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Emergence of two novel recombinant porcine reproductive and respiratory syndrome viruses 2 (lineage 3) in Southwestern China

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ABSTRACT

The lineage 3 of porcine reproductive and respiratory syndrome virus 2 (PRRSV-2) was first reported in mainland China in 2010 and it has spread rapidly in recent years. Here, two novel lineage 3 strains of PRRSV-2 were isolated from diseased pigs in Southwestern China during 2017–2018, and were designated as GZgy17 and SCya18. The complete genomes of the two isolates were then determined, and sequence alignment revealed that GZgy17 had the same discontinuous 30-amino acid (aa) deletion in NSP2 as JXA1, while SCya18 contained the discontinuous 131-aa deletion in NSP2 identical to that of NADC30, when compared to the strain VR-2332. Notably, GZgy17 contained an additional 19-aa deletion in NSP2, and SCya18 had a unique 3-nt deletion in its 3'UTR. Homology and phylogenetic analysis showed that GZgy17 and SCya18 shared low nucleotide homology (91.2–92.0%) with QYYZ and were classified into a new cluster of lineage 3 strains based on ORF5 genotyping. Recombination analyses revealed that GZgy17 and SCya18 both originated from a SH/CH/2016-like (lineage 3) strain and had recombined with a JXA1-like (lineage 8) and a NADC30-like (lineage 1) strain, respectively. Furthermore, we compared the virulence of the two strains in 4-week-old piglets. The results showed that GZgy17 caused mortality rates of 20% and exhibited higher pathogenicity in piglets compared to SCya18. Our findings suggest that recombination might be responsible for the variations in pathogenicity of lineage 3 strains of PRRSV-2 and highlight the importance of surveillance of this lineage in China.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV), a member of the genus *Porartevirus*, family *Arteriviridae* and the order *Nidovirales* (Adams et al., 2017), causes clinical disease characterized by reproductive failure in pregnant sows including late-term abortions, stillborns, mummified and weak-born pigs, and respiratory disease, such as interstitial pneumonia, in growing pigs (Benfield et al., 1992; Wensvoort et al., 1991). PRRSV has been recognized as a major swine pathogen that is endemic worldwide and leads to serious economic losses since its first recognition in the United States in the late 1980s (Keffaber, 1989). PRRSV is an enveloped, single-strand, non-segmented, and positive-sense RNA virus, which is divided into two major genotypes, namely the PRRSV-1 (European type) and PRRSV-2 (North American type) genotypes (Murtaugh et al., 1995; Nelson et al., 1993). Furthermore, each genotype can be divided into several clades. The Lelystad virus (LV) and VR-2332 serve as the representative strains of PRRSV-1 and PRRSV-2, respectively (Nelsen et al., 1999; Nelson et al., 1993).

The genome of PRRSV is approximately 15 kilobases (kb) in length, is capped at the 5'-end and polyadenylated at the 3'-terminus. It contains at least ten overlapping open reading frames (ORFs), designated as ORF1a, ORF1b, ORF2a, ORF2b, and ORF3–7, including ORF5a (Fang et al., 2012; Johnson et al., 2011). ORF1a and ORF1b are located downstream of the 5'-untranslated region (UTR) and encode replication-related polymerase proteins that can be autoproteolytically cleaved into at least 16 nonstructural proteins (NSPs): NSP1α, NSP1β, and NSP2–12, including NSP2TF, NSP2N, NSP7α, and NSP7β. ORF2–7

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are located at the 3'-end of the genome and encode six membrane-associated structural proteins (GP2, E, GP3, GP4, GP5, and M), and the nucleocapsid (N) protein (Dokland, 2010; Kappes and Faaberg, 2015; Lunney et al., 2016). Among these ORFs, ORF5 encodes the major virion surface glycoprotein GP5, which plays an important role in viral assembly, infectivity, and the induction of neutralizing antibodies (Ansari et al., 2006; Ostrowski et al., 2002). Owing to its marked genetic variation, the ORF5 gene has been extensively used in molecular epidemiologic research, as well as for the classification of PRRSV field strains (Gao et al., 2017; Guo et al., 2018; Shi et al., 2010).

PRRSV has been documented in China since late 1995 (the representative strain CH-1a) (Guo et al., 1996), and it has been a major disease problem for the Chinese swine industry over the past two decades. To date, the majority of PRRSV-2 strains in China can be classified into four lineages (sublineages) based on the global PRRSV classification system: lineage 1 (1.9), lineage 3, lineage 5 (5.1), and lineage 8 (8.7) (Guo et al., 2018; Shi et al., 2010). Among these four lineages, lineage 8 strains were the most significant variant due to the clinical severity of the diseases they caused in their hosts. A striking example was the highly pathogenic PRRSV (HP-PRRSV, JXA1-like) that caused a large-scale outbreak in China in 2006 affecting more than 20 million pigs (Tian et al., 2007). Although the lineage 5 strains (VR-2332-like) emerged as early as 1996, they have not been pandemic in China (Guo et al., 2018). Lineage 1, also known in China as NADC30-like strains, has been prevalent since 2013 and led to pandemics in large areas of China between 2014 and 2016 (Li et al., 2016a; Zhao et al., 2015; Zhou et al., 2015). Lineage 3 (QYYZ-like) was first reported in Guangdong province of mainland China in 2010 (Lu et al., 2015) and was originally considered to be a novel lineage due to its low sequence identity with other PRRSV lineages from China. Currently, lineage 3 PRRSV mainly occurs sporadically in southeastern areas of China (Gao et al., 2017). However, this lineage has spread rapidly in recent years and merits special attention in terms of control and vaccine strategies.

In the current study, porcine alveolar macrophage cells (PAMs) were used to isolate two novel recombinant PRRSV strains, GZgy17 and SCya18, from diseased piglets in Southwestern China, during 2017–2018. Although the two newly-emerged PRRSV isolates were both classified as lineage 3 of PRRSV-2 based on the global PRRSV classification system, they exhibited different recombination patterns. Meanwhile, unique point mutations and deletions had occurred in their genomes. Furthermore, we assessed the virulence properties of the two new isolates in 4-week-old piglets. Our study indicates that both recombination and mutations are involved in the evolution of PRRSV and highlights the importance of the surveillance of lineage 3 of PRRSV-2 in China.

2. Materials and methods

2.1. Sample collection and virus isolation

Clinical samples (lung tissues) were collected from suspected cases of PRRSV in non-vaccinated piglets from two independent pig farms in Sichuan province and Guizhou province of Southwestern China from 2017 to 2018. Clinical signs were characterized by high fever (40.3–41.5 °C), blue ears, respiratory distress, and eventual death. Mortality rates on the two pig farms were 12.9% (45/350) (a pig farm in Guizhou province) and 2.7% (30/1100) (a pig farm in Sichuan province). The clinical samples were collected from dead pigs from the two farms and then tissue samples were homogenized in RPMI-1640 medium (Transgene, Beijing, China) using TissueLyser (Beijing, China) for RNA extraction and virus isolation. The tissue homogenates were centrifuged at 5000 × *g* for 10 min. The suspensions were passed through 0.22-μm filters and transferred to PAMs. After incubation for 1 h at 37 °C, the suspensions were replenished with new RPMI-1640 medium containing 10% FBS (HyClone, South Logan, USA) at 37 °C in a humidified 5% CO_2 atmosphere for 3–5 days and monitored daily for cytopathic effects (CPE). Indirect immunofluorescence assay (IFA) was conducted as previously described (Zhou et al., 2018b). A specific PRRSV monoclonal antibody against the PRRSV N protein (GeneTex, Irvine, USA) labeled with fluorescein isothiocyanate (FITC) (Proteintech, Rosemont, USA) was used to detect PRRSVs. Viruses from both strains were purified by plaque assay as previously described (Zhou et al., 2018a), and the virus titers were performed using PAM cells and calculated according to the Reed–Muench method as previously described (Reed and Muench, 1938). Then the purified viral isolates were used to sequence their whole genomes.

2.2. Viral genome extraction, RACE and RT-PCR

Total RNA was extracted from the suspensions of PRRSV infected cells using the Trizol Reagent (Invitrogen, Carlsbad, USA) following the manufacturer's instructions. Reverse transcription reactions were performed at 37 °C for 15 min and at 85 °C for 5 s using the PrimeScript™ RT Reagent Kit (TaKaRa, Dalian, China). Twelve pairs of primers spanning the entire viral genome were used for amplifying the complete genome, as previously described (Zhou et al., 2018a). The amplified PCR products were purified using a Gel Extraction Kit (OMEGA, USA). The purified PCR products were ligated to a pMD19T-simple vector (TaKaRa, Dalian, China). For each fragment, at least 3 positive recombinant clones were selected for plasmid extraction and sent to the TsingKe Biological Technology (Beijing, China) for sequencing to determine a consensus sequence. The extreme 5′ and 3′ termini of the viral genome were determined by using a 5′/3′ RACE kit (TaKaRa, Dalian, China) following the manufacturer's instructions. The PRRSV strain isolated from Guizhou province in 2017 was designated as GZgy17 and the other virus isolated from Sichuan province in 2018 was designated as SCya18.

2.3. Genome alignment and phylogenetic analysis

The full-length genomic sequences of GZgy17 and SCya18 that were obtained were assembled using the SeqMan program of DNAstar software, version 7.0 (DNASTAR Inc., Madison, USA). The sequences of PRRSV genomes, ORFs, and deduced proteins were analyzed by the EditSeq and MegAlign programs of DNAstar. To analyze the evolutionary relationship of the two novel isolates with other PRRSV isolates, a total of 54 full-length representative PRRSV sequences were available from the GenBank database, including PRRSV-2 isolates obtained in China from 1996 to 2016 (n = 46), North America (n = 7), and PRRSV-1 from Europe (n = 1) (Supplementary Table S1). Multiple sequence alignments were conducted using ClustalW in the MEGA software (version 6, Tempe, AZ, USA), and unrooted phylogenetic trees were constructed based on the nucleotide sequences using the neighbor-joining method. The bootstrap values of the phylogenetic tree were evaluated with 1000 replicates. The classification of lineages was established according to the description of Shi et al. (2010) (Shi et al., 2010).

2.4. Recombinant analysis

To detect potential within-gene recombination events of the two PRRSV field strains, GZgy17 and SCya18, their complete genome sequences as well as those of 54 other reference strains were subjected to recombination screening using the Recombination Detection Program 4 (RDP4, v4.24) software. Seven detection methods, including RDP, Bootscan, GENECONV, MaxChi, Chimaera, SiScan, and 3Seq, were used to confirm the recombination events. The Default settings were used for all methods and the recombination events were considered significant (p-value $< 1 \times 10^{-5}$) when confirmed by at least five of the aforementioned methods. To visualize the recombination events and breakpoints, a similarity analysis was implemented in the SimPlot software (v3.5.1, Baltimore, MD, USA) with a 200-bp window width

and a 20-bp step size. Nucleotide identity was analyzed by the Kimura (2-parameter) method with a transition–transversion (T/t) ratio of 2. Furthermore, a series of phylogenetic trees based on each of the recombinant fragments was constructed to confirm these putative recombination events.

2.5. Animal challenge experiment

In total, 14 4-week-old healthy piglets were obtained from the Sichuan Animal Science Academy (Chengdu, China). The piglets were confirmed not to be infected with PRRSV, porcine circovirus type 2 (PCV2), classical swine fever virus (CSFV), pseudorabies virus (PRV), or swine influenza virus (SIV) by PCR or RT-PCR. All animals were randomly divided into three groups that were kept separate in different rooms. The piglets in the first test group ($n = 5$) were infected with GZgy17, those in the second test group ($n = 5$) were infected with SCya18, and the piglets in the control group ($n = 4$) were sham infected with uninfected RPMI-1640 medium. Two mL (2×10^5 $TCID_{50}$/mL) of PRRSV isolates was intranasally administered to each piglet in each of the two test groups. After inoculation, the clinical signs and rectal temperatures of the inoculated piglets were recorded daily throughout the experiment. A scoring system developed by Li et al. (2014) (Li et al., 2014) was applied to score the clinical symptoms ranging from 0 to 20. The clinical scoring included the gross clinical score (GCS), respiratory clinical score (RCS) and nervous signs score (NSS). Total scores for each piglet represented the sum of the GCS, RCS and NSS. A humane end point was determined when pigs showed neurological signs like high fever hyperspasmia and ataxia, or noticeable slowing of breathing. All the surviving pigs were humanely euthanized at 14 days post-inoculation (dpi). The animal experiment in this study was approved by the Animal Ethics Committee (AEC) of the College of Life Sciences, Sichuan University (license: SYXK-Chuan-2013 − 185). All experimental procedures and animal welfare standards strictly followed the guidelines of Animal Management at Sichuan University.

2.6. Serological and viremia test

For the detection of PRRSV-specific antibodies and the viremia test, serum samples were collected on 0, 3, 5, 7, 10, and 14 dpi. PRRSV-specific ELISA antibody titers were measured using the commercial enzyme-linked immunosorbent assay (ELISA) kit, IDEXX HerdChek ELISA (Westbrook, ME, USA). The threshold for seroconversion was set at a sample-to-positive (s/p) ratio of 0.4 according to the manufacturer's instructions.

Total RNA was extracted from serum samples using Trizol Reagent (Invitrogen, Carlsbad, USA), and then total RNA was retro-transcribed into cDNA using the PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). The real-time quantitative RT-PCR (qRT-PCR) was performed to quantify the copy numbers of PRRSV genomic cDNA. A standard curve was constructed with 10-fold serially diluted ORF7-cloned plasmid standards of 10^1–10^8 copies/μL. The conditions for qRT-PCR were 95 °C for 4 min., followed by 40 cycles of 95 °C for 8 s, 55 °C for 10 s, and 72 °C for 30 s. The viral loads in the sera of the inoculated pigs were calculated by the $2^{-\Delta\Delta Ct}$ method.

2.7. Lung gross pathological examination

Lung gross pathological examination was performed immediately once the piglets died during the experiment, or the surviving pigs were autopsied at 14 DPI. The details of scoring system we applied were as previously described (Halbur et al., 1996). Each piglet was scored from 0 to 100.

2.8. Lung histopathology and immunohistochemistry (IHC) staining

A histopathological assay and hematoxylin and eosin (H & E) staining of the lung tissues were performed for histopathological examinations. Briefly, lung samples were collected at necropsy and immediately fixed in 10% neutral buffered formalin, dehydrated in alcohol, and embedded in paraffin. The H & E-stained lung sections were blindly evaluated and the distribution and severity of interstitial pneumonia were scored as 0 (no microscopic lesions), 1 (mild, focal to multifocal interstitial pneumonia), 2 (moderate, multifocal to coalescing interstitial pneumonia), 3 (severe, patchy to coalescing and extensive interstitial pneumonia), or 4 (severe and diffuse interstitial pneumonia) (Halbur et al., 1996). The IHC was performed to detect the distribution of PRRSV antigen in the lung samples. The specific PRRSV monoclonal antibody against the PRRSV N protein was used for IHC. Each slide was assigned an estimated score based on the number of positive cells as follows: 0 (no positive cells), 1 (1–10 positive cells), 2 (11–30 positive cells), 3 (31–100 positive cells), or 4 (> 100 positive cells) (Zhou et al., 2017). The slides were visualized by 200× and 400× microscope photographs.

2.9. Statistical analysis

Statistical analysis in this study was performed using one-way ANOVA with a Tukey's *t*-test in GraphPad Prism software 5.0 (San Diego, CA, USA) and were considered statistically significant at a *p*-value of < 0.05. The measured values were expressed as the mean with standard deviations (SD).

3. Results

3.1. The lineage 3 strains of PRRSV-2 were distributed among at least nine provinces of mainland China since 2010

Lineage 3 strains of PRRSV-2 were initially recognized in Hong Kong and Taiwan, China (Brar et al., 2014); however, no isolation has been documented for this lineage after 2005. Until 2010, lineage 3 strains were first reported in Guangdong province, mainland China (Lu et al., 2015). According to the recent epidemiological investigation of PRRSVs during 1996–2016 by Gao et al. (2017) (Gao et al., 2017), lineage 3 strains of PRRSV-2 were detected in seven provinces (autonomous cities or regions), including Guangdong, Fujian, Guangxi, Jiangxi, Shanghai, Xinjiang, and Henan. Among these provinces, 73.8% of lineage 3 strains of PRRSV-2 were isolated in Guangdong province. In this study, lineage 3 strains of PRRSV-2 were detected in another two provinces (Sichuan and Guizhou) from 2017 to 2018 (Fig. 1). Therefore, the lineage 3 strains of PRRSV-2 spread gradually in mainland China and have been detected in at least nine provinces since 2010.

3.2. Both GZgy17 and SCya18 strains belong to PRRSV-2, but exhibit genetic variation within their genomes

The complete genomic sequences of GZgy17 and SCya18 were determined and deposited in the GenBank database under the accession numbers of MK144542 and MK144543, respectively. The genomes of GZgy17 and SCya18 were 15,263 and 15,014 nucleotides (nt) in length, respectively, excluding the poly(A) tail at the 3' end. GZgy17/SCya18 shared 91.0%/82.9%, 86.0%/83.0%, 84.5%/81.0%, 81.7%/91.0%, and 88.9%/82.4% sequence identity with JXA1 (lineage 8), VR-2332 (lineage 5), QYYZ (lineage 3), NADC30 (lineage 1), and SH/CH/2016 (lineage 3), respectively (Table 1). Their sequence identity with LV (PRRSV-1) was only 44.3%/44.4% (Table 1), indicating that both GZgy17 and SCya18 belong to the PRRSV-2 strains. Additionally, GZgy17 had slightly higher homology to the MLV vaccine strain, JXA1-80 (91.1%), than to its parental strain, JXA1 (91.0%) (Table 1).

To further characterize the genomic variation in the two newly-emerged PRRSV-2 isolates, the nucleotide homology of the 5'UTR, Nsps, ORFs, and 3'-UTR genes in the two isolates and in seven PRRSV representative strains (JXA1, VR-2332, QYYZ, NADC30, SH/CH/2016,

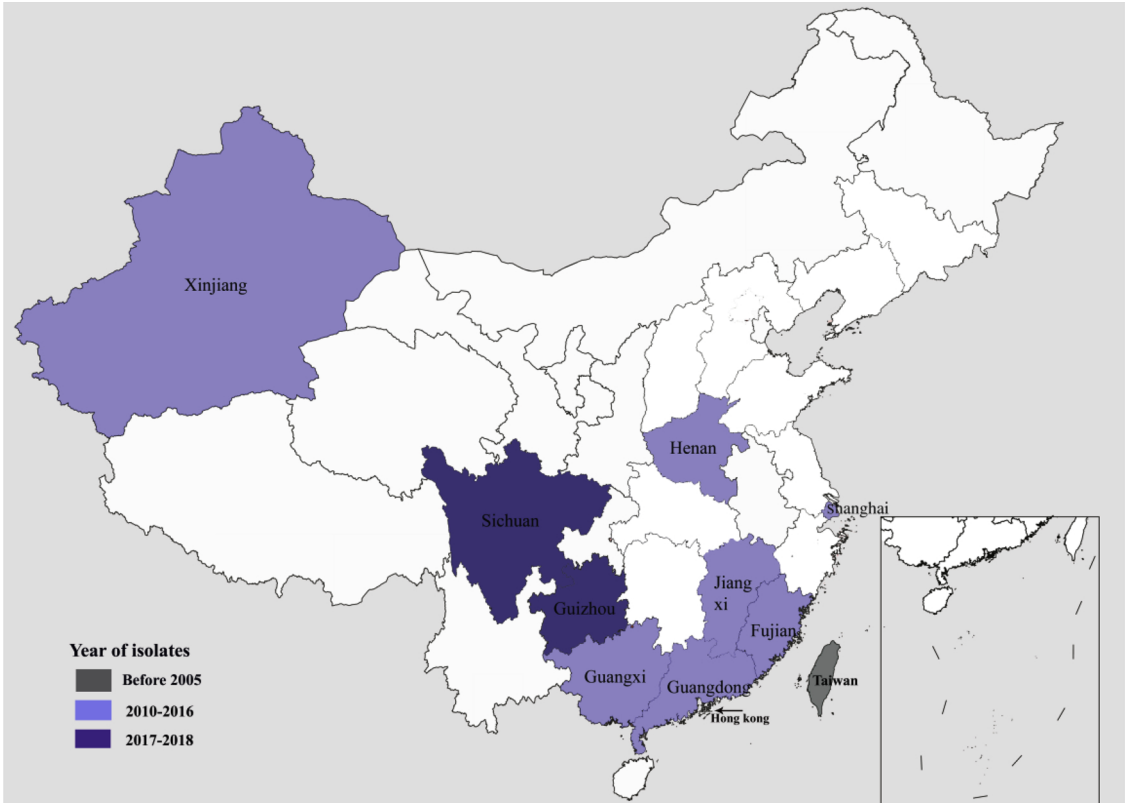

Fig. 1. Geographic distribution of lineage 3 of PRRSV-2 in China. Lineage 3 of PRRSV-2 was reported in Hong Kong and Taiwan before 2005 (gray). In mainland China, this lineage was first recognized in Guangdong province in 2010. Previously reported seven provinces were positive for lineage 3 PRRSV during 2010–2016, including Guangdong, Fujian, Guangxi, Jiangxi, Shanghai, Xinjiang, and Henan (light blue). The two lineage 3 PRRSV-positive provinces (Sichuan and Guizhou) in this study during 2017–2018 were indicated by dark blue (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 1
Nucleotide identity of GZ17 and SCya18 compared with six PRRSV reference strains.

Region	Nucleotide Identity % (GZ17/SCya18)						
	JXA1 (Lineage 8)	VR-2332 (Lineage 5)	QYYZ (Lineage 3)	NADC30 (Lineage 1)	SH/CH/2016 (Lineage 3)	LV (PRRSV-1)	JXA1-P80 (Vaccine)
Complete genome	91.0/82.9	86.0/83.0	84.5/81.0	81.7/**91.0**	88.9/82.4	44.3/44.4	**91.1**/82.7
5'UTR	**99.5**/93.7	92.1/90.1	94.8/92.1	91.6/89.0	95.3/92.1	46.6/47.7	**99.5**/**94.2**
ORF1a	**92.1**/78.4	83.0/77.9	79.7/73.6	77.0/**90.9**	88.8/78.4	34.9/33.3	**92.1**/77.9
Nsp1α	94.3/**93.1**	89.3/88.7	90.4/90.0	87.8/86.9	93.3/92.2	43.3/47.6	**94.4**/93.0
Nsp1β	90.8/**90.8**	82.6/82.1	81.0/82.4	77.3/77.8	87.0/87.4	24.9/20.2	**91.1**/91.1
Nsp2	**90.9**/70.3	78.8/69.3	74.6/63.5	70.2/**91.3**	87.6/71.6	22.4/24.2	90.7/68.8
Nsp3	**94.6**/83.1	88.7/86.5	80.9/81.2	81.6/**93.8**	90.7/81.8	35.9/36.4	**94.6**/83.2
Nsp4	**92.2**/84.2	86.8/84.8	81.9/83.5	84.8/**94.9**	87.9/83.3	47.4/43.0	**92.2**/83.8
Nsp5	92.7/86.9	86.5/88.8	81.2/81.6	87.6/**94.5**	89.8/85.7	48.8/46.9	**93.1**/87.6
Nsp6	**97.9**/91.7	93.8/91.7	95.8/89.6	91.7/**97.9**	95.8/93.8	54.1/66.0	**97.9**/91.7
Nsp7	**94.2**/80.2	87.0/84.8	89.7/79.8	82.0/**93.1**	90.7/80.1	35.6/41.8	**94.2**/80.3
Nsp8	92.5/87.2	91.0/88.7	89.5/88.0	86.5/**94.0**	91.0/87.2	50.8/48.9	**93.2**/88.0
ORF1b	**94.3**/86.3	88.9/86.8	88.0/84.9	86.3/**93.4**	87.9/83.4	50.0/51.4	94.2/86.3
Nsp9	**95.1**/85.0	89.2/87.0	88.5/84.0	85.4/**95.3**	93.2/84.4	55.7/57.1	94.9/84.9
Nsp10	**92.7**/84.6	88.5/85.9	88.2/84.6	84.9/**95.1**	79.3/73.4	44.5/47.5	**92.7**/84.6
Nsp11	**94.9**/92.1	89.1/87.1	87.6/85.8	90.3/89.8	91.5/**92.5**	66.8/53.7	94.8/91.9
Nsp12	**94.6**/86.5	87.6/87.4	85.6/86.5	87.4/85.4	84.7/**93.0**	22.6/17.6	**94.6**/86.5
ORF2	89.6/88.2	90.9/91.2	**92.5**/92.1	86.4/86.6	92.3/**92.0**	54.2/54.1	89.4/88.7
ORF3	83.8/90.2	86.1/**90.6**	84.7/88.0	84.2/86.0	**89.9**/89.8	54.1/54.2	84.3/89.9
ORF4	86.6/88.6	88.1/88.3	87.0/89.0	86.0/88.5	**91.1**/**90.5**	55.7/58.4	87.2/88.8
ORF5	83.9/85.2	84.2/86.6	92.0/91.2	85.1/86.2	**92.1**/**92.9**	48.7/49.8	83.6/85.1
ORF6	89.3/89.7	92.0/91.8	**92.2**/**93.1**	89.7/91.4	92.0/92.0	58.0/60.2	89.0/89.3
ORF7	91.4/89.8	**93.3**/91.4	91.7/87.6	89.5/**96.5**	90.3/86.8	54.3/52.9	91.4/89.8
3'UTR	87.3/85.8	90.3/89.6	**91.0**/86.6	87.3/**91.0**	85.1/82.8	54.0/47.1	85.0/85.7

Bold face numbers depict the highest percentage identity.

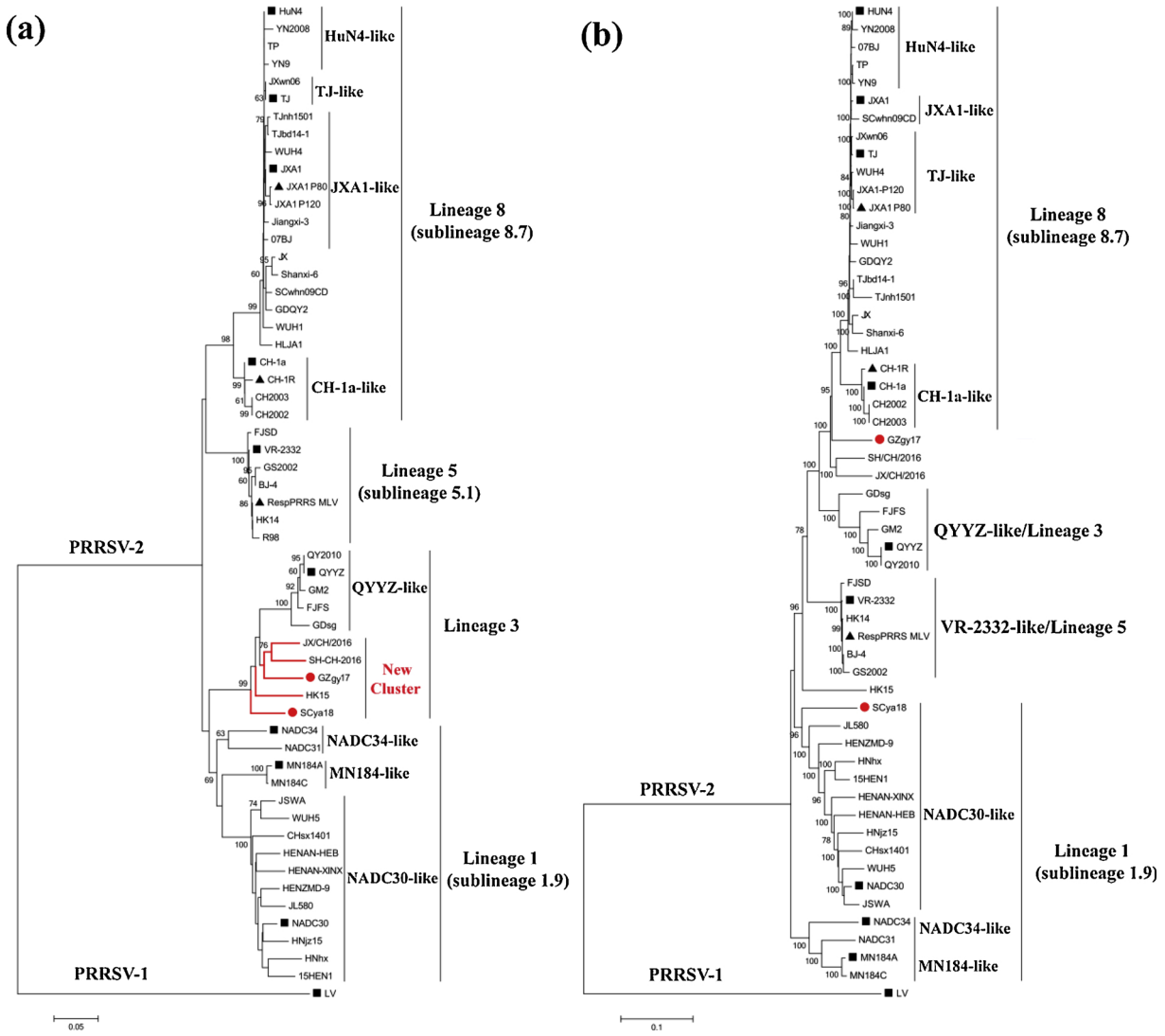

Fig. 2. Phylogenetic trees based on ORF5 (a) and full-length genomic sequence (b) of GZgy17 and SCya18 isolates with 54 PRRSV reference strains available in GenBank. The two isolates in this study are labeled with "red circles". The representative strains are labeled with "black squares"; while the modified live attenuated vaccines are labeled with "black triangles". The phylogenetic tree is constructed by using the neighbor-joining method (1000 bootstrap) in MEGA6. Numbers along branches are bootstrap values. Scale bar indicates nucleotide substitute per site (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

LV, and a MLV vaccine strain JXA1-P80) were compared. The results showed that 5'UTR, ORF1a (Nsp1α, Nsp1β, Nsp2–8), and ORF1b (Nsp9–12) of GZgy17 shared 90.7–99.5% nucleotide homology with JXA1 or JXA1-P80, which was higher than the homology shared with the other five strains. ORF2, ORF6, and 3'UTR of GZgy17 shared 91.0–92.5% nucleotide identity with QYYZ, a higher percentage than that with the other strains, and ORF3–5 of GZgy17 shared 89.9–92.1% nucleotide identity with SH/CH/2016, which was higher than those with the others. In addition, its ORF7 region shared 93.3% nucleotide homology with VR-2332, which was higher than that with the other six strains. The SCya18 strain's 5'UTR, Nsp1α, and Nsp1β shared 90.8–94.2% nucleotide homology with JXA1 or JXA1-P80, which was higher than that with the other strains, whereas SCya18's Nsp2–10, ORF7 and 3'UTR shared 91.0–97.9% nucleotide identity with NADC30, which was higher than that with the others. SCya18's Nsp11–12, ORF2, and ORF4–5 shared 90.5–93.0% nucleotide identity with SH/CH/2016. In addition, its ORF3 region shared 90.6% nucleotide homology with VR-2332, which was higher than that with the other six strains (Table 1).

3.3. GZgy17 and SCya18 exhibit different phylogenetic relationships based on the ORF5 gene and their whole genomes

To establish the genetic relationships of GZgy17 and SCya18 with other PRRSV isolates, we constructed phylogenetic trees based on their ORF5 and the complete genomic sequences. The results showed that all of the PRRSV-2 strains in China belonged to one of four lineages based on the ORF5 genotyping: lineage 8 (JXA1-/HUN4-/TJ-/CH-1a-like), lineage 1 (NADC30-/NADC34-/MN184-like), lineage 5 (VR-2332-like), and lineage 3 (QYYZ-like) (Fig. 2). The lineage 3 PRRSV strains reported in mainland China since 2010showed evidence of evolutionary divergence, and have been further classified into two clusters: a QYYZ-like cluster and a new cluster (SH/CH/2016-like). The two new PRRSV isolates, GZgy17 and SCya18, shared low nucleotide homology (91.2–92.0%) (Table 1) with QYYZ-like strains, and were classified in a new cluster, together with another three strains (SH/CH/2016, JX/CH/2016 and HK15). Notably, the GZgy17 and SCya18 isolates were classified in lineage 3 based on ORF5 genotyping, whereas SCya18 belonged to lineage 8 and GZgy17 belonged to an inter-lineage between lineage 8 and 3, based on their whole genomes (Fig. 2). These results

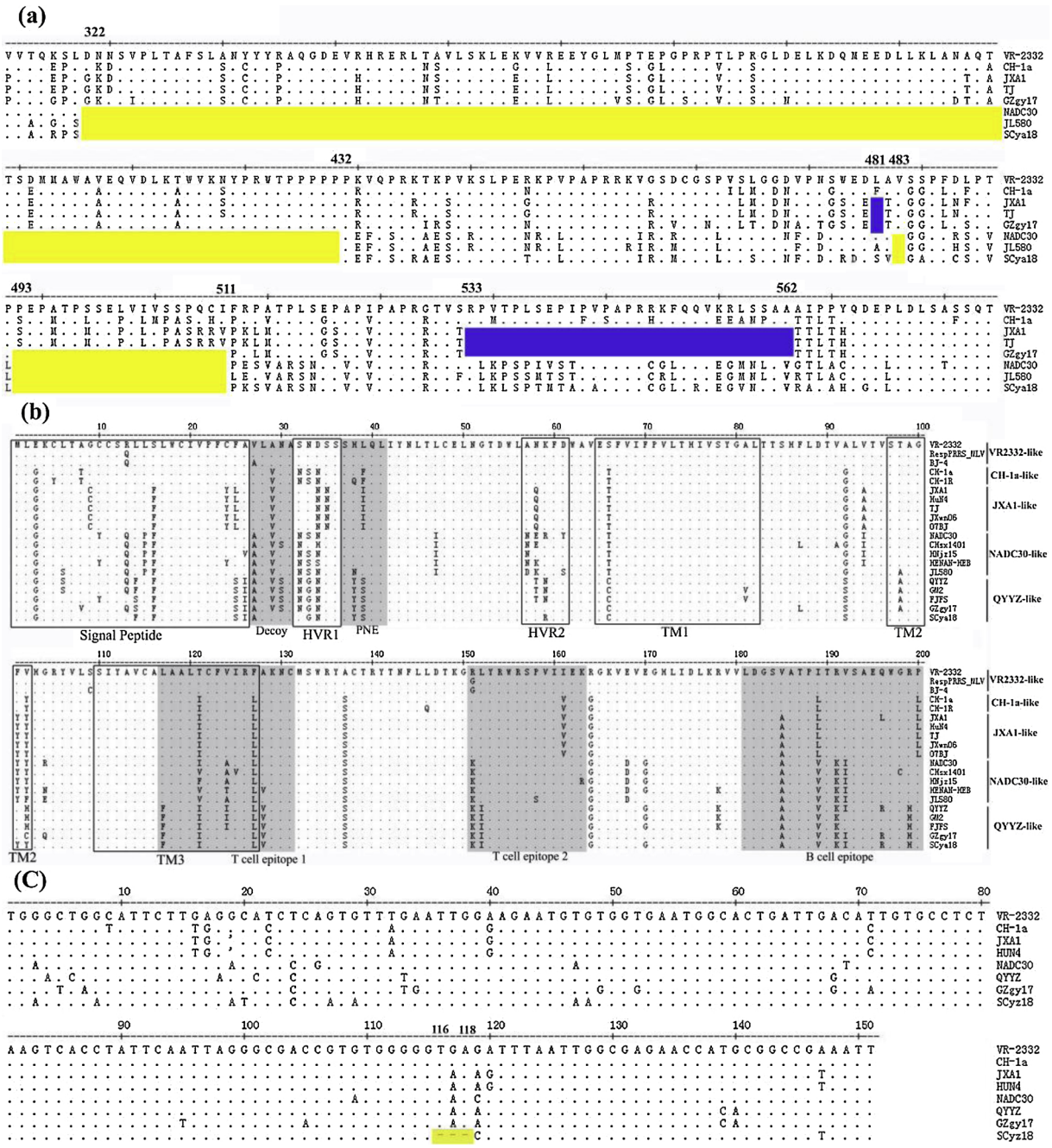

Fig. 3. Multiple sequences alignment of Nsp2, GP5, and 3′UTR. (a) Two discontinuous amino acid deletions at positions 481 and 533–562 (blue regions) in NSP2 of GZgy17 and HP-PRRSV-like strains, and an additional 19-deltion at positions 493–511 (yellow regions) in NSP2 are also observed in GZgy17. Three discontinuous amino acid deletions at positions 322–432, 483, and 493–511 (yellow regions) in NSP2 of SCya18 and NADC30-like strains. (b) Multiple alignment of GP5 amino acid sequences of GZgy17 and SCya18 and eighteen PRRSV reference strains. Black boxes indicate the regions of signal peptide, hypervariable regions (HVR1 and 2), and transmembrane domains (TM1, 2 and 3). Light shadows indicate the amino acid residues in the decoy epitope, primary neutralizing epitope (PNE), T cell and B cell epitopes. (c) Three continuous nucleotides deletion in 3′UTR at positions 116–118 is observed in SCya18 (yellow region) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

may indicate that mosaic recombination events occurred in the genomes of the GZgy17 and SCya18 isolates.

3.4. Novel amino acid deletions or substitutions were detected in the Nsp2, GP5, and 3′UTR

Amino acid alignment of the nonstructural protein 2 (NSP2) of GZgy17 and SCya18 isolates with the representative strains showed that the GZgy17 had the same amino acid (aa) deletion in NSP2 as JXA1 (an HP-PRRSV strain reported in China in 2006), while SCya18 contained the discontinuous aa deletion in NSP2 identical to that of NADC30 (a moderately pathogenic strain identified in the USA in 2008). These deletions were identified as a discontinuous 30-aa deletion (1-aa at position 481, and 29-aa at positions 533–562) and a discontinuous 131-aa deletion (111-aa at positions 322–432, 1-aa at position 483, and 19-aa at positions 493–511), respectively, when compared with the sequence of VR-2332 (Fig. 3a). Interestingly, GZgy17 contained an additional 19-aa deletion in NSP2 from positions 493–511, the same position as in a 19-aa deletion in NADC30-like strains, which has never been described. Comparisons of the amino acid analyses of GZgy17 and

Table 2
Information on recombination events of PRRSV isolates GZgy17 and SCya18 detected by RPD4 software.

Recombinant strain	Breakpoints (position in alignment)		Major (similarity)	Minor (similarity)	*p*-value of the detection methods						
	Beginning	Ending			RDP	GENECONV	BootScan	MaxChi	Chimaera	SiScan	3Seq
GZgy17	12,053	14,698	JXA1(94.3%)	SH/CH/2016 (90.9%)	3.819×10^{-14}	NS	3.243×10^{-46}	1.261×10^{-18}	5.847×10^{-27}	NS	3.746×10^{-40}
SCya18	1663	10,927	NADC30 (93.7%)	SH/CH/2016 (90.9%)	4.339×10^{-47}	9.277×10^{-16}	6.263×10^{-42}	4.849×10^{-39}	1.141×10^{-17}	1.204×10^{-84}	4.490×10^{-74}
	14,529	15,406	NADC30 (95.0%)	SH/CH/2016(90.9%)	2.733×10^{-14}	2.407×10^{-09}	9.165×10^{-16}	2.512×10^{-09}	2.949×10^{-10}	4.381×10^{-10}	1.711×10^{-05}

NS: not significant.

SCya18 GP5 with those of the other representative strains showed that extensive amino acid substitutions lay within the signal peptide, hypervariable regions (HVR1 and HVR2) and the potential neutralization epitope (PNE) domain of the protein (Fig. 3b). Several unique amino acids substitutions, such as $L^{14}\rightarrow F/S^{14}$, $F^{25}\rightarrow S^{25}$, $A^{26}\rightarrow I^{26}$, $N^{33}\rightarrow G^{33}$, $H^{38}\rightarrow Y^{38}$, $A^{92}\rightarrow S^{92}$, $L^{117}\rightarrow F^{117}$, $L^{152}\rightarrow I^{152}$ and $R^{119}\rightarrow H^{199}$, were only identified in QYYZ-like strains, including GZgy17 and SCya18 (Fig. 3b). In addition, SCya18 exhibited a novel continuous 3-nt deletion in its 3′UTR at positions 116–118, which is reported here for the first time (Fig. 3c).

3.5. GZgy17 and SCya18 both originated from a lineage 3 strain (SH/CH/2016-like) and had recombined with a lineage 8 (JXA1-like) and a lineage 1 strain (NADC30-like), respectively

RDP4 and SimPlot analysis were performed to identify possible recombination events based on the multiple alignment of representative PRRSV genomes. The analysis revealed that GZgy17 and SCya18 sequences showed remarkably high degrees of certainty, with *p*-values of $\leq 1 \times 10^{-5}$, according to the results of at least five detection methods (Table 2). From the similarity plot, two recombination breakpoints (position in alignment) within the GZgy17 genome were identified, which were located in ORF2 (nt 12,053) and ORF6 (nt 14,698) (Fig. 4a). The breakpoints in GZgy17 separated its genome into three regions, where region A (nt 1–12,053) was closely related to the JXA1-like strain and region B (nt 12,054–14,698) was closely related to QYYZ-like strain, whereas region C (nt 14,698–3′UTR) was divided into a separate branch (Fig. 4c). Three recombination breakpoints were identified in SCya18, which were located in Nsp2 (nt 1663), Nsp11 (nt 10,927), and ORF6 (nt 14,529) (Fig. 4b). The breakpoints in SCya18 separated its genome into four regions, where region A (nt 1–1663) was closely related to the JXA1-like strain, region B (nt 1664–10,927) was closely related to the NADC30-like strain, region C (nt 10,928–14,529) was closely related to the SH/CH/2016-like strain, and region D (nt 14,530–3'UTR) was closely related to NADC30-like strain (Fig. 4d). These results indicated that the GZgy17 and SCya18 strains exhibited different recombination patterns. GZgy17 is a recombinant virus from JXA1-like (lineage 8) and SH/CH/2016-like (lineage 3) isolates, whereas SCya18 is a product of recombination event between NADC30-like (lineage 1) and SH/CH/2016-like isolates.

3.6. Pathogenicity analysis

3.6.1. Pigs infected with GZgy17 exhibited more severe clinical manifestations than pigs infected with SCya18

We compared the pathogenicity of GZgy17 and SCya18 strains in 4-week-old piglets. After PRRSV challenge, the piglets infected with GZgy17 showed a febrile response (40.6 °C) starting 1 dpi, and hovered over 40 °C from 4 to 14 dpi with a peak of 40.9 °C at 9 dpi (Fig. 5a). Clinical signs, such as cough, sneezing and anorexia, began to appear within 2–3 dpi. More severe symptoms, characterized by dyspnea, tachypnea and shivering were observed from 7 dpi to 14 dpi. One GZgy17-infected piglet died at 13 dpi (Fig. 5b) and others were moribund at 14 dpi. Piglets infected SCya18 showed fever (40.4 °C) starting 1 dpi, and exhibited transient fever (above 40 °C) for 7 days starting 4–5 dpi and running to 10–14 dpi. The clinical signs were milder in SCya18-infected piglets; cough, anorexia, dyspnea and tachypnea were observed within 5–14 dpi. All piglets in the SCya18-infected group survived until the end of study (Fig. 5b). The average body temperature of the pigs infected with GZgy17 was significantly higher than that of the SCya18-infected pigs at 7, 8, 9, 11, and 12 dpi ($p < 0.05$). The average rectal temperature of piglets in the control group remained at 39.0 °C–39.4 °C, and no obvious clinical signs were observed throughout the experiment (Fig. 5a). The average clinical scores of GZgy17-infected group were significantly higher than those of SCya18-infected group ($p < 0.05$) from 7 to 14 dpi (Fig. 5c). At 7 dpi, the body weight gains

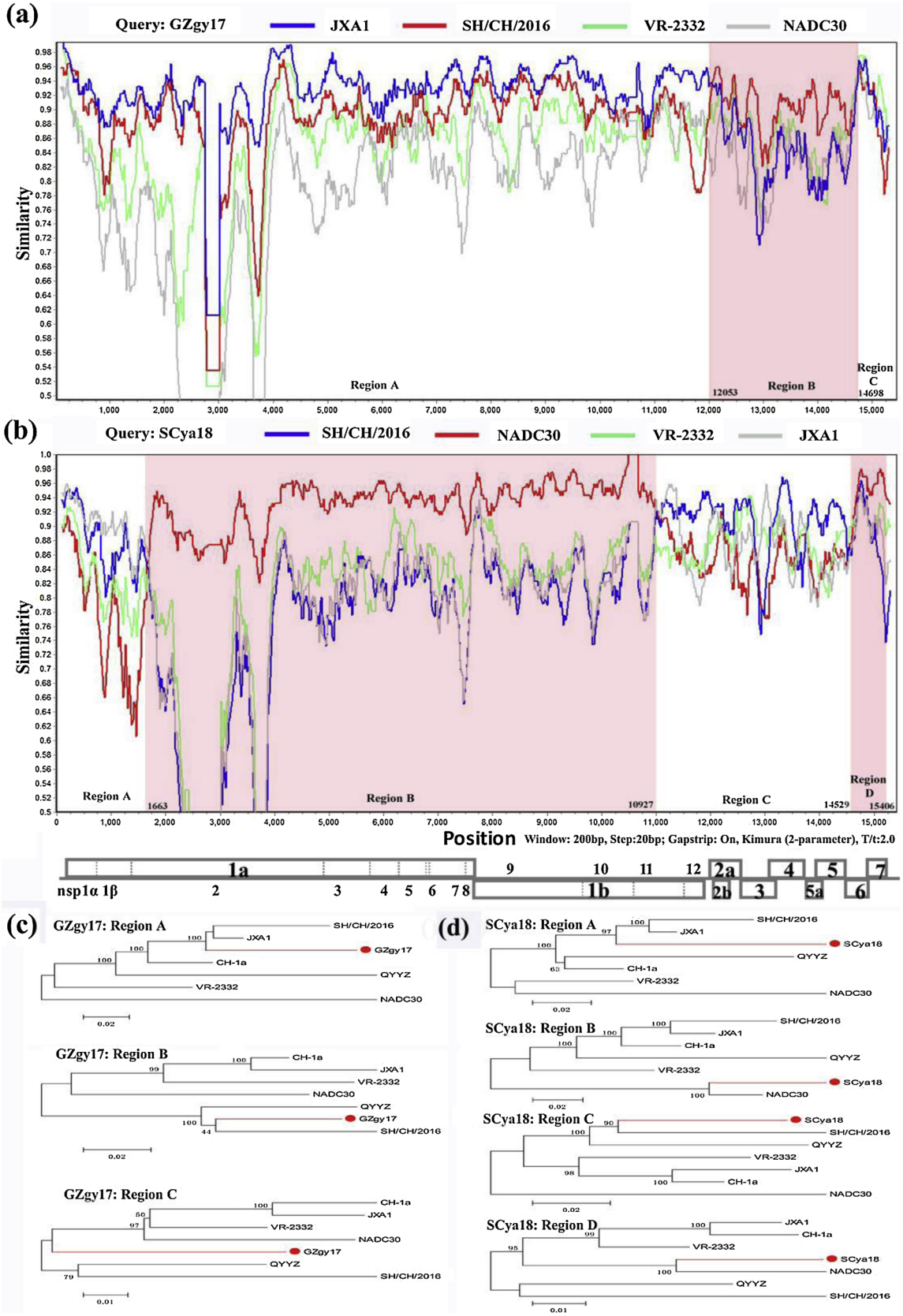

Fig. 4. Genome recombination analysis of PRRSV isolates GZgy17 and SCya18. The y-axis indicates the percentage similarity between the query sequence (GZgy17/SCya18) and four representative sequences. (a) Genome scale similarity comparisons of GZgy17 (query) with JXA1 (blue), SH/CH/2016 (red), VR-2332 (green), and NADC30 (gray). (b) Genome scale similarity comparisons of SCya18 (query) with SH/CH/2016 (blue), NADC30 (red), VR-2332 (green), and JXA1 (gray). The supposed recombination regions are shown with pink regions, and the recombination breakpoints are marked at the bottom with nucleotide sites and viral genome structure referenced to VR-2332. (c) Phylogenetic trees based on each recombinant region (Regions A–C) of GZgy17. (d) Phylogenetic trees based on each recombinant region (Regions A–D) of SCya18 (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

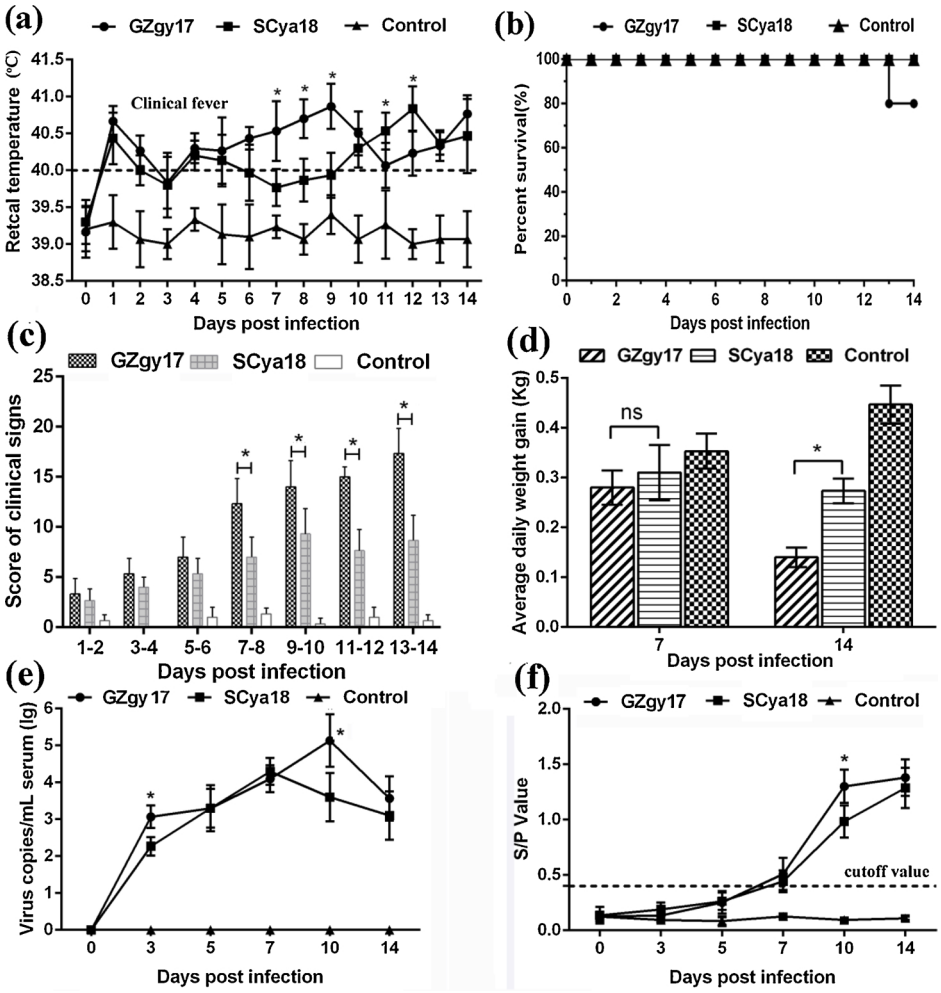

Fig. 5. The rectal temperature, survival rate, clinical sign scores, weight gain, and viremia and antibody levels of piglets during the challenge experiment. (a) Rectal temperatures of piglets inoculated with GZgy17, SCya18, and RPMI-1640 medium. The clinical fever cut-off value was set at 40.0 °C. (b) The survival and mortality curves of the inoculated piglets. (c) The scores of clinical signs of the inoculated piglets. (d) Average daily weight gain of the inoculated piglets during each week of the challenge study. (e) The PRRSV RNA copy numbers in serum of challenged pigs at different days post challenge were detected by qRT-PCR. (f) PRRSV-specific antibodies in serum of challenged pigs at different days post challenge. The cut-off value for seroconversion was set at a sample-to-positive (s/p) ratio of 0.4. The measured values in this study were expressed as the mean ± standard deviations (SD). Asterisk indicates significant differences between the GZgy17- and SCya18-inoculated groups (*$P < 0.05$).

of the GZgy17-infected piglets and the SCya18-infected piglets were 0.281 kg (± 0.107) and 0.310 kg (± 0.177), respectively, with no significant difference. However, the GZgy17-infected piglets had gained significantly less weight at 14 dpi compared with the SCya18-infected piglets ($p < 0.05$) (Fig. 5d).

3.6.2. The GZgy17-infected group showed higher level of viremia and PRRSV-specific antibodies than the SCya18-infected group

After PRRSV challenge, pig serum samples were collected at 0, 3, 5, 7, 10 and 14 dpi for viremia and PRRSV-specific antibodies measurement. As illustrated in Fig. 5e, the viral titers of GZgy17- and SCya18-infected groups reached peak levels at 10 dpi and 7 dpi, respectively. The serum virus RNA copy numbers in the GZgy17-infected pigs were significantly higher than those in the SCya18-infected pigs at 3 and 10 dpi ($p < 0.05$). No viremia was detected in the serum samples from the control group throughout the study. Meanwhile, PRRSV-specific antibodies were measured using an IDEXX ELISA kit. As shown in Fig. 5f, all pigs in the two challenged groups seroconverted at 7 dpi. The antibody titer of pigs in GZgy17-infected group was significantly higher than in the SCya18-infected group at 10 dpi ($p < 0.05$). There was no significant difference in antibody titers between the two challenge groups at 0, 3, 5, 7 and 14 dpi. The control group remained negative for PRRSV-specific antibodies throughout the experimental period.

3.6.3. Pigs inoculated with GZgy17 developed more severe interstitial pneumonia than pigs infected with SCya18

At necropsy, the major pathological lesions in the PRRSV-challenged piglets were characterized by pulmonary consolidation, edema, and interstitial pneumonia (Fig. 6a-b). The lung gross lesion scores of SCgy17-infected pigs were significantly higher than those of SCya18-infected pigs ($p < 0.05$) (Fig. 7a). Microscopic histopathological examination showed that all pigs inoculated with GZgy17 developed interstitial pneumonia characterized by marked thickening of the alveolar septa, vacuolar degeneration of alveolar epithelial cells, and severe inflammation characterized by infiltrating neutrophils and lymphocytes (Fig. 6d). The lung sections of pigs inoculated with SCya18 developed milder interstitial pneumonia with compensating emphysema and pulmonary artery necrosis (Fig. 6e). The microscopic lung lesion scores of GZgy17-infected pigs were significantly higher than those of SCya18-infected pigs ($p < 0.05$) (Fig. 7b). No obvious macroscopic or microscopic pathological lesions were observed in the lungs of piglets in the control group (Fig. 6c and f).

IHC staining of lungs in each group of piglets was also performed to detect viral antigen. As shown in Fig. 6g–h, viral antigen was mainly distributed in the bronchial epithelial cells and the macrophages in lungs of the PRRSV-challenged piglets. The lung IHC scores of GZgy17-infected pigs were significantly higher than those of SCya18-infected pigs ($p < 0.05$) (Fig. 7c). No positive staining was detected in the control group (Fig. 6i and c). Meanwhile, the viruses were recovered from lung tissues collected from the two PRRSV-challenge groups, and ORF5 gene was sequenced to confirm that they were the original viruses used in the challenges.

4. Discussion

PRRSV has been documented in China since 1995 (Guo et al., 1996);

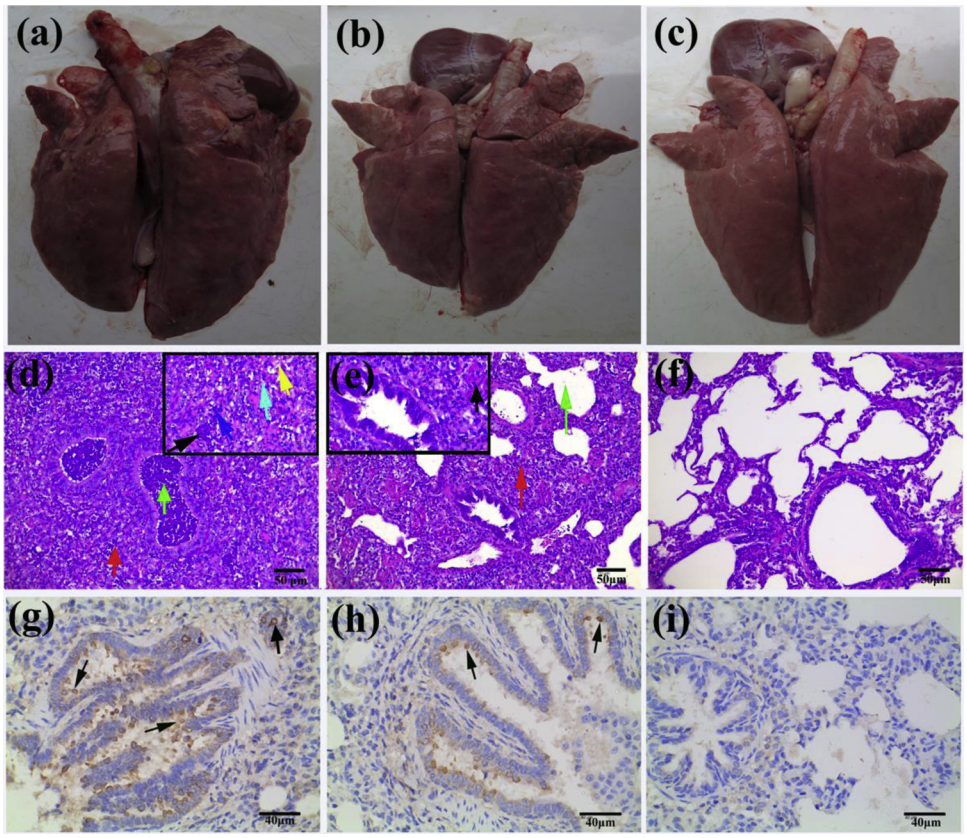

Fig. 6. Gross and microscopic lung lesions observation of the inoculated piglets. (a) Severe interstitial pneumonia with consolidation, and pulmonary edema were observed in GZgy17-inoculated pigs. (b) Moderate interstitial pneumonia with consolidation was observed in SCya18-inoculated pigs. (d) Interstitial pneumonia characterized by marked thickening of the alveolar septa (red arrow), vacuolar degeneration of alveolar epithelial cells (light blue arrow), foci of necrosis (yellow arrow), and severe inflammation (green arrow) characterized by infiltrating neutrophils (black arrow) and lymphocytes (dark blue arrow) could be observed in GZgy17-inoculated pigs. (e) Milder interstitial pneumonia (red arrow) with compensating emphysema (green arrow) and pulmonary artery necrosis (black arrows) could be observed in SCya18-inoculated pigs. No macroscopic and microscopic pathological lesions were observed in the control groups (c and f). Original magnification, 200× and 400×. Viral antigen was mainly distributed in the bronchial epithelial cells and the macrophages (black arrows) in lungs of the GZgy17- (g) and SCya18-challenged (h) piglets. No positive staining was detected in the control group (i). Original magnification, 400× (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

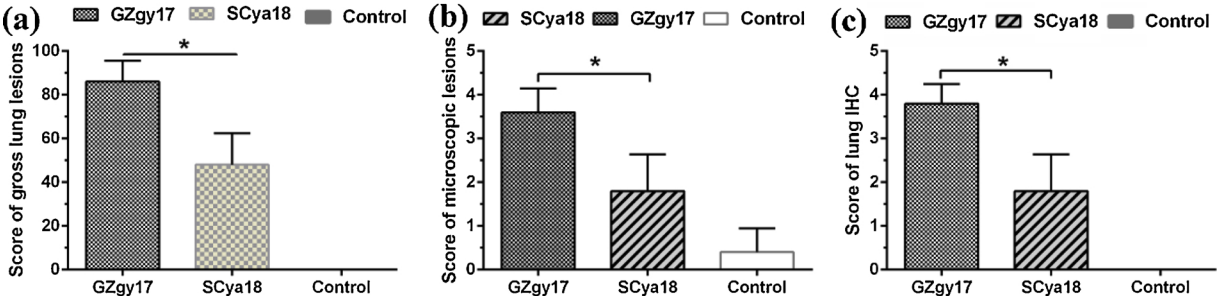

Fig. 7. Scores of lung gross and microscopic lesions and IHC examination of the inoculated piglets. (a) The scores of lung gross lesions, which were graded based on percent lung area affected. (b) The scores of lung microscopic lesions, which were graded based on the distribution and severity of interstitial pneumonia. (c) The scores of numbers of PRRSV-positive cells in lungs. Asterisk indicates significant differences between the GZgy17- and SCya18-inoculated groups (*$P < 0.05$).

however, variant strains of PRRSV associated with large-scale outbreaks of high fever with 50–100% morbidity and 20–100% mortality in growing pigs have emerged in China since 2006 and pose a serious problem for the swine industry of China (Tian et al., 2007). Although several imported and domestic commercial modified live virus (MLV) vaccines were widely used in the field, PRRS still remains a problem in the swine industry and has caused severe economic losses in China. According to the recent investigation of genetic diversity of PRRSV based on the global genotyping, five lineages (lineage 1, 3, 5, 8, and 9) of PRRSV-2 were identified in China (Gao et al., 2017). Among these five lineages, lineage 8 (JXA1-/HUN4-/TJ-like) and lineage 1 (NADC30-like) strains have received great attention in recent years due to their high pathogenicity and high incidence of recombination, respectively (Guo et al., 2018; Li et al., 2016a; Zhao et al., 2015; Zhou and Yang, 2010). Less attention has been paid to other lineages (lineage 3, 5 and 9) in China due to their limited geographical distribution and/or low pathogenicity (Gao et al., 2017; Guo et al., 2018). However, the lineage 3 PRRSV spreads rapidly and its proportion among circulating PRRSVs has significantly increased in recent years (Gao et al., 2017).

In this study, two new PRRSV isolates, GZgy17 and SCya18, were isolated from Guizhou and Sichuan province, respectively, southwestern China. The two isolates were found to divide into a new cluster of lineage 3, based on phylogenetic analysis of ORF5 gene sequences, indicating that lineage 3 of PRRSV-2 strains underwent evolutionary divergence from 2010 to 2018. We previously identified lineage 3 strains in Sichuan province in 2016 (Zhou et al., 2018d), while this lineage was reported for the first time in Guizhou province in 2017. Recently, two new lineage 3 PRRSV strains were reported in Guangdong and Fujian provinces, southern China (Sun et al., 2018), and HP-PRRSV (JXA1) provides most of their complete genomes. However, the two strains in our study showed different recombination patterns, HP-PRRSV (JXA1) and NADC30 provide most of the genomes of strains GZgy17 and SCya18, respectively. Therefore, the recent emergence of new lineage 3 PRRSVs in southwestern and southern China at almost the same time highlights the importance of monitoring this lineage in China.

Recombination is considered to be the most important mechanism in the evolutionary history of PRRSV. With the emergence of NADC30-

like PRRSV strains in China since 2013, numerous PRRSV-2 recombination events between different genetic sublineages have taken place in the field. For example, lineage 1 PRRSV strains were reported to recombine with lineage 8 strains (HENAN-HEB, JL580, FJ1402 and HNhx) or lineage 5 strains (CHsx1401, HENAN-XINX and HNyc15) (Li et al., 2016a, 2017; Li et al., 2016b; Zhang et al., 2016; Zhao et al., 2015). Recently, lineage 3 PRRSV strain was reported to recombine simultaneously with lineage 1 and lineage 8 (SCcd16) (Zhou et al., 2018d). Moreover, a new PRRSV strain (FJLIUY-2017) with a complex genome recombination between lineage 1, 3, 5, and 8 strains was identified in Fujian province, China (Liu et al., 2018). In this study, recombination analysis based on the whole genome sequences revealed that SCya18 is likely a product of a recombination event between SH/CH/2016-like and NADC30-like (lineage 1) strains. To our knowledge, a lineage 3 PRRSV that has only recombined with a lineage 1 strain has never been previously reported.

Mutation is another important mechanism of PRRSV evolution. Chinese HP-PRRSVs were characterized by a discontinuous 30-aa deletion (1aa + 29aa) in the NSP2-coding region (Tian et al., 2007), while NADC30-like PRRSVs showed a discontinuous 131-aa deletion (111aa + 1aa + 19aa) in NSP2 relative to the sequence of VR-2332 (Brockmeier et al., 2012), which differ from the molecular marker of Chinese HP-PRRSVs. Here, we found that GZgy17 contained a new deletion pattern of "1aa + 19aa + 29aa" in its NSP2-coding region. Interestingly, the "1aa + 29aa-deletion" of GZgy17 is located in the same positions as in HP-PRRSVs, whereas the "19aa-deletion" is located in the same position as in NADC30-like PRRSVs, which reported here for the first time. In addition, a novel continuous 3-nt deletion in 3'UTR at positions 116–118 was found in SCya18, which is different from the recently reported nt-deletions at positions (nt 118–120) of two recently described PRRSV strains (LNWK96 and SCN17) (Zhang et al., 2018; Zhou et al., 2018c). Previous study revealed that the sequences of 3'UTR are involved in viral virulence (Sun et al., 2010); whether the deletion in 3'UTR of SCya18 is associated with its virulence requires further study by using reverse genetic system.

The QYYZ-like PRRSV strains (QYYZ and GM2) were demonstrated to be of low pathogenicity with mild clinical presentations in animal experiments (Lu et al., 2015). However, recent evidence has revealed that increased PRRSV virulence is related to recombination among different strains. Two QYYZ-like PRRSV strains, GD1404 and GDsg, which recombined with a JXA1-like strain, were demonstrated to be highly pathogenic strains in experiments in animals (Dong et al., 2017; Zhang et al., 2017). Particularly, the virus GDsg could cause serious hemorrhage and microscopic lesions in the brain (Dong et al., 2017). In this study, we tested the pathogenicity of the two newly-emerged PRRSVs in 4-week-old piglets. The results revealed that the strain SCya18, which recombined with NADC30-like strain, was of moderate pathogenicity with mild clinical presentations, whereas strain GZgy17, which recombined with HP-PRRSV-like strain (JXA1), was a highly pathogenic strain with persistent fever, higher viremia and antibody levels, higher scores of lung lesions, and a higher mortality rate (20%). The results showed that GZgy17 exhibited greater pathogenicity compared with the SCya18 in piglets, and shared a similar degree of virulence with GD1404 and GDsg (Dong et al., 2017; Zhang et al., 2017). However, no pathological lesions were observed in the brains of piglets in the GZgy17/SCya18-challenged groups (data not shown). The results in the present study suggesting that recombination might be responsible for the variation in pathogenicity in lineage 3 strains of PRRSV-2.

In summary, two PRRSV strains, GZgy17 and SCya18, were isolated from lung tissues of piglets in Sichuan and Guizhou provinces, southwestern China. Phylogenetic and recombination analysis revealed that the two SH/CH/2016-like (lineage 3) isolates exhibited different recombination patterns. Meanwhile, multiple novel aa/nt deletions were observed within their genomes. Furthermore, PRRSV challenge experiments in piglets showed that GZgy17 is a highly virulent strain with greater pathogenicity than the strain SCya18. Our study suggests that recombination might responsible for the variations in pathogenicity of lineage 3 strains of PRRSV-2 and highlights the importance of monitoring lineage 3 of PRRSV-2 strains in China.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.01.026.

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