, are the most variable [16]. On the basis of such differences in the viral genome sequences and the pathogenicity and antigenic characteristics of the virus, PRRSV falls into two major genotypes: the European type (genotype 1) and the North American type (genotype 2) [1, 10, 19]. The nucleotide sequence identity between these two genotypes is only approximately 60% [18, 20].

Since its first isolation in North America in 1987, PRRSV has been isolated in the Netherlands [29] and the USA [2], and it has impacted the global pig industry for nearly 40 years [21, 33]. In 2006, outbreaks of highly pathogenic (HP) PRRSV began to spread in China [27]. Clinically, PRRS causes high mortality in pig herds of all ages and also causes severe reproductive disorders in pregnant sows. The molecular characteristics of the HP-PRRSV genome include a unique 30 (1+29) discontinuous amino acid (aa) deletion in NSP2. The clinical features of this infection include pyrexia (40 °C–42 °C), a high incidence rate (50%–100%), and a high pathogenicity rate (20%–100%) [6]. Isolates representing the HP-PRRSV type include HUN4 and JXA1 [35]. That these HP strains are now predominant in China has seriously impacted China's pig farming industry.

Many aspects of PRRSV, such as its pathogenic mechanisms, are not well understood. Therefore, establishing a reverse genetics system for this pathogen would help to clarify many outstanding issues at the molecular level.

Many full-length infectious cDNA clones of RNA viruses have been successfully constructed [J et al., 1998, [22, 28, 31]. Most of the reverse genetics platforms for PRRSV are based on the classical European and North American PRRSV strains, and there have been few reports on infectious clones of HP-PRRSV strains [14, 36].

In this study, we successfully isolated a strain of HP-PRRSV and named it GDzj. We investigated the genetic relationships of GDzj by performing molecular evolutionary analysis and nucleotide sequence comparisons, and we examined its pathogenicity using challenge infections in piglets. We also constructed a full-length infectious cDNA clone of the GDzj strain using the bacterial artificial chromosome (BAC) system, providing an efficient platform for further research on the biological characteristics of this virus.

Materials and methods

Cells, clinical samples, strains and vectors

PRRSV cultures were passaged in Marc-145 cells. Lung samples were collected from sows on a commercial pig farm in Guangdong Province, China, that displayed clinical signs of PRRS. These sows had undergone stillbirths and showed signs of pyrexia, dyspnea, and spontaneous abortion. Trans5 α competent cells and BAC vector plasmids (pSMART BAC v2.0; *Bam*HI linearized) were preserved in the Institute of Swine Disease Control and Prevention, College of Animal Science, South China Agricultural University. BAC competent cells were purchased from Bomad Biotechnology (Gu'an, China) Co., Ltd. The pEASY-Blunt vector was purchased from Beijing TransGen Biotechnology (Beijing, China) Co., Ltd.

Main reagents

DNA and RNA extraction kits were purchased from Magen (Shanghai, China). Reverse transcription reagents, agarose gel DNA recovery kits, and plasmid extraction kits were all purchased from Promega (USA). High-fidelity DNA polymerase was purchased from Baori Biotechnology Co., Ltd. (Beijing, China) DS2000 and DS15000 DNA markers were purchased from Guangzhou Dongsheng Biotechnology Co., Ltd. Restriction endonucleases, T4 DNA ligase, Lipofectamine LTX, and associated reagents were purchased from Thermo Fisher Scientific (USA). Homologous recombinase and 2 \times Taq Plus Master Mix were purchased from Nanjing Novezin Biotechnology Co., Ltd. Fetal bovine serum, trypsin, DMEM high-glucose medium, and opti-MEM were purchased from GIBCO (USA). The PRRSV N protein monoclonal antibody was purchased from Beijing Yiqiao Shenzhou Technology Co., Ltd., and the Coralite

Table 1 Primers for amplification of PEDV PCV2 and PCV3

Primer	Sequence (5'-3')	Length (nt)
ORF3-F	CGGTGCTTGTTTTTCAGGTTG	814
ORF3-R	CAATTGGACGAAGGTAATGCT	
PCV2-F	CTGTTTTTCGAACGCAGTGCC	486
PCV2-R	GCATCTTACCCACACCCGCCT	330
PCV3-F	CCACAGAAGGGCGCTATGTC	
PCV3-R	CCGCATAAGGGTCGTCTTG	

F, forward PCR primer; R, reverse PCR primer

488-conjugated Affinipure Goat Anti-Rabbit IgG (H+L) secondary antibody was purchased from Guangzhou Guanjing Trading Co., Ltd.

Virus isolation and viral RNA extraction

The lung tissues with lesions used in this study were collected from a pig farm with suspected cases of PRRS in Guangdong Province. Individual lung tissues were cut into pieces, mixed with phosphate-buffered saline (PBS), and ground. After centrifugation, the supernatant was filtered through a 0.22- μ m filter. Marc-145 cells were used to isolate PRRSV. When approximately 80% of the virus-infected cells showed a cytopathic effect (CPE), the virus was obtained from them by freeze-thawing and then passaged blindly on Marc-145 cells for three generations. Viral RNA was extracted in accordance with the instructions from the DNA/RNA extraction kit (Magen). The extracted RNA was amplified by reverse transcription polymerase chain reaction (RT-PCR) using NSP2 primers to confirm the presence of PRRSV, and by PCR testing to ensure that porcine epidemic diarrhea virus (PEDV) and porcine circovirus (PCV) 2 and 3 were not present (Table 1 and Fig. 1).

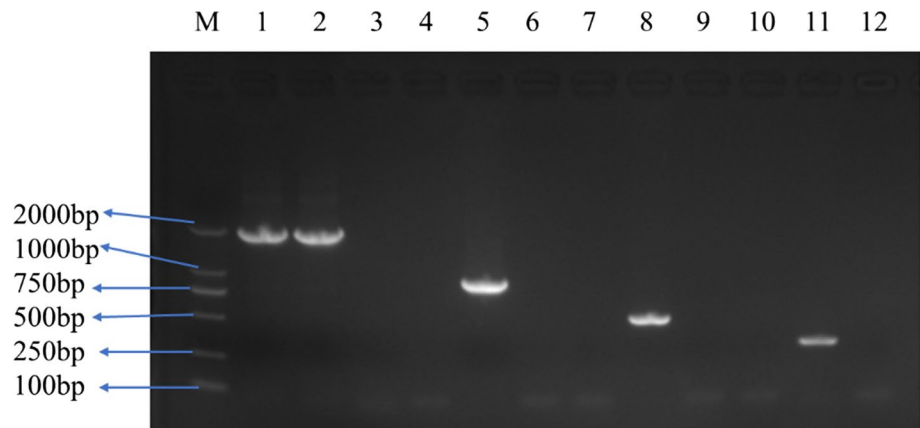
The extracted viral RNA was reverse transcribed using a reverse transcription kit (Promega) with random primers, and each reaction included 6 μ l of RNA template, 4 μ l of reaction buffer, 2 μ l of enzyme mix, and RNase-free water added to a total volume of 20 μ l. The resulting cDNA was PCR-amplified according to the operating instructions for the high-fidelity DNA polymerase, and the purified PCR product was ligated to the pEAST-Blunt vector and used to transform Trans5 α competent cells. Positive bacteria were selected for gene sequencing, and the sequencing products were automatically spliced together *in silico* using DNASTAR (USA) software to obtain the whole genome sequence of the virus. The complete genome sequence was named GDzj and was submitted to the GenBank database under the accession number MF772778.1.

Identification and sequence analysis of the isolated viruses

The virus was inoculated onto a monolayer of Marc-145 cells at a multiplicity of infection (MOI) of 0.1, and the cells were incubated at 37 °C with 5% CO₂. These cells were fixed with 4% paraformaldehyde for 30 min at 48 hours postinfection (hpi), permeabilized using 0.5% Triton X-100, washed three times with PBS for 10 min at room temperature, and then incubated with anti-PRRSV-N-protein antibody diluted 1:500 at 4 °C. After again washing three times with PBS, the cells were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG at 37 °C for 1 h. Finally, after three washes, the cells were observed by fluorescence microscopy.

We used Lasergene 7.1 (USA) and MEGA 6.0 (USA) software to compare the sequences of the sequenced strains to those of representative strains from China and abroad. We constructed phylogenetic trees based on the aa sequences of the NSP2 and GP5 genes of the GDzj strain and reference strains. Gene homology analysis was carried out using

Fig. 1 Detection of PRRSV, PEDV, PCV2, and PCV3. M, Marker DS2000 bp DNA; lanes 1–3, PRRSV NSP2 PCR products; lanes 4–6, PEDV ORF3 PCR products; lanes 7–9, PCV2 PCR products; lanes 10–12, PCV3 PCR products; lanes 2, 5, 8, and 11, positive control; lanes 3, 6, 9, and 12, negative control



SimPlot (USA) software using representative PRRSV sequences from the GenBank database (Table 2).

Animal experiments

To further study the pathogenic characteristics of the GDzj strain, an animal challenge experiment was performed using 21-day-old SPF weaned piglets. Laboratory tests for PRRSV, PCV2, PEDV, and *Mycoplasma pneumoniae* were all negative. The piglets, which were divided into two groups, were kept in separate spaces with separate water, food, and heating. One group ($n = 3$) was used as a control group, and the other group ($n = 5$) was used as the challenge infection group. Piglets in the control group were inoculated with 2 ml of porcine alveolar macrophage culture supernatant. Piglets in the infection challenge group were inoculated intranasally with 2 ml of the virus at a concentration of 10^5 TCID₅₀/ml. The clinical effects of PRRSV infection were monitored, including changes in food intake, mental state, breathing, eyelid edema and secretions, exercise status, and skin and mucous membrane color. A separate thermometer was used to measure the rectal temperature of each pig once a day, and the weight of each pig was determined once every 3 days. Serum, which was collected from the piglets on days 0, 3, 5, 7, 10, 14, 17, and 21 post-challenge, was used to measure antibody and antigen levels and was also used for RT-qPCR to determine the viral load in each piglet.

During the experiments, piglet survival was recorded and a survival curve was drawn. Dead piglets were necropsied, and the remaining live piglets were euthanized at 21 dpi. To investigate tissue damage in the piglets and to determine the location of PRRSV in the lung tissues after challenge infection, the lungs from the piglets in each challenge group were collected, the regions showing obvious lesions were fixed, the tissues were sectioned, and immunohistochemical analysis was performed.

Construction of a full-length cDNA infectious clone of PRRSV

The scheme used for construction of a full-length cDNA clone of the GDzj strain is shown in Fig. 2. Briefly, the construction involved transforming competent cells with a low-copy-number BAC plasmid, designing and synthesizing a gene containing a CMV promoter, a multi-enzyme cleavage site, a hepatitis D virus ribozyme, and a BGH termination signal sequence, as shown in Fig. 2. The construct was ligated to the BAC plasmid via restriction enzyme digestion and ligation, and positive plasmids were identified by screening. A recombinant plasmid verified by sequencing was named pBAC.

The cDNA obtained by reverse transcription was amplified by RT-PCR using the primers shown in Table 3, and

four fragments were obtained. The fusion PCR method was used to mutate position 3407 in the GDzj genome, resulting in a cysteine-to-alanine mutation and introducing a single restriction site for *Bst*BI as a genetic marker for rescuing the virus. The amplified PCR products were recovered and purified separately, and the pBAC plasmid was digested using the restriction sites shown in Fig. 2. Four fragments were obtained, and fragments Bzj1 and Bzj4 contained portions of the pBAC plasmid. After double digestion of the pBAC vector with the appropriate restriction enzymes, the fragment Bzj1 was inserted into the pBAC vector using a homologous recombination kit according to the instructions, and the positive pBAC-Bzj1 plasmid was confirmed by sequencing. The other three fragments were inserted sequentially into the plasmid in the same way, resulting in a full-length cDNA plasmid clone, which was confirmed by sequencing and named pBAC-GDzj.

Rescue and identification of the virus

Marc-145 cells were transfected with the pBAC-GDzj plasmid when the cell density reached 70%–80%, using Lipofectamine LTX. At 96 h post-transfection, 90% of the cells contained lesions. After three repeated freeze-thaw cycles, the cell culture medium was collected and inoculated onto a monolayer of Marc-145 cells. After adsorption at 37°C for 1 h, and the supernatant was discarded, the cells were washed three times with PBS buffer, DMEM cell maintenance medium containing 2% fetal bovine serum (FBS) was added, and the plate was placed in an incubator at 37°C with 5% CO₂. The cells were examined daily for CPE. Approximately 90% of the lesion-containing regions were freeze-thawed to harvest the virus. The rescued virus was named RvGDzj. The experiment was set up with the parental GDzj virus and untransfected Marc-145 cells as controls.

Indirect immunofluorescence assay (IFA), RT-PCR, single enzyme digestion identification, and sequence determination were used to identify the third-generation (P3) rescued virus. The third-generation RvGDzj-P3 (from the rescued virus) and the third-generation GDzj-P3 (from the parental virus) were inoculated separately onto a Marc-145 cell monolayer, and the plate was placed in an incubator at 37°C with 5% CO₂. After 48 h, IFA was conducted in accordance with standard procedures, and a PRRSV N protein antibody was used for IFA detection. For RT-PCR identification, 200 µL of the rescued virus suspension was used for viral RNA extraction using a DNA/RNA extraction kit, and RT-PCR amplification was performed with PRRSV GP5- and NSP2-specific primers (Table 4). The amplified PCR products were subjected to agarose gel electrophoresis and then sequenced. In these experiments,

Table 2 Information on representative PRRSV strains

No.	Strain	Source	Year	Accession no.
1	LV	Netherlands	1993	NC_043487.1
2	CH-1a	Beijing, China	2000	AY032626
3	HB-1_sh_	Hebei, China	2002	AY150312
4	VR-2332	America	2002	AY150564.1
5	RespPRRS-MLV	America	2005	AF066183
6	JXA1	Jiangxi, China	2006	EF112445.1
7	JXWN06	Jiangxi, China	2006	EF641008.1
8	HUN4	Hunan, China	2007	EF635006.1
9	WUH4	Hubei, China	2007	EU187484.1
10	GD	Guangdong, China	2008	EU825724.1
11	CWZ-1-F3	Chongqing, China	2009	FJ889130.1
12	JXA1-P80	Guangdong, China	2009	FJ548853.1
13	GM2	Guangdong, China	2011	JN662424.1
14	QYYZ	Guangdong, China	2011	JQ308798.1
15	QY2010	Guangdong, China	2012	JQ743666.1
16	JXA1-P160	Beijing, China	2013	KC422731.1
18	CHsx140	Beijing, China	2015	KP861625.1
19	NVDC-CQ4-2012	Beijing, China	2015	KP771777.1
24	CH	Taiwan, China	2015	KP998416.1
17	HENXX-1	Henan, China	2016	KU950372.1
20	GDZS2016	Guangdong, China	2016	MH046843.1
21	JSWA	Shandong, China	2016	KY373214.1
22	SCN17	Sichuan, China	2017	MH078490.1
23	NADC30	Shandong, China	2018	MH500776.1

Fig. 2 pBAC-GDzj plasmid construction strategy. The fragment that includes the unique restriction enzyme site is shown in the illustration. The four overlapping fragments from the full-length GDzj genome produced by PCR amplification are shown in the middle section.

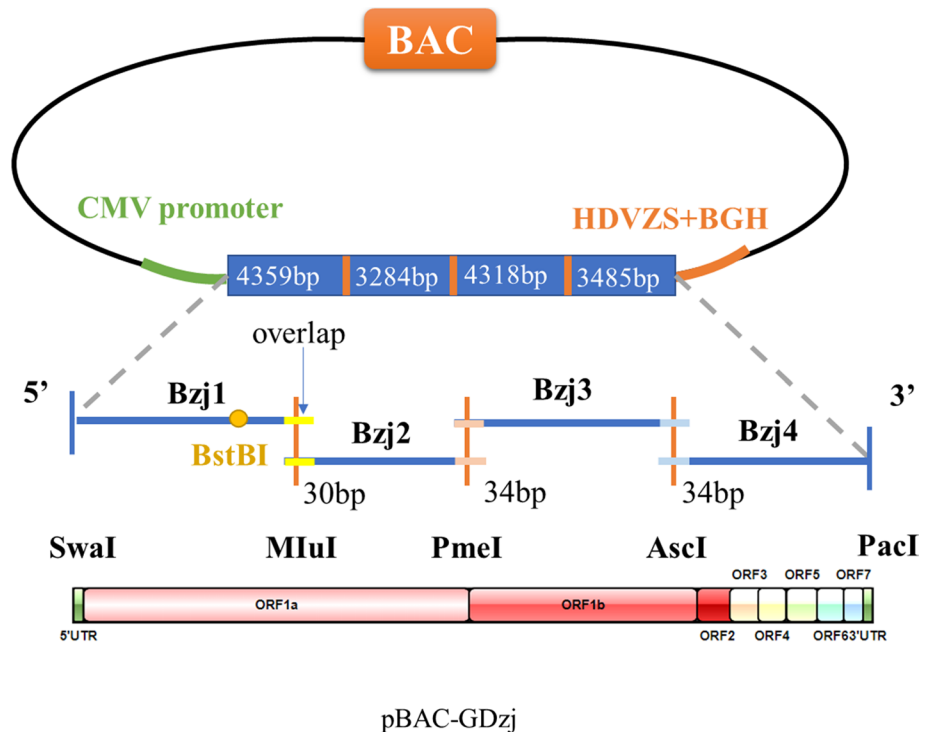


Table 3 Primers used for amplification of the genomic cDNA of PRRSV strain GDzj

Primer	Sequence (5'-3')	Length (nt)
BzjF1	TGGTTTAGTATTTAAATCTCGAGATGACGTATAGGTGTTGGCTCTATGC (Swa I)	4359
BzjR1	CGTGTCCCTTTTACGCGTGCGACCTTAATTAAGGGTCGGCATGGCATCTC (MluI, PacI)	
BzjF2	TTCAACGTGTTCCCTTTTACGCGTGCGACCAGGTCGTCACCTCGTCGACCT (MluI)	3284
BzjR2	AAGGAGCAGTGTTTAAACTGCTAGCCTTAATTAAGGGTCGGCATGGCATCTC (PmeI, PacI)	
BzjF3	GCCTAACTAAGGAGCAGTGTTTAAACTGCTAGCCGCCAGCGGCTTGACCCG (PmeI)	4318
BzjR3	TGCGTTTCGGGCGCGCCAGAAAGGGTTAATTAAGGGTCGGCATGGCATCTC (AscI, PacI)	
BzjF4	TTACAATGATCGTTCGGGCGCGCCAGAAAGGGAAAATTTATAAAGCTAA (AscI)	3485
BzjR4	CCATGCGGCCGTAATTAATAAAT TAATTAAGGGTCGGCATGGCATCTC (PacI)	

F, forward PCR primer; R, reverse PCR primer. The underlining indicates the recognition sites for the restriction enzymes shown in parentheses.

Table 4 Primers for amplification of PRRSV GP5 and Nsp2

Primer	Sequence (5'-3')	Length (nt)
GP5F	GATATCATGTTGGAGAAATGVTTGACCG	603
GP5R	GGATCCCTAAGGACGACCCCATTTGTTCC	
Nsp2F	GGCTCCCTGCGGCCCTTGGACAG	1872
Nsp2R	GGCGGCGGTGTCTCGAGAATCATCT	

F, forward PCR primer; R, reverse PCR primer

the parental GDzj virus and untransfected Marc-145 cells were used as positive and negative controls, respectively.

Virus growth kinetics

Growth curves for the third-generation RvGDzj virus and the third-generation parental GDzj virus were determined using Marc-145 cells. Marc-145 cells were pre-cultured in a 12-well cell culture plate to obtain a monolayer, and the third-generation RvGDzj virus and the third-generation GDzj parental virus were inoculated separately at an MOI of 0.1 onto the cell monolayer and incubated in an incubator at 37°C with 5% CO₂ for 1 h. Three replicates were performed for each virus. After incubation, the supernatant was discarded, the cells were washed three times with PBS buffer, DMEM cell maintenance medium containing 2% FBS was added, and the cells were cultured in an incubator at 37°C with 5% CO₂. The supernatant was collected at 12, 24, 36, 48, 60, and 72 hpi.

Marc-145 cells were seeded onto a 96-well cell culture plate. When the cell confluence exceeded 90%, the rescued virus obtained was diluted in a tenfold series. For each dilution, four wells of a plate seeded with Marc-145 cells were

inoculated separately with either virus (100 µL per well), and the blank control was not inoculated. The cell culture plate was incubated at 37 °C with 5% CO₂. The cells were observed continuously for 2–7 days, the number of cytopathic wells at each dilution was determined, and the 50% tissue culture infectious dose (TCID₅₀) was calculated for the viral supernatant. Finally, the infection time was used as the abscissa, and the TCID₅₀ measured at this time point was used as the ordinate for drawing a growth curve for the virus, which enabled the growth characteristics of the rescued and parental viruses to be compared in Marc-145 cells.

Statistical analysis

Numerical data are expressed as the mean ± SD and were analyzed using GraphPad Prism software (version 8.0.1 for Windows; GraphPad Software Inc.). Differences between groups were assessed using ANOVA. Differences were considered statistically significant when the *P*-value was < 0.05 and extremely significant when the *P*-value was < 0.01 or *P* < 0.001.

Results

Isolation and identification of viruses

Obvious cytopathic changes were observed microscopically after the diseased material was inoculated onto Marc-145 cells for 72 hours, whereas the uninfected Marc-145 control cells showed no such changes (Fig. 3A and B). IFA showed that the virus-inoculated cells were observable by fluorescence microscopy. A lack of green fluorescence in the blank Marc-145 control cells showed that they did not specifically react with the anti-PRRSV N protein antibody, thereby confirming that virus isolation was successful (Fig. 3C and D).

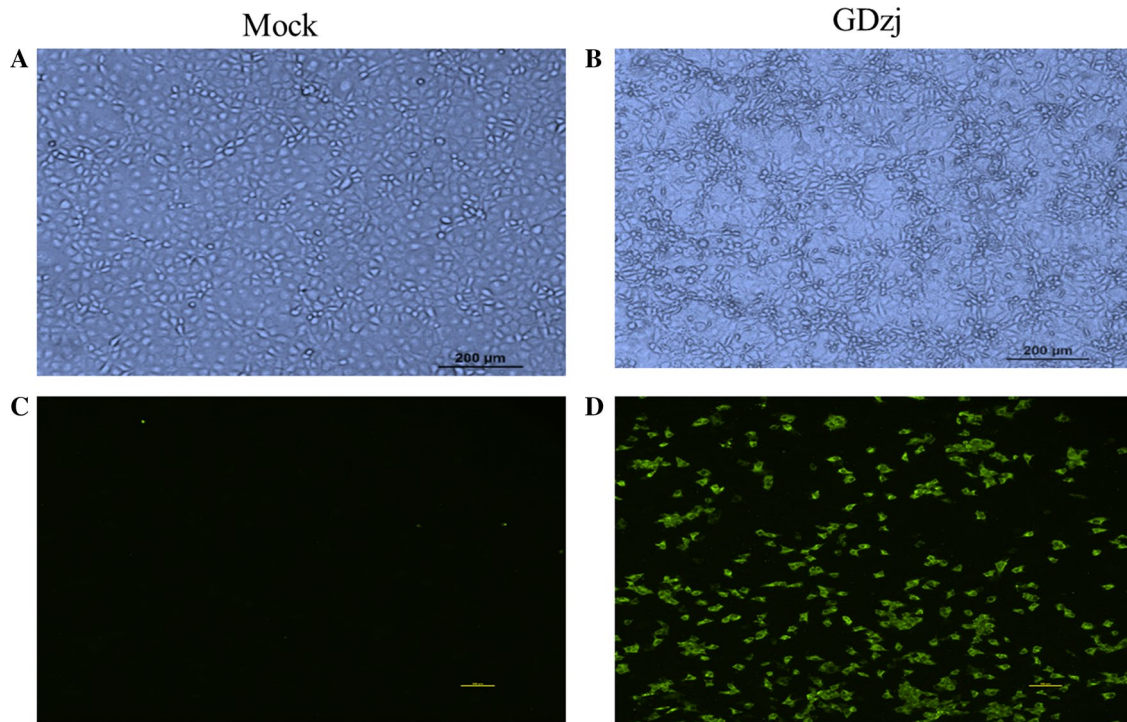


Fig. 3 Virus isolation and identification. (A and B). Cytopathic effect in the Marc-145 cells infected with GDzj at 72 hours post-infection (hpi). (C and D). Indirect immunofluorescence of GDzj-infected and

mock-infected Marc-145 cells at 72 hpi using a monoclonal antibody against the PRRSV N protein.

Sequence analysis

We used DNASTAR 7.1 software to analyze the whole genome of the GDzj strain and representative strains from China and abroad and constructed an evolutionary tree from the data. The results showed that the GDzj strain belongs to lineage 8.7 (represented by the JXA1-like strain) and shares the highest nucleotide sequence similarity with HUN4; therefore, GDzj is an HP-PRRSV strain (Fig. 4A). The sequence of the whole genome of the GDzj strain is 58.5% identical to that of the representative European LV strain, 88.4% identical to that of the representative North American VR-2332 strain, and 98.2% and 97.9% identical to those of the Chinese domestic HUN4 and JXA1 strains, respectively (Fig. 4B). This shows that the GDzj strain belongs to the North American type and is closely related to the HUN4 strain and the JXA1 strain. The levels of the whole gene sequences from the GDzj strain and the reference JXA1 and HUN4 strains (Fig. 4C) indicate that GDzj is an HP strain.

The NSP2 and GP5 sequences from the GDzj strain were further analyzed and compared with those of the reference strain. We identified two non-contiguous aa deletions in the NSP2 gene of GDzj, a finding that is consistent with the known deletion sites in the HP strain (Fig. 4D). The GP5 gene contains an N34G mutation at position 34, an I94A mutation at position 94, and a Q196R mutation at position

196 (Fig. 4E). Based on these genetic differences, our aa-based phylogenetic tree showed that NSP2 and GP5 belong to the branch represented by the HP strains HuN4, JXA1, and JXwn06 (Fig. 4F and G).

Clinical manifestations in piglets and their lung and hilar lymph node characteristics in PRRSV challenge experiments

In our challenge infections, from the second day of the challenge, piglets in the experimental group appeared to have messy fur, a depressed mood, and decreased food intake, accompanied by temperature rise and fever. In the later part of the challenge infections, some piglets died and the body temperatures of some of them dropped slightly.

The mean body temperature of the GDzj challenge group was significantly higher than that of the control group after challenge, with the highest temperature reaching 41.5°C, a fever state (Fig. 5A). The score relating to the clinical signs in the GDzj challenge group was significantly higher than that of the control group (Fig. 5B), the weight gain was significantly lower than that of the control group (Fig. 5C), and the mortality rate reached 40% (Fig. 5D).

After the challenge infections, serum antibodies and antigen levels in the GDzj group and the control group were

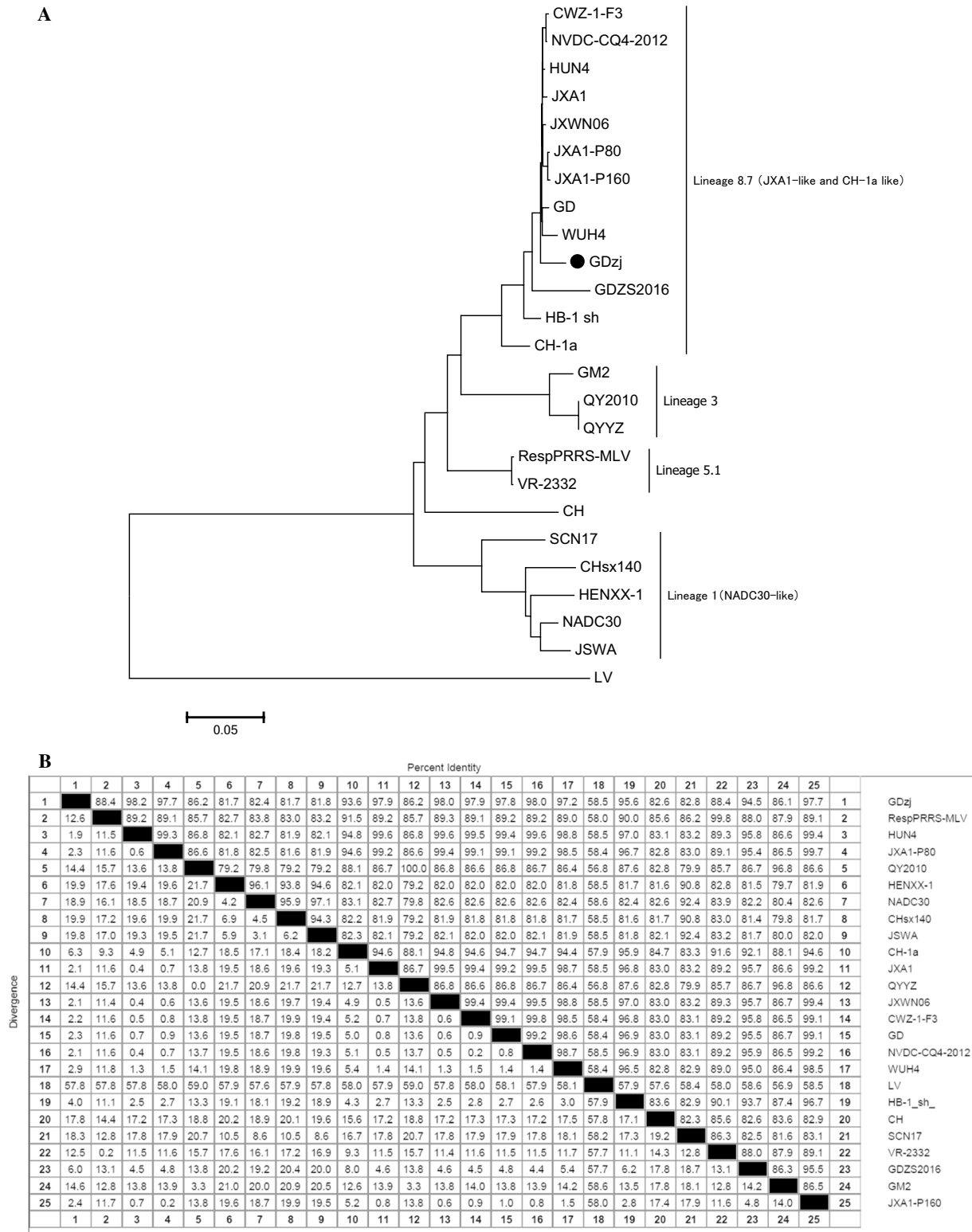
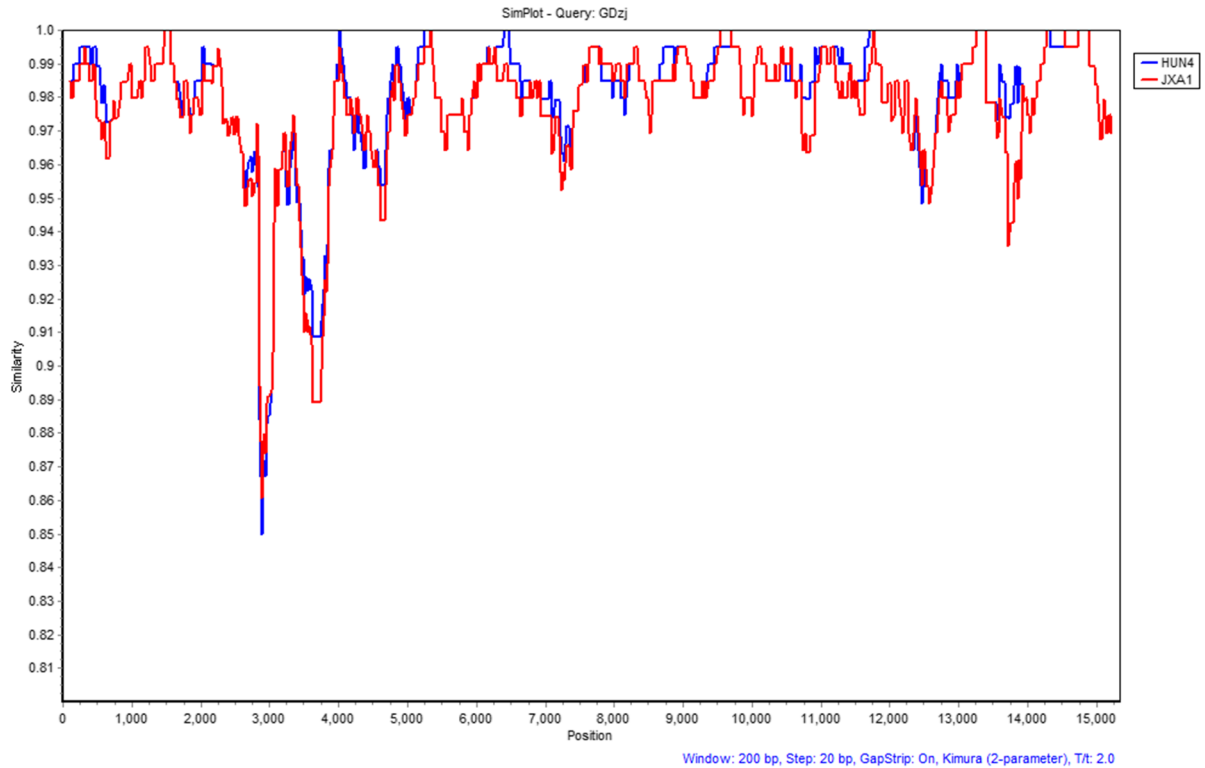


Fig. 4 Phylogenetic analysis (A) nucleotide sequence comparisons (B), and homologous recombination analysis (C) of whole viral genome sequences. The similarity plot was generated using GDzj as the query sequence against those of JXA1 (blue) and HUN4 (red). (C and E) Amino acid sequence alignment and evolutionary tree analysis

of the GP5 (F) and NSP2 (G) genes of strain GDzj. The phylogenetic tree was constructed by the neighbor-joining method using Lasergene 7.1 and MEGA 6.06 software. The genome sequence comparisons were conducted using SimPlot 3.51 software.

C



D

#NADC30	EPVFPVAPRR	TVSRPKPSPI	VSTPVPAPRC	GL-----	-----	---QQVEGMNL	AVGTILACQDE	LLDLSTSSQT	EYEASPLALP	QSEDALAVRR
#HUN4	...LM..S.-	-FK.MTEPLS	G.A.....R	TV-----	-----	-----	-TT..TH...	P...PA...	...F...FS	.NMGI.EAGG
#CH-1a	...LM..SQ-	-IF.FVTEPLS	GPA.....R	TVSRPMTEPLS	EPIFVSAPRH	KF...EA.P	.AT..TY...	P...AF...	...C...FL	.NMGI.EAGG
#CH-1R	...LM..SQ-	-IF.FVTEPLS	GPA.....R	TVSRPMTEPLS	EPIFVSAPRH	KF...EA.P	.AT..TY...	P...AF...	...C...FL	.NMGI.EAGG
#VR-2332	.L.I.SS.Q-	-IF.PATEPLS	EPA.I....G	TVSRPVTEPLS	EPIFVPAPRR	KF...KRLSS	.AAIPPY..N	P...A...	...P..P..A	.GGV.G.EG
#JXA1	...L...S.-	-FK.MTEPLS	G.A.....R	TV-----	-----	-----	-TT..TH...	P...A...	...F...FS	.NMGI.EAGG
#CHex1401	...L...S.-	-FK.MTEPLS	G.A.....R	TV-----	-----	-----	-TT..TH...	P...A...	...F...FS	.NMGI.EAGG
#HENAN-HEB	...L...S.-	-FK.MTEPLS	G.A.....R	TV-----	-----	-----	-TT..TH...	P...A...	...F...FS	.NMGI.EAGG
#JXA1-P80	...L...S.-	-FK.MTEPLS	G.A.....R	TV-----	-----	-----	-TT..TH...	P...A...	...F...FS	.NMGI.EAGG
#RespPRRS-MLV	.L.I.SS.Q-	-IF.PATEPLS	EPA.I....G	TVSRPVTEPLS	EPIFVPAPRR	KF...KRLSS	.AAIPPY..N	P...A...	...P..P..A	.GGV.G.EG
#JXwn06	...L...S.-	-FK.MTEPLS	G.A.....R	TV-----	-----	-----	-TT..TH...	P...A...	...F...FS	.NMGI.EAGG
#GDzj	...LM..S.-	-FK.MTEPLS	G.A.....R	TV-----	-----	-----	-TT..TH...	P...A...	...F...FS	.NMGI.EAGG

E

#NADC30	MLGKCLTAGY	CSQLPFLWCI	VPFCAALVN	ANSNSSSHLQ	LIYNLTICEL	NGTDWLNERF	YWAVETVFI	PVLTHIVSYG	ALTTSHFLDT	VGLITVSTAG
#HuN4	...CC..R.L...	...YL.V...	.SN.N...I.	...L...	...AQK.	D.....A.....
#CH-1a	...T.C..R.R.LS...	...V...	...F...	...L...	...ANK.	D.....V.....
#CH-1R	...Y.T.C..R.R.LS...	...V...	...QF...	...L...	...ANK.	D.....V.....
#VR2332	...E...C..R.R.LS...	...V.A.	.SNS.....	...L...	...AN.	D...S...A.V.....
#JXA1	...CC..R.L...	...YL.V...	.SN.N...I.	...L...	...AQK.	D.....A.....
#CHex1401	...C.....S	...NH...	...K.	D.....	...L.	A.....A.....
#HENAN-HEB	...Y.....S	...S.	...KK.	D.....	...A.A.....
#JXA1-P80	...CC..R.L...	...YL.V...	.SN.N...I.	...L...	...AQK.	D.....A.....
#MLV	...E...C..R.R.LS...	...V.A.	.SND.....	...L...	...ANK.	D...S...A.V.....
#JXwn06	...CC..R.L...	...YL.V...	.SN.N...I.	...L...	...AQK.	D.....A.....
#GDzj	...CC..R.L...	...YL.V...	.SN.N...I.	...L...	...AQK.	D.....A.....

#NADC30	YHRRYVLS	IYAVCALAAL	ICFAIRLAKN	CMSWRYSCTR	YNFLDITKG	KLYRWRSPVI	IEKGGKVDVG	GHLIDLKRVV	LDGSAATFVT	KISAEQWGRP
#HuN4	...G.....	...V.....	...R.....	...V.....	...R.....	...V.....	...E.E	...L.RV.....	...L	...L
#CH-1a	F..G.....	...V.....	...R.....	...V.....	...R.....	...V.....	...E.E	...V..L.RV.....	...L	...L
#CH-1R	F..G.....	...V.....	...Q.....	...R.....	...V.....	...E.E	...V..L.RV.....	...L	...L	...L
#VR2332	FV.G.....	...T.V.F...	...A...	...R.....	...R.....	...R.....	...E.E	...V..I.RV.....	...L	...L
#JXA1	...G.....	...V.....	...R.....	...R.....	...V.....	...E.E	...L.RV.....	...L	...L	...L
#CHex1401	...G.....	...V.....	...R.....	...R.....	...V.....	...E.E	...L.RV.....	...L	...L	...L
#HENAN-HEB	...N.....	...V.T.V...	...K.	...R.....	...R.....	...E.E	...L.RV.....	...L	...L	...L
#JXA1-P80	...G.....	...V.....	...R.....	...R.....	...V.....	...E.E	...L.RV.....	...L	...L	...L
#MLV	FV.G.....	...T.V.F...	...A...	...G.....	...R.....	...R.....	...E.E	...V..I.RV.....	...L	...L
#JXwn06	...G.....	...V.....	...R.....	...R.....	...V.....	...E.E	...L.RV.....	...L	...L	...L
#GDzj	...G.....	...V.....	...R.....	...R.....	...V.....	...E.E	...L.RV.....	...L	...L	...L

Fig. 4 (continued)

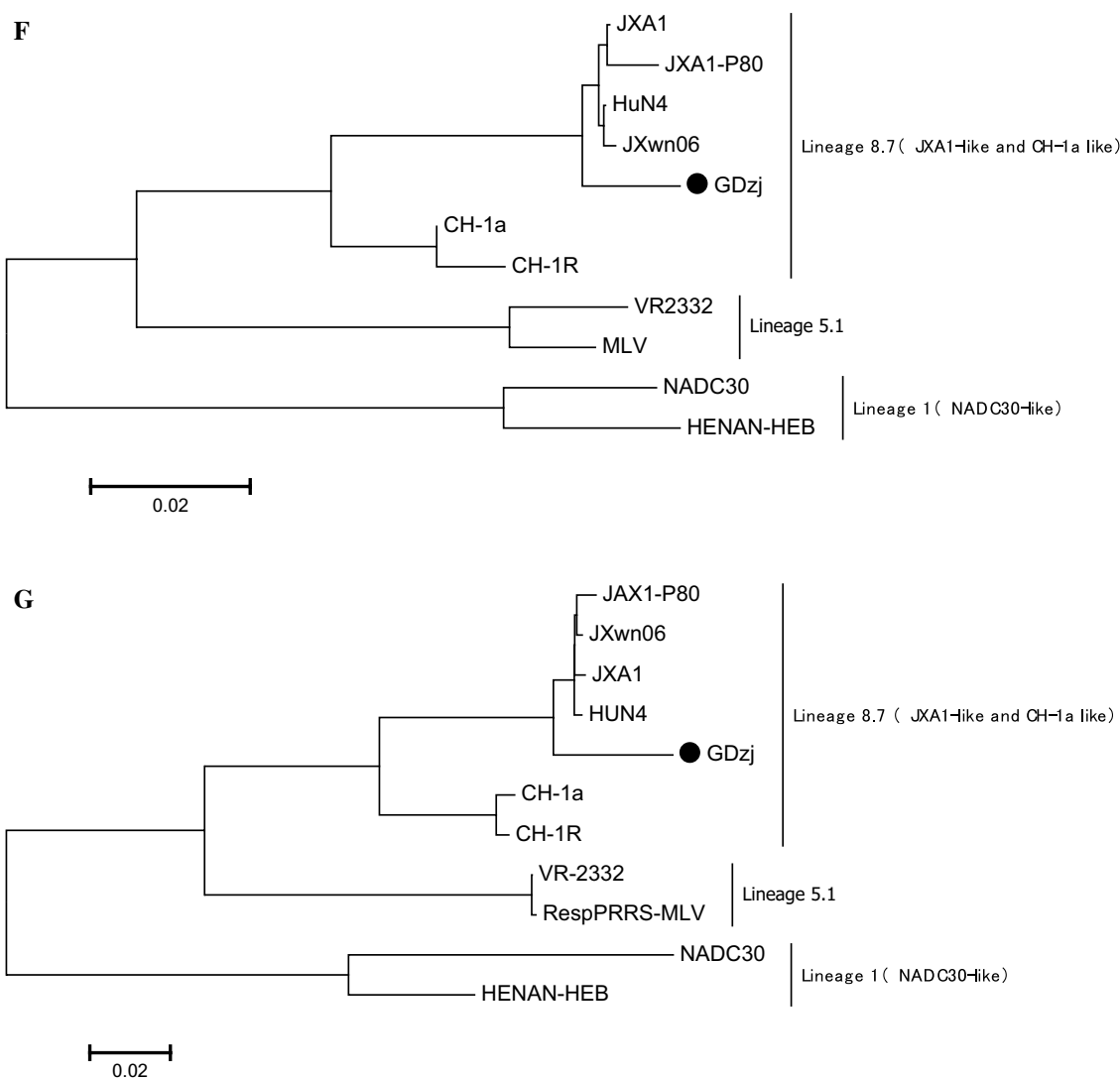


Fig. 4 (continued)

measured. In the GDzj group, antibodies became detectable at 7 days after challenge, and the antibody level gradually increased and antigen became detectable at 1 day after challenge (Fig. 5E). Serum antigen levels in the GDzj group were highest at 7 days post-challenge but then gradually decreased and stabilized. Piglets in the control group were negative for both antigen and antibodies (Fig. 5F).

The lungs from the piglets in the challenge group showed obvious edema, with "meat-like changes" occurring in many places and being almost diffuse throughout the lungs, and the interlobular interval was significantly widened, showing the typical signs of interstitial pneumonia (Fig. 5G). Analysis of the prepared pathological tissue sections from the autopsied material showed that the lung tissue from the piglets in the infection challenge group showed obvious signs of pneumonia, as mainly manifested by thickening of the alveolar septum, a reduction in the alveolar cavity

volume, and proliferation of inflammatory cells. The prepared immunohistochemical sections showed obvious tan-colored positive signals in the lung tissues from the piglets in the challenge group, and tracer virus particles were mainly located in alveolar macrophages and epithelial cells, but these signals were not seen in the control group (Fig. 5H).

Rescue and virus identification

Marc-145 cells were transfected with the full-length cDNA pBAC-GDzj plasmid, and after 96 hours, 90% of the cells displayed cytopathic changes and the virus was harvested by freeze-thawing. This virus suspension was inoculated onto Marc-145 cells, and CPE was observed. The results showed that, microscopically, the CPE was similar to that seen with the parental GDzj virus, and the rescued virus was named RvGDzj. As shown in Fig. 6A, compared with the control

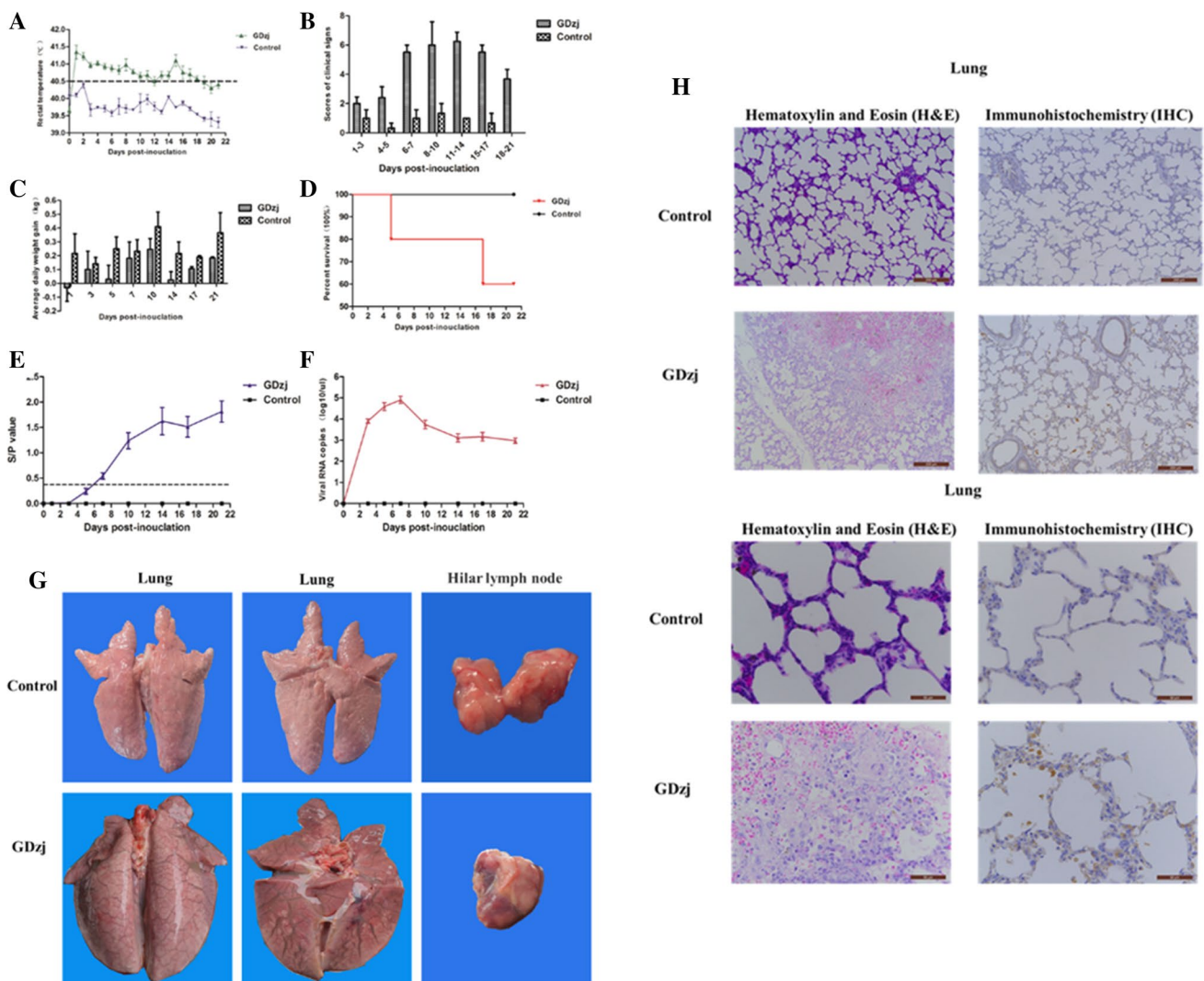


Fig. 5 Clinical scores and post-mortem tissue changes in the experimental animals after virus challenge. (A) Rectal temperatures recorded for 21 days after virus challenge. Data are the mean \pm standard deviation (error bars). (B) Clinical scores after virus challenge. Data were recorded as described previously [15], and the mean values were calculated on days 1–3, 4–5, 6–7, 8–10, 11–14, 15–17, and 18–21 postinfection (p.i.). Asterisks indicate significant differences between the GDzj-inoculated group and the control group (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). (C) Average daily weight gain in the laboratory animals after viral challenge. Data were recorded

and the mean values were calculated on days 1, 3, 5, 7, 10, 14, 17, and 21 p.i. (D) Survival of laboratory animals after virus challenge. (E and F) Serum antigen (E) and antibody (F) status of the experimental animals after infection challenge. (G) Lungs and hilar lymph nodes of infected and uninfected animals. (H) Pathological sections in the lungs from the experimental animals after viral challenge. Lung sections were stained with hematoxylin and eosin (H & E) and also examined by immunohistochemistry (IHC) using monoclonal antibodies specific for the N protein of PRRSV.

cells (i.e., uninfected Marc-145 cells), the cells inoculated with the rescued virus or the parental virus showed evidence of shrinkage and accumulated protrusions, whereas the control cells always appeared normal.

The rescued RvGDzj was serially passaged in Marc-145 cells. We took the culture medium from the third-generation rescued virus (RvGDzj-P3), extracted RNA from it, and used PRRSV GP5 and NSP2 primers for RT-PCR tests. Upon agarose gel electrophoresis, a strong PCR amplicon

was detected. Sequencing of this amplicon showed that the GP5 and NSP2 sequences from the rescued virus were identical to those of the parent strain (Fig. 6B).

To distinguish the parental virus from the rescued virus, genome position 3407 was mutated, a *Bst*BI restriction endonuclease site was added for cloning purposes, and the rescued virus fragment was amplified by RT-PCR. *Bst*BI was used to distinguish the two viruses by restriction digestion, and two fragments were only obtained from the rescued virus (Fig. 5C).

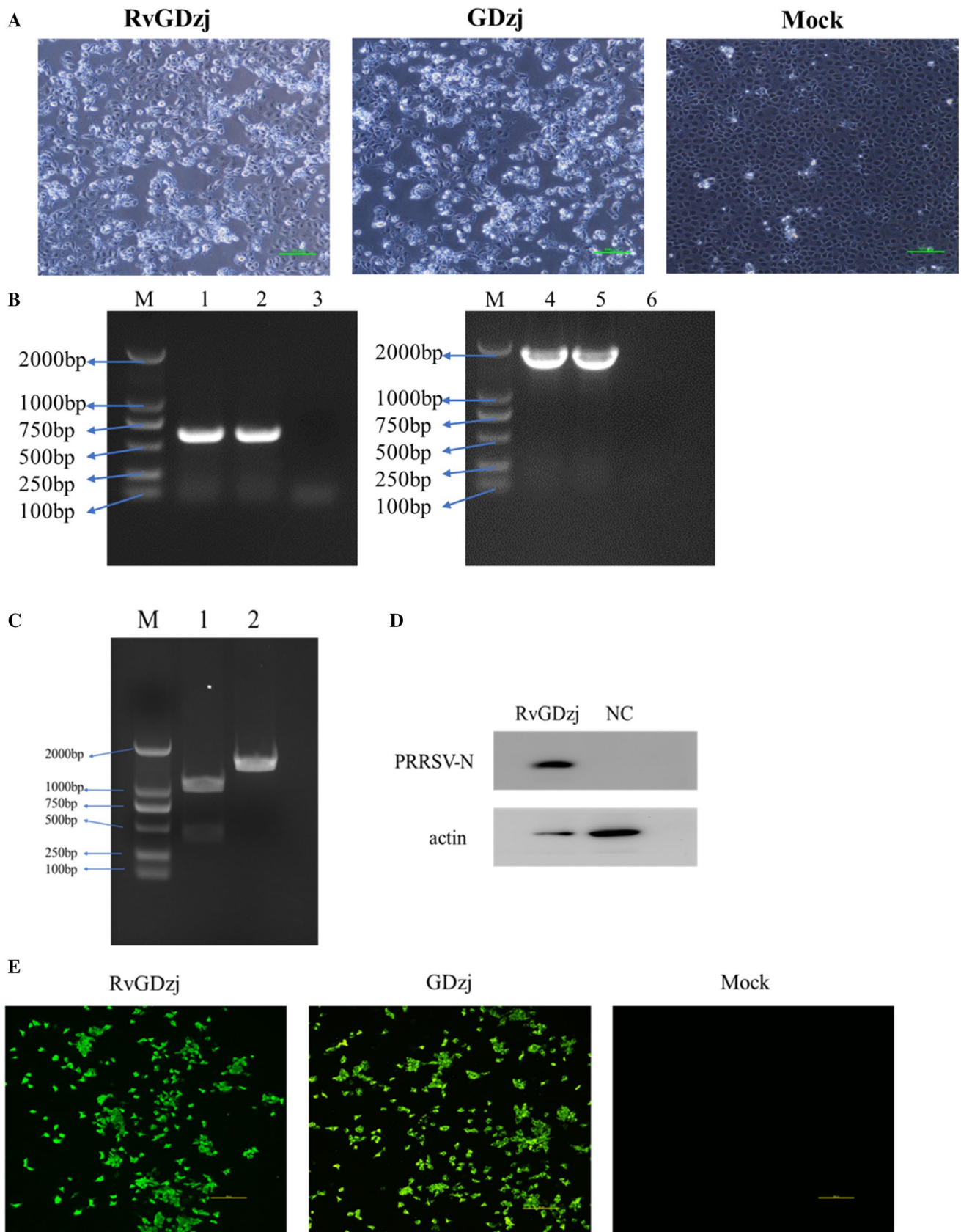


Fig. 6 Rescue and identification of viruses. (A) Cytopathic effects in Marc-145 cells infected with the rescued viruses at 36 hpi. (B) PCR identification of the rescued virus. M, Marker DS2000 bp DNA; lane 1, GP5 fragment from the rescued virus (RvGDzj); lane 2, GP5 fragment from the parent virus (GDzj); lane 3, negative control; lane 4, NSP2 fragment from the rescued virus (RvGDzj); lane 5, NSP2 fragment from the parent virus (GDzj); lane 6, negative control. Restriction enzyme digestion, IFA, and western blotting were used to identify the rescued virus (RvGDzj). (C) Restriction enzyme digestion and identification of PCR products from the rescued viruses. M, Marker DS2000bp DNA, lane 1, restriction fragment from the rescued virus (RvGDzj); lane 2, restriction fragment from the parent virus (GDzj). (D) Western blot identification of the rescued virus (RvGDzj). (E) IFA identification of the rescued virus

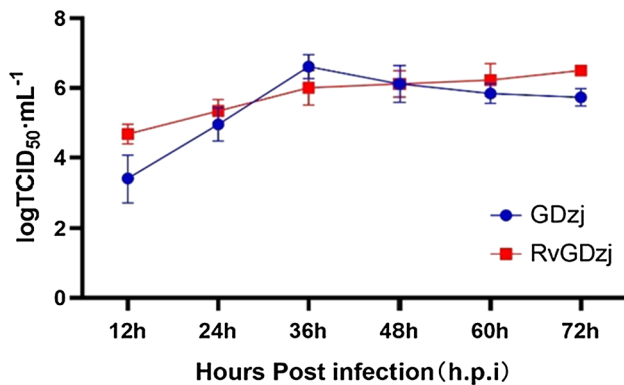


Fig. 7 Growth curves for the rescued and parental viruses. Marc-145 cells were infected with GDzj or RvGDzj at a MOI of 0.1. Supernatants from the infected cells were collected at the time points indicated (12, 24, 36, 48, 60, and 72 hpi). Viral titers were determined by a TCID₅₀ assay in Marc-145 cells and used to produce growth curves.

A monoclonal antibody targeting the PRRSV N protein was used in IFA to distinguish cells infected with the third generation of the rescued virus (RvGDzj-P3) from mock-infected cells (Fig. 6D). Marc-145 cells inoculated with the parental virus or with the rescued virus displayed the specific green fluorescence characteristic of anti-PRRSV N-protein positivity under a fluorescence microscope, whereas the blank control cells did not, which shows that the virus rescue was successful and that the rescued virus was recognized by this antibody (Fig. 6E).

Comparison of the *in vitro* growth kinetics of the parental strain and the rescued virus

The growth curves of the third-generation rescued virus (RvGDzj-P3) and the third-generation parental virus (GDzj-P3) were compared and found to be similar. However, the viral titer of the parental virus reached a peak at 36 hpi and decreased thereafter, while that of the rescued virus peaked

at 72 hpi with a titer higher than at 12, 24, 60, and 72 hpi, but the difference was not significant (Fig. 7).

Discussion

PRRSV is a major disease afflicting the global swine industry. Despite PRRSV becoming more prevalent over the last 35 years, it has not yet been substantively controlled globally, so its economic impact and harm to the swine industry remain unchanged. PRRSV is an RNA virus with an extremely high mutation rate and propensity for fast evolution. The increasing diversity of strains and the increasing frequency of the appearance of new strains and recombinant strains have made its prevention and control difficult [5, 9, 25]. The in-depth study of this virus largely depends on the ability to construct infectious virus clones. In 2006, an outbreak of PRRSV caused by HP-PRRSV occurred in China. Some laboratories have isolated several HP-PRRSV strains and established reverse genetic systems [7, 8, 17, 24].

Reverse genetics is a virus rescue technology that copies the virus from a cDNA clone of the viral genome. The strategy to rescue infectious viruses from plasmids encoding a cloned cDNA corresponding to the viral genome is one of the most important technological advances in experimental virology systems. As a technology platform, it can be used to study the genome structure, gene functions, and pathogenic mechanisms of RNA viruses [3]. As one of the basic technology platforms employed by many molecular biology laboratories, reverse genetics technology has been widely used in various fields of life science research. Its application scope includes not only the study of gene replication and regulation of gene expression in animal viruses and the interactions occurring between viruses and their hosts but also research on antiviral strategies and gene therapy, the development of new genetically engineered vaccines, and the construction of new target viral vectors [34]. This technology can also provide a basic technical resource for the prevention and control of RNA viruses causing major diseases in various animals, such as African swine fever, peste des petits ruminants, and Schmallenberg disease [11, 12, 23].

In the process of constructing infectious clones of PRRSV, early research mainly adopted the establishment of a virus rescue system by placing clones containing the full-length cDNA of the PRRSV genome under a prokaryotic transcription promoter such as a phage T7 or SP6 promoter. The necessity to generate full-length RNA PRRSV transcripts by *in vitro* transcription, however, means that this process has many operational limitations. To circumvent this, we combined a BAC-based system with homologous recombination technology to construct a full-length

cDNA infectious clone of PRRSV. This system offers several advantages over similar systems. First, because the BAC plasmid can accommodate up to 350 kb of foreign DNA, it can be used to clone large foreign genes. It is also suitable for the construction of infectious clones of viruses with large genomes. Second, CMV is widely recognized as the most powerful promoter for initiating eukaryotic gene expression. A BAC construct made by placing the clone of the full-length viral genome under the control of the CMV promoter can be used directly to transfect eukaryotic cells without *in vitro* transcription, thereby saving time. Third, the use of homologous recombination technology eliminates the need for restriction enzyme sites, greatly improving the efficiency of construction of full-length cDNA clones and reducing costs in the process.

The ability to construct full-length infectious cDNA clones mainly hinges on whether a faithful whole-genome fragment can be obtained for the virus of interest. In the case of PRRSV, because its genome is relatively large, obtaining a full-length cDNA sequence is difficult. Therefore, we avoided introducing gene mutations and deletions during the construction of the full-length infectious cDNA clones by dividing the whole genome from the HP-PRRSV GDzj strain into four fragments during the construction process. High-fidelity enzymes were used for amplification to reduce the number of mutations during the RT-PCR process. To ensure the fidelity of the constructed infectious molecular clone, we also sequenced the full length of the plasmid. Thus, we successfully constructed a full-length infectious clone from the GDzj strain, and a virus (RvGDzj) was rescued. It is worth noting also that in the present study the rescued recombinant PRRSV and the parental virus displayed similar proliferation ability *in vitro*. This infectious clone thus provides a powerful tool for further investigating the pathogenesis of PRRSV.

In summary, we isolated a PRRSV strain GDzj from lung tissue from a pig with suspected PRRS infection. In experimental challenge infections of weaned piglets, this virus caused severe fever, difficulty in breathing, loss of appetite, and other clinical manifestations in the piglets and some infections were fatal. Necropsy revealed the presence of lesions in the lung tissue and hilar lymph nodes. Our next experiments, which involved constructing a full-length cDNA infectious clone of GDzj using the BAC system, showed that the rescued virus displayed *in vitro* proliferation characteristics similar to those of the parental PRRSV. The outcome of our research not only provides a useful reverse genetics platform for exploring the pathogenesis of HP-PRRSV but also lays a foundation for exploring and studying other RNA viruses with even larger genomes.

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Author contributions SW, JD, and LY contributed equally to the work; SW, LY, and PZ performed the assays and performed the data analyses; TL, LZ, PL, and LW performed the sequence alignment; and JD, ZX and CS wrote the manuscript. All authors reviewed and approved the final form of the manuscript.

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Availability of data and materials Genetic data presented in this paper are publicly available via GenBank.

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical approval and consent to participate All of the samples were collected according to the animal ethics regulations of the National Engineering Center for Swine Breeding Industry (NECSBI 2015-16).

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