A novel NADC34-like porcine reproductive and respiratory syndrome virus 2 with complex genome recombination is highly pathogenic to piglets

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Abstract

Background: The NADC34-like porcine reproductive and respiratory syndrome virus 2 (PRRSV-2) first emerged in China in 2017 and has the potential to become the dominant PRRSV strain in China. This study aimed to analyze the genetic features and investigate the pathogenicity of a novel NADC34-like PRRSV in southwest China.

Results: In this study, a novel PRRSV-2, SCcd2020, was isolated from diseased piglets in Sichuan province, southwest China in 2020. The complete viral genome was determined and analyzed. An ORF5-based phylogenetic analysis showed that SCcd2020 clustered with NADC34-like strains, whereas the genome sequence clustered the isolate with NADC30-like viruses and it contains a discontinuous 131-aa deletion in NSP2 when compared to NADC30 strain. Notably, recombination analyses indicated that SCcd2020 is a multiple recombinant virus from NADC30-like, NADC34-like and JXA1-like strains, which is the first description of Chinese domestic HP-PRRSV involving the recombination event of an NADC34-like strain. Evolutionary analyses conducted on ORF5 sequences using Bayesian phylodynamic models estimated the time of emergence of NADC34-like strains in China was as early as July 2013, not long before its first detection in the United States in 2014. Importantly, an animal challenge study in 4-week-old piglets showed that SCcd2020 causes high fever and severe hemorrhagic pneumonia with pulmonary consolidation and edema, and it has a high mortality rate (60%), which indicated that SCcd2020 is a highly pathogenic PRRSV strain.

Conclusions: This is the first report of the emergence of a novel highly pathogenic NADC34-like recombinant strain, and it highlights the importance of monitoring newly emerging PRRSV strains in China.

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is an economically important and devastating infectious disease in commercial pig farming. This viral disease was first reported in the United States in the late 1980s [1] and quickly spread worldwide. The etiological agent, PRRS virus (PRRSV), is an enveloped, single-strand, non-segmented, positive-sense RNA virus [2]. According to the latest PRRSV classification criteria of the International Committee on Taxonomy of Viruses (ICTV), the virus is divided into two separate species: *Betaarterivirus suid 1* (former PRRSV-1, European type) [3] and *Betaarterivirus suid 2* (former PRRSV-2, North American type) [1], which belong to the genus *Betaarterivirus*, subfamily *Variartevirinae* family *Arteriviridae*, and order *Nidovirales* [4].

The genome of PRRSV is approximately 15 kb, comprising 10 overlapping open reading frames (ORFs) [2]. ORF1a and ORF1b encode replication-related polymerase proteins that can be autoproteolytically cleaved into at least 16 nonstructural proteins (NSPs): NSP1α, NSP1β, NSP2TF, NSP2N, NSP2−6, NSP7α, NSP7β, and NSP8−12. ORF2−7 encode seven membrane-associated structural proteins (GP2a, E, GP3, GP4, GP5, 5a and M), and a nucleocapsid (N) protein [5−7]. Among these ORFs, ORF5 has been extensively used in molecular epidemiologic research, as well as for the classification of PRRSV field strains due to its marked genetic variation [8−11]. PRRSV-2 strains around the world show considerable diversity, which allows them to be further classified into nine lineages (L1−9) with >11% diversity levels based on ORF5 sequences [8].

PRRSV-2 has been the dominant strain in China for decades since it was first documented in the country in late 1995 (the representative strain CH-1a) [12]. To date, four major known lineages (sublineages) of PRRSV-2 have been identified in China: lineage 1 (L1.8 and L1.5), lineage 3, lineage 5 (L5.1), and lineage 8 (L8.7) [13, 14]. Among the four lineages, lineage 1, NADC30-like and NADC34-like, strains are presumed to have spread to China from the USA because they are most homologous with American NADC30 and NADC34 strain, respectively [15, 16]. Lineage 3 (QYYZ-like) strains were first reported in mainland China in 2010 and began to spread widely in recent years [17, 18]. Lineage 5 (VR-2332-like) strains emerged as early as 1996 [9]; however, they have not been pandemic in China. Lineage 8 contains the Chinese highly pathogenic PRRSVs (HP-PRRSVs, JXA1-like), which caused a large-scale outbreak in China in 2006, and low pathogenic strains (LP-PRRSVs, CH-1a-like) [19].

The NADC34-like PRRSV was first identified in Liaoning province, China in 2017 [16]. Subsequently, the virus was detected in three provinces (Heilongjiang, Fujian, and Henan) in China during 2018−2019 and has the potential to be the next endemic strain, in view of the wide prevalence of the NADC30-like strains in China [16, 20, 21]. In this study, we are the first to report a novel NADC34-like PRRSV variant isolated from Sichuan province of China in 2020 that is a natural recombinant virus with a complex genome resulting from recombination among multiple PRRSV strains. In addition, we explored the evolution of the new isolate. Furthermore, an animal challenge experiment showed that the newly emerged strain is highly pathogenic for piglets.

Results

Geographical distribution and tMRCA estimations of NADC34-like strains in China

The NADC34 strains of PRRSV-2 were initially recognized in the USA in 2014 [24]. In 2017, two NADC34-like strains (LNWK96 and LNWK130) were first detected in Liaoning province of China [16]. According to the recent epidemiological investigation of PRRSVs during 2018−2019, the NADC34-like strains were subsequently reported in three other provinces, Heilongjiang, Fujian, and Henan [20, 21, 28, 29]. In 2020, Sichuan was the fifth province to detect an NADC34-like strain. Recently, a NADC34-like PRRSV strain (JS2021NADC34) was identified in Jiangsu province in 2021 [30]. Therefore, the NADC34-like strains of PRRSV-2 spread gradually in China and have been detected in at least six provinces since 2017 (Fig. 1a). Furthermore, the estimated time to tMRCA suggests the emergence of the NADC34-like strains in China was as early as July 2013, not long after their first detection in 2014 in the USA (Fig. 1b). In accordance with the detection time and the phylogenetic classification of NADC34-like strains in China, we speculate that the FJ0908 strain was transmitted from Liaoning to Fujian province, and the SCcd2020 strain was transmitted from Heilongjiang to Sichuan province (Fig. 1a and b).

Isolation and identification of SCcd2020

For viral isolation, PAMs were used to isolate the new PRRSV strain. At 48 h after viral inoculation, scanning electron microscope of the infected PAMs showed marked morphological changes under, resulting in collapsing and shedding. No obvious CPE was observed in the uninfected cells. IFA was used to visualize the presence of the N protein of SCcd2020 in the infected PAMs. At 24 hpi viral protein in green was observed in infected cells while none was observable in mock-infected cells (data not shown). The $TCID_{50}$ value of SCcd2020 strain was determined as $1 \times 10^{9.19}$/mL using PAMs. The strain failed to replicate in MARC-145 cells.

Full-length genomic characterization of the NADC34-like PRRSV strain SCcd2020

The genome of SCcd2020 was 15,019 nucleotides (nt) in length excluding the poly(A) tail. The sequences were deposited in the GenBank database under the accession numbers of MW803134. SCcd2020 shared 82.5%, 82.7%, 79.8%, 89.2%, and 86.2% sequence identity with JXA1 (L8), VR-2332 (L5), QYYZ (L3), NADC30 (L1), and NADC34 (L1), respectively (Table 1). Its sequence shared 43.6% nucleotide identity with PRRSV-1 LV strain, indicating that SCcd2020 belonged to PRRSV-2.

Table 1
Nucleotide identity of SCcd2020 compared with eleven PRRSV reference strains.

Nucleotide Identity % (SCcd2020)											
Region	VR-2332	BJ-4	JXA1	QYYZ	FJFS	NADC30	CHsx1401	ISU30	LNWK96	IA/2014/NADC34	FJ0908
	VR-2332-like		JXA1-like	QYYZ-like		NADC30-like			NADC34-like		
	(Lineage 5)		(Lineage 8)	(Lineage 3)		(Lineage 1)			(Lineage 1)		
Complete genome	82.7	82.6	82.5	79.8	79.9	**89.2**	89.2	86.4	85.0	86.2	85.5
5'UTR	93.7	93.7	92.7	91.1	90.6	**99.0**	97.4	90.1	94.8	95.3	93.2
ORF1a	78.1	77.9	78.3	74.0	73.9	**87.3**	87.6	83.9	80.3	80.8	80.5
ORF1b	87.0	86.9	87.3	85.5	85.9	**87.8**	92.1	88.9	87.4	88.0	87.6
nap1α	87.8	87.6	84.6	85.9	83.5	**89.3**	88.5	88.9	88.7	88.7	91.5
nap1β	78.7	78.3	77.2	74.5	76.5	**87.4**	87.4	79.3	79.1	80.0	79.5
nsp2	69.7	69.4	66.9	64.2	64.1	**89.3**	89.3	84.6	77.0	77.4	76.9
nsp3	85.4	85.5	87.1	79.6	79.7	**88.4**	87.7	81.4	83.9	85.8	85.9
nsp4	88.9	88.9	**94.6**	83.2	84.2	82.0	82.8	82.5	84.2	83.5	84.2
nsp5	85.9	86.1	**90.8**	81.0	81.2	84.3	86.1	86.7	83.9	84.7	84.1
nsp6	97.9	97.9	**97.9**	95.8	95.8	91.7	85.4	93.8	89.6	89.6	87.5
nap7	87.0	86.7	**95.2**	90.9	89.8	80.3	81.6	81.0	80.1	80.3	80.3
nsp8	92.5	92.5	**95.5**	92.5	90.3	85.1	87.3	88.1	87.3	90.3	89.6
nsp9	88.2	88.2	**88.8**	86.7	87.5	91.6	91.6	89.4	87.3	88.2	87.6
nsp10	84.8	84.7	**84.6**	84.0	83.7	93.0	93.1	91.7	89.3	89.8	89.6
nsp11	88.0	87.9	**88.5**	86.1	86.2	86.5	88.3	84.6	86.1	86.5	86.1
nsp12	88.5	88.2	**89.3**	86.1	85.6	93.7	95.0	85.4	85.0	85.0	84.7
ORF2a	87.4	87.0	84.7	85.0	83.0	84.0	84.3	84.4	94.4	**95.6**	94.6
ORF2b	88.7	88.3	85.6	87.4	81.1	86.9	87.4	86.9	96.8	**97.3**	96.8
ORF3	83.4	83.4	82.2	81.8	82.6	83.1	83.9	84.3	91.1	**94.6**	92.0
ORF4	86.2	85.8	85.1	84.4	84.9	92.2	92.4	94.0	92.7	**95.7**	93.3
ORF5	86.7	86.6	85.7	86.1	87.2	84.7	84.4	86.1	85.7	**97.0**	95.0
ORF5a	87.2	89.1	85.9	89.1	86.5	89.1	89.1	89.1	89.7	**96.8**	95.5
ORF6	89.0	88.8	88.4	88.8	89.7	94.1	94.3	94.7	94.7	**95.4**	94.5
ORF7	100.0	90.6	89.0	87.1	89.0	95.2	93.5	92.5	93.0	**94.1**	91.9
3'UTR	92.7	94.0	89.4	90.1	87.4	97.4	98.0	95.4	94.0	**96.0**	95.4
Bold face numbers depict the highest percentage identity.											

Furthermore, the genome of the SCcd2020 isolate was compared with 11 PRRSV representative strains (VR-2332, BJ-4, JXA1, QYYZ, FJFS, NADC30, CHsx1401, ISU30, NADC34, LNWK96, and FJ0908). The 5'-UTR, ORF1a (Nsp1α, Nsp1β, Nsp2−3) of SCcd2020 shared 88.4−99.0% nt homology with NADC30, which was higher than the homology shared with the other 11 strains. ORF1b (nsp4−12) of SCcd2020 shared a 84.6−97.9% nt identity with JXA1, a higher percentage than that with the other strains, and ORF2−7 of SCcd2020 shared a 94.1−97.3% nt identity with NADC34, which was higher than those with the other strains (Table 1). These results indicate that mosaic recombination events occurred in the genome of SCcd2020.

An amino acid (aa) alignment of the NSP2 hypervariable (HV) region of SCcd2020 with those of the representative strains showed that the strain contained the same discontinuous aa deletion in NSP2 identical to that of NADC30, which identified in the United State in 2008. These deletions were identified as a molecular marker with a discontinuous 131-aa deletion (111-aa deletion at positions 323−433, 1-aa deletion at position 483, and 19-aa deletion at positions 508−526), when compared with the sequence of VR-2332 (Fig. 2a). SCcd2020 GP5, when compared with those of the other representative strains, showed extensive aa substitutions within the signal peptide, hypervariable regions (HVR1 and HVR2) and transmembrane regions (TM2 and TM3) domain (Fig. 2b). Several unique aa substitutions, such as 23F→S, 33N/D/G→T, 34D/N/H/G→S, 38H/Y→R, 59K/H/N→S, 102V/Y/H→C, 104G/K→Q, 132→M/L, and 167V→I were identified in the NADC34-like strains. Among them, only the unique 33N/D/G→T substitution was identified in the SCcd2020 strain (Fig. 2b).

Phylogenetic analysis

To establish the genetic relationships between SCcd2020 and other PRRSV isolates, we constructed phylogenetic trees based on their ORF5 and the complete genomic sequences using MEGA-X software. All of the PRRSV-2 strains in China belonged to one of four lineages on the basis of ORF5 genotyping: lineage 8 (JXA1-/CH-1a-like), lineage 1 (NADC30-/NADC34-like), lineage 5 (VR-2332-like), and lineage 3 (QYYZ-like) (Fig. 3). The sub-lineage 1.5 (NADC34-like) PRRSV strains showed evidence of evolutionary divergence, and they were mainly classified into two groups: A and B. Interestingly, the SCcd2020 isolate was classified in the NADC34-like cluster based on ORF5 genotyping (Fig. 3b), whereas it was classified in the NADC30-like cluster based on the full-length genomic sequence (Fig. 3a). This indicates that SCcd2020 may be a recombinant strain.

Recombination analysis

To account for possible recombination events, the multiple alignment of SCcd2020 and reference PRRSV sequences was screened using RDP4 and SimPlot software. The different methods available in RDP4 strongly supported SCcd2020 being a natural recombinant virus from three isolates with NADC30 serving as the major parental strain, and JXA1 and NADC34 serving as the minor parental strains. In addition, the multiple crossover events were further confirmed using SimPlot and phylogenetic analysis. From the similarity plot, five recombination breakpoints (positions in the alignment) within the SCcd2020 genome were identified. They were located in ORF1a (nt 3,866, 4,388, and 5,211), ORF1b (nt 7,957) and ORF2 (nt 11,975) (Fig. 4a). The breakpoints separated the genome into six regions, with region A (nt 3,866−4,388 and 5,211−7,957) being closely related to JXA1-like strain, region B (nt 11,975−3'-UTR) being closely related to NADC34-like strain. Region C (nt 1−3,866, 4,388−5,211, and 7,957−11,975) was divided into the NADC30-like cluster (Fig. 4b). Therefore, the natural recombinant virus SCcd2020 identified in this study was recombined from three PRRSV-2 strains.

Pathogenicity of the NADC34-like PRRSV strain SCcd2020 in piglets

Clinical manifestations

In the SCcd2020-challenged group, all the pigs showed a febrile response (above 40.0 °C) starting from 1 dpi, and exhibited slight clinical signs within 1−4 dpi, such as depression, action retardation, cough, sneezing, and anorexia. More severe clinical manifestations, including high fever, shivering, hyperspasmia, and respiratory distress, were manifested within 5−14 dpi. Three piglets were moribund, with their rectal temperature falling below 37.0 °C from 10 to 11 dpi, and were euthanized immediately (Fig. 5a and 5d). The remaining two pigs were moribund and euthanized at 14 dpi. The average rectal temperature of the RPMI-1640-inoculated pigs remained below 40 °C and exhibited no obvious clinical signs throughout the experiment (Fig. 5a). The body weights of the inoculated pigs decreased significantly from 6 to 14 dpi, and showed negative growth within 6−11 dpi (Fig. 5b). The average clinical scores of the inoculated pigs were significantly higher than the RPMI-1640-inoculated group ($p < 0.05$) starting from 1 dpi until the end of the experiment (Fig. 5c).

Kinetics of viremia and antibody response

After challenge, pig serum samples were collected for detection of viremia and PRRSV-specific antibody. The viral loads in sera of SCcd2020-infected pigs increased sharply from 3 dpi and peaked at 10 dpi (Fig. 5e). Meanwhile, PRRSV-specific antibodies were measured using an IDEXX ELISA kit. The pigs in the challenged groups seroconverted at 7 dpi and the antibody titer peaked at 10 dpi (Fig. 5f). Animals from the RPMI-1640-inoculated group remained negative for viremia and PRRSV-specific antibodies until the end of the experiment.

Pathological examination and immunohistochemistry

At 14 dpi, all the pigs were euthanized and necropsied. All the pulmonary tissues in the PRRSV-challenged animals were deep red, with some black necrotic foci. The major pathological lesions were characterized by pulmonary consolidation, edema, and large areas of hemorrhage in the dorsal and ventral sides of the lungs. The hemorrhagic regions were mainly distributed in the apical, cardiac, and intermediate lobes (Fig. 6a and 6b). Microscopic histopathological examinations showed that all the PRRSV-challenged animals developed interstitial pneumonia characterized by marked thickening of the alveolar septa, the hyperplasia and necrosis of the alveolar epithelium, and severe inflammation characterized by infiltrating neutrophils and lymphocytes. Fibrous tissue hyperplasia and a small amount of red blood cells were observed in the lung stroma (Fig. 6e). No obvious macroscopic or microscopic pulmonary lesions were found in the lungs of the control group (Fig. 6c, 6d, and 6f). PRRSV-specific staining was detected in the lungs of SCcd2020-infected pigs (Fig. 6g), and no positive staining was detected in the control group (Fig. 6h). The virus antigen was mainly distributed in the cytoplasm of macrophages in the bronchioles

and within the alveolar wall cells. Furthermore, the isolate was re-isolated from lung tissues of the PRRSV-challenged animals, and RT-PCR was performed to confirm that they were the original viruses used in the animal experiment.

Discussion

PRRSV, with the representative strain CH-1a, showed low pathogenicity when it was first documented in China in 1995 [12]. However, HP-PRRSV strains 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 [19] and pose a serious threat to the swine industry. Since 2011, several modified live-attenuated vaccines (MLVs) against HP-PRRSV strains have been developed and widely used in the field, and the infectious disease was brought under temporary control in the following years [9]. Nevertheless, PRRS disease still remains a problem in the swine population due to the poor cross-protection of these MLVs, especially against the NADC30-like strains that emerged in China in 2013 [18, 31, 32]. Recently, another lineage 1 PRRSV-2 strain (NADC34-like or ORF5 RFLP 1-7-4-like) appeared in several provinces of China, and it has the potential to become the dominant strain in China [21]. In the present study, a novel NADC34-like PRRSV strain (SCcd2020), was isolated in 2020 in Sichuan province of China, and its genomic characteristics were analyzed. SCcd2020 is a multiple recombinant virus from NADC34-like, NADC30-like, and domestic Chinese HP-PRRSV (JXA1-like) strains. To our knowledge, the novel isolate displayed a different recombination pattern compared with the previously reported NADC34-like strains isolated from 2017 to 2021 in China [16, 20, 21, 28–30], indicating the emergence of a new PRRSV recombination variant in China.

Bayesian phylodynamic models have been used to test research hypotheses related to the evolution of infectious viruses [24]. In this study, evolutionary analyses conducted on ORF5 sequences using Bayesian phylodynamic models estimated that the time of emergence for NADC34-like strains in China was as early as July 2013, not long after the first detection in the United States in 2014. Although the short timeframe and limited quantity of NADC34-like PRRSVs circulating in China might have led to an inaccurate estimation of the tMRCA, our estimative of the tMRCA places a shared common ancestor for NADC34-like strains in China about four years prior to its first detection in 2017. We speculate that the American NADC34-like strains spread to China near the time of the outbreak in 2014, but the virus was not identified because of its mild virulence [28, 29] and limited transmission range.

Recombination is one of the principal mechanisms of PRRSV evolution [33]. With the emergence of NADC30-like (lineage 1) strains in 2013, a great number of PRRSV strains in China were reported to show different patterns of recombination among members of lineages/sublineages [34–36]. However, unlike NADC30-like PRRSVs, few recombination events have been reported in NADC34-like strains. To date, only five NADC34-like strains in China (LNWK96, LNWK130, FJ0908, LNDZD10-1806, and HLJDZD32-1812) have recombined with other strains [16, 20, 21]. Interestingly, the recombination could occur between the parental NADC34 strain with NADC30 and/or ISU30, but no evidence of an NADC34-like strain recombining with local strains in China has been reported to date. According to our study, the multiple recombination events of the novel PRRSV isolate SCcd2020 occurred among NADC30, NADC34, and a Chinese local HP-PRRSV (JXA1-like) strain, which is reported here for the first time.

Increased PRRSV virulence is related to recombination among different strains [37]. The IA/2014/NADC34 strain was initially identified in the United States in 2014 and was demonstrated to be a virulent strain with 14.3% (2/14) mortality that produced serious clinical symptoms in piglets [38]. However, recent studies have revealed that two non-recombinant NADC34-like PRRSV strains (HLJDZD32-1901 and PRRSV-ZDXYL-China-2018-1) in China were demonstrated to be moderately pathogenic strains in piglets [28, 29]. In this study, SCcd2020 infection caused a persistent high fever, severe interstitial pneumonia and hemorrhagic pneumonia, and it increased the viremia and antibody levels in the inoculated piglets. The virus exhibited high pathogenicity in piglets, resulting in 100% morbidity and a mortality rate of 60%. The results indicated that SCcd2020 is a highly pathogenic PRRSV strain. Unlike other reported NADC34-like PRRSVs in China [28, 29], SCcd2020 had recombinant with the domestic Chinese HP-PRRSV strain (JXA1-like strain), which might be responsible for the increased pathogenicity of this virus.

NADC34-like PRRSV was first discovered in China in 2017, but it has not spread as fast as NADC30-like viruses in the field. This may be related to the strong epidemic prevention and control measures taken by the Ministry of Agriculture and Rural Affairs of the People's Republic of China after the outbreak of African classical swine fever in August 2018 [39]. A large-scale ban on pig transport and widespread culling may have affected the spread of NADC34-like PRRSVs [21]. However, the emergence and recombination of new viral strains bring great challenges to the prevention and control of PRRS. NADC34-like strains could enhance the pathogenicity through genetic recombination with local Chinese strains, resulting in highly pathogenic strains. Therefore, the NADC34-like PRRSV still requires significant attention.

Conclusion

In conclusion, an NADC34-like PRRSV-2 strain was isolated from clinical samples collected from Sichuan province of southwestern China in 2020. Genome characterization of the newly emerging strain revealed that it is a recombinant strain resulting from complex recombination events between NADC30-like, NADC34-like, and Chinese HP-PRRSV-like strains. In addition, the estimated time to tMRCA suggests an emergence of the NADC34-like strains in China as early as 2013. Further animal study in 4-week-old piglets indicated that SCcd2020 is a highly pathogenic PRRSV strain. Our study is the first to report the emergence of a novel highly pathogenic NADC34-like recombinant strain, and it highlights the importance of monitoring the newly emerging PRRSV strains in China.

Materials And Methods

Sample collection and virus isolation, plaque purification

In 2020, an outbreak of respiratory symptoms in a non-PRRSV-vaccinated pig farm in Sichuan province in southwest China. The diseased pigs in the farm were characterized by high fever (40.5–41.7°C), blue ears, respiratory distress, and eventual death. The morbidity was about 75% (150/200), with a mortality

of 15% (30/200). Samples of lung tissues were collected from dead pigs and then tissue samples were immersed with RPMI-1640 medium (Transgene, Beijing, China) using a QIAGEN TissueLyser (QIAGEN, Germany). The tissue homogenates were centrifuged at 5000 × g for 10 min and the supernatants were filtered through 0.22-μm membrane and transferred to pulmonary alveolar macrophages (PAMs) and MARC-145 cells for virus isolation. The inoculated cells were maintained at 37°C in a humidified 5% CO_2 atmosphere for 2−4 days and monitored daily for cytopathic effects (CPE). RT-PCR and indirect immunofluorescence assay (IFA) were conducted to identify PRRSV as previously described [22]. The isolate was purified by plaque assay and the virus titers were calculated according to the Reed-Muench method [23]. Then the purified viral isolate was used to sequence its full-length genome and was denominated as SCcd2020.

Viral genome extraction and RT-PCR

Total RNAs were extracted from the PRRSV infected PAMs using the Trizol (Thermo, Waltham, MA, USA) and were transcribed to cDNA using the PrimeScript™ RT Reagent Kit (TaKaRa, Dalian, China) following the manufacture instruction. Complete genome sequences were obtained with a genome walking strategy using 9 pairs of primers that cover the entire coding sequence by producing overlapping fragments (Table S1). The amplified PCR products were then purified and cloned into the pMD19-T vector (TaKaRa, Dalian, China). Three positive clones from each fragment were subjected to Sanger sequencing with the amplification primers (TsingKe Biological Technology Co., Ltd). The 5'-untranslated region (UTR) and 3'-UTR of the viral genome were determined by using a 5'/3' RACE kit according the manufacturer's instructions (TaKaRa, Dalian, China). The complete genome sequence of SCcd2020 as well as its annotations were deposited into NCBI GenBank.

Divergence-Time estimation of NADC34-like strains in China

To determine the temporal structure of NADC34-like strains in China, a total of 116 ORF5 sequences (length 603 nucleotides) were downloaded from NCBI database. The information on the sampling month/year was attributed for each sequence in the ORF5 data set. The Bayesian Markov chain Monte Carlo (MCMC) method was selected to infer the divergence time of ORF5 gene strains in BEAST (v1.8.0) by using a molecular clock and a coalescence-based method as previous described [24]. In brief, the sampling time/year of the non-contemporaneous sequences was used to calibrate the molecular clock. The times to most recent common ancestor (tMRCA) for the NADC34-like strains were calculated using the Bayesian MCMC techniques [25].

Genome alignment and phylogenetic analysis

The whole-genome sequences of SCcd2020 were assembled using the SeqMan tool in the Lasergene DNASTAR™ software (DNASTAR Inc). Multiple sequence alignments were carried out using the MegAlign program of DNASTAR™ software (DNASTAR Inc). For the phylogenetic analysis, a total of 86 full-length PRRSV genomic and 116 ORF5 sequences were retrieved from GenBank. These nucleotide sequences were used to generate a neighbour-joining phylogenetic tree using the ClustalW alignment tool in MEGAX software (Tempe, USA). An unrooted phylogenetic tree was built using the Kimura 2-parameter substitution model and 1,000 bootstrap replicates. The classification of lineages was established according to the description of Shi et al. (2010) [8].

Recombinant analysis

To detect potential recombination events of the SCcd2020 isolate, seven methods (RDP, Bootscan, GENECONV, MaxChi, Chimaera, SiScan, and 3Seq) available in the Recombination Detection Program 4 (RDP4, v4.24) software were used to screen the alignment for recombination events with the following parameters: sequences were set to linear, Bonferroni correction, highest acceptable p-value of 0.05 and 100 permutations. Only recombination events detected by at least five of the aforementioned methods were considered. The detected recombination events were further confirmed by SimPlot software (v3.5.1, Baltimore, MD, USA) using the Kimura (2-parameter) method with a transition-transversion (T/t) ratio of 2. In addition, phylogenetic trees based on each recombinant fragment was constructed to confirm the accuracy of recombination events.

Experimental PRRSV inoculation of piglets

To evaluate the pathogenicity of SCcd2020 isolate, animal challenge experiment was performed. Ten 4-week-old healthy piglets obtained from Sichuan Animal Science Academy (Chengdu, China) were randomly divided into two groups. The piglets were confirmed to be negative for PRRSV, African swine fever virus (ASFV), classical swine fever virus (CSFV), pseudorabies virus (PRV) or porcine circovirus type 2 (PCV2) by PCR or RT-PCR. Five piglets in Group 1 (the challenge group) were each intranasally and intramuscularly inoculated with 2 mL $10^{9.19}$ $TCID_{50}$ SCcd2020 strain, while the remaining five piglets in Group 2 were inoculated with uninfected RPMI-1640 medium with the same dose to serve as the negative control. The experiments were approved by the Animal Ethics Committee of the College of Animal & Veterinary Sciences, Southwest Minzu University (registration protocol 201401002). Clinical signs and rectal temperatures of pigs were recorded daily throughout the study. Serum samples collected on 0, 3, 5, 7, 10, and 14 days post-inoculation (dpi) were subjected to PRRSV-specific antibodies using the commercial ELISA kit (IDEXX HerdChek ELISA, USA). The threshold for seroconversion was set at sample-to-positive (s/p) ratio of 0.4. The dynamics of viremia were tested by real-time quantitative RT-PCR (qRT-PCR) as previous described [26]. All the surviving pigs were humanely euthanized at 14 dpi. After euthanized, portions of lung tissue samples were immersed in formalin for histopathological and immunohistochemical examinations as previous described [27].

Statistical analysis

Significant differences between two groups were determined using a Tukey's t-test in GraphPad Prism software 5.0 (San Diego, CA, USA). The difference in values was considered statistically significant or highly significant if the associated p value was < 0.05 or < 0.01. The measured values of rectal temperature, weight gain, clinical sign scores, virus load, and antibody titers were expressed as the mean with standard deviations (SD).

Abbreviations

PRRSV

Porcine Reproductive and Respiratory Syndrome Virus
ICTV
International Committee on Taxonomy of Viruses
NSP
Nonstructural Proteins
IFA
Indirect Immunofluorescence Assay
CPE
Cytopathic Effects
MCMC
Markov Chain Monte Carlo
tMRCA
The Times to Most Recent Common Ancestor
RDP4
Recombination Detection Program 4
ASFV
African Swine Fever Virus
CSFV
Classical Swine Fever Virus
PRV
Pseudorabies Virus
PCV2
Porcine Circovirus Type 2
SD
Standard Deviations.

Declarations

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Author' Contributions

Long Zhou conceived and designed the experiments. Jifeng Yu, Jun Zhou, Yaoping Long, Lu Xiao, Yandi Fan and Danjiao Yang performed the experiments. Yaoping Long and Jifeng Yu analyzed the data. Long Zhou, Jie Liu and Zhidong Zhang wrote the paper. Long zhou, Bin Zhang and Zhidong Zhang checked and finalized the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The genome sequences of SCcd2020 have been deposited in the GenBank database under the accession number of MW803134.

Ethics approval and consent to participate

The study was approved by the Animal Care and Use Committee of Southwest Minzu University, Chengdu, Sichuan. No specific permissions were required for the collection of samples. All experimental procedures and animal welfare standards strictly followed the guidelines of Animal Management at Southwest Minzu University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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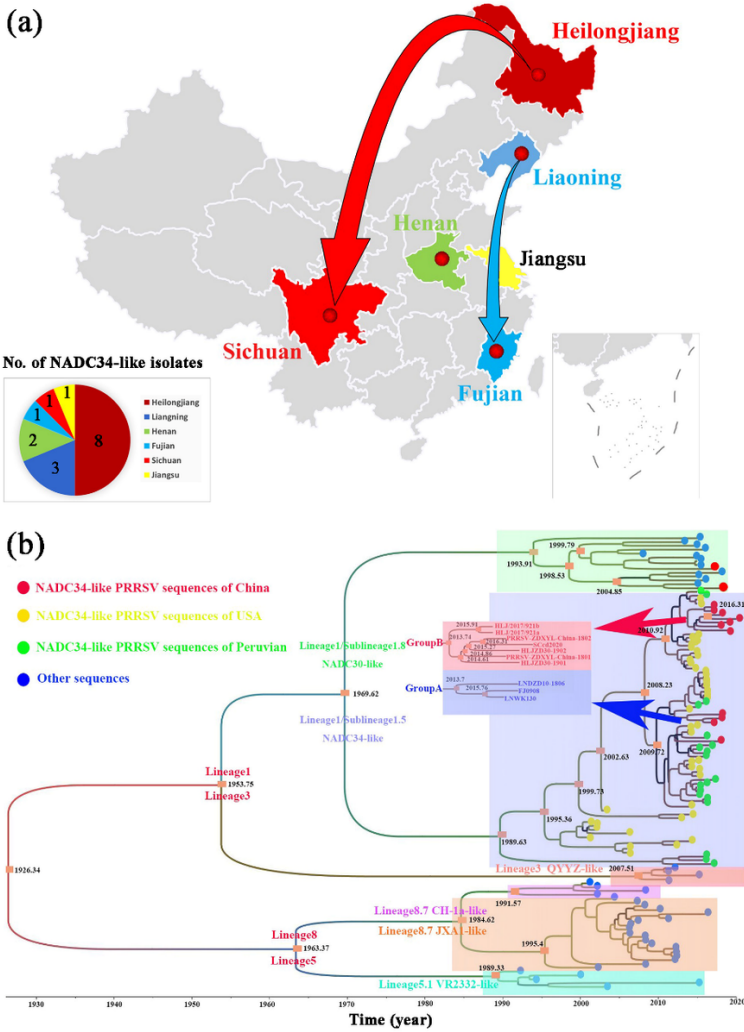
Figures

Figure 1

Geographic distribution and tMRCA estimation of NADC34-like PRRSVs in China. (a) In mainland China, NADC34-like strain was first recognized in Liaoning province in 2017 (dark blue). Previously reported four provinces were positive for NADC34-like PRRSV during 2018–2021, including Heilongjiang (dark red), Fujian (bright blue), Henan (green), and Jiangsu (yellow). The NADC34-like PRRSV-positive province (Sichuan) in this study in 2020 were indicated by bright red. (b) The divergence time of PRRSV strains to tMRCA was determined using the MCMC in BEAST. The NADC34-like PRRSV strains from China are marked with red dots, NADC34-like PRRSV strains from the United States are marked with yellow dots, NADC34-like PRRSV strains from Peru are marked with green dots, and the other remaining strains are indicated by blue dots. The divergence nodes are marked with orange rectangle and the divergence time are shown at each divergence nodes. The branch lengths in years are indicated by scale bars.

Figure 2

Multiple amino acid sequences alignment of NSP2 and GP5. (a) Three discontinuous amino acid deletions at positions 323–433, 483, 508 –526 (purple regions) in NSP2 of SCcd2020 and NADC30-like strains. The light red regions indicate the amino acid deletions of Chinese HP-PRRSV (JXA1-like) strains and the green region indicates the amino acid deletions of NADC34-like strains. (b) Multiple alignment of GP5 amino acid sequences of SCcd2020 and twelve PRRSV reference strains. Red box indicates the region of signal peptide, purple boxes indicate the hypervariable regions (HVR1 and 2), and the green boxes indicate the transmembrane domains (TM1, 2 and 3). Three linear antigenic epitopes (A: decoy epitope, B: primary neutralizing epitope (PNE) and C: homologous neutralizing epitope) are indicated in light blue shadows.
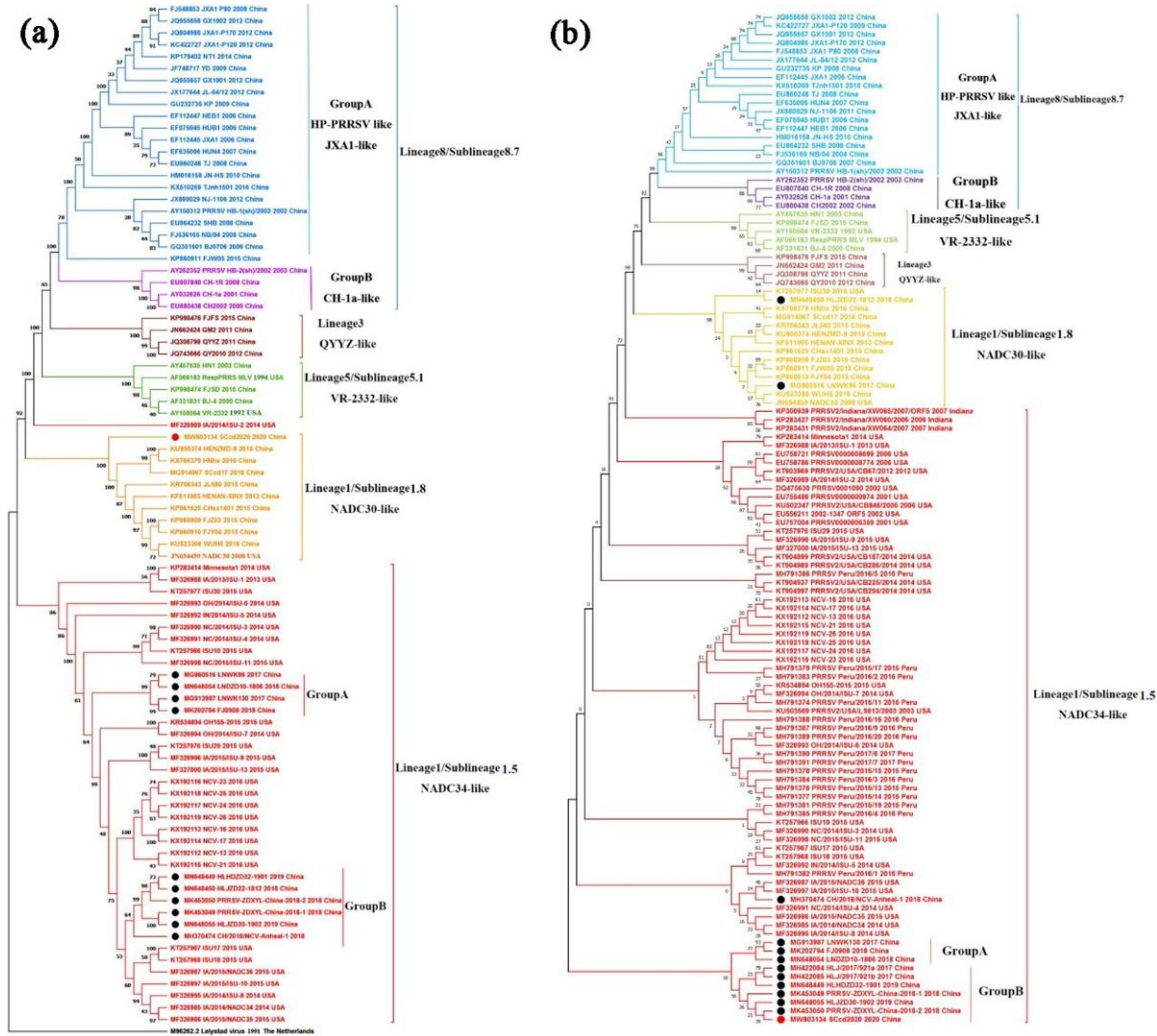

Figure 3

Phylogenetic trees based on full-length genomic (a) and ORF5 sequence (b) of SCcd2020 isolate with 86 whole genomic and 117 ORF5 PRRSV reference strains available in GenBank. The SCcd2020 isolate in this study is labeled with "red circle". The NADC34-like strains in China are labeled with "black circles". The phylogenetic tree is constructed by using the neighbor-joining method in MEGA-X software with bootstrap values calculated for 1,000 replicates. Numbers along branches are bootstrap values.
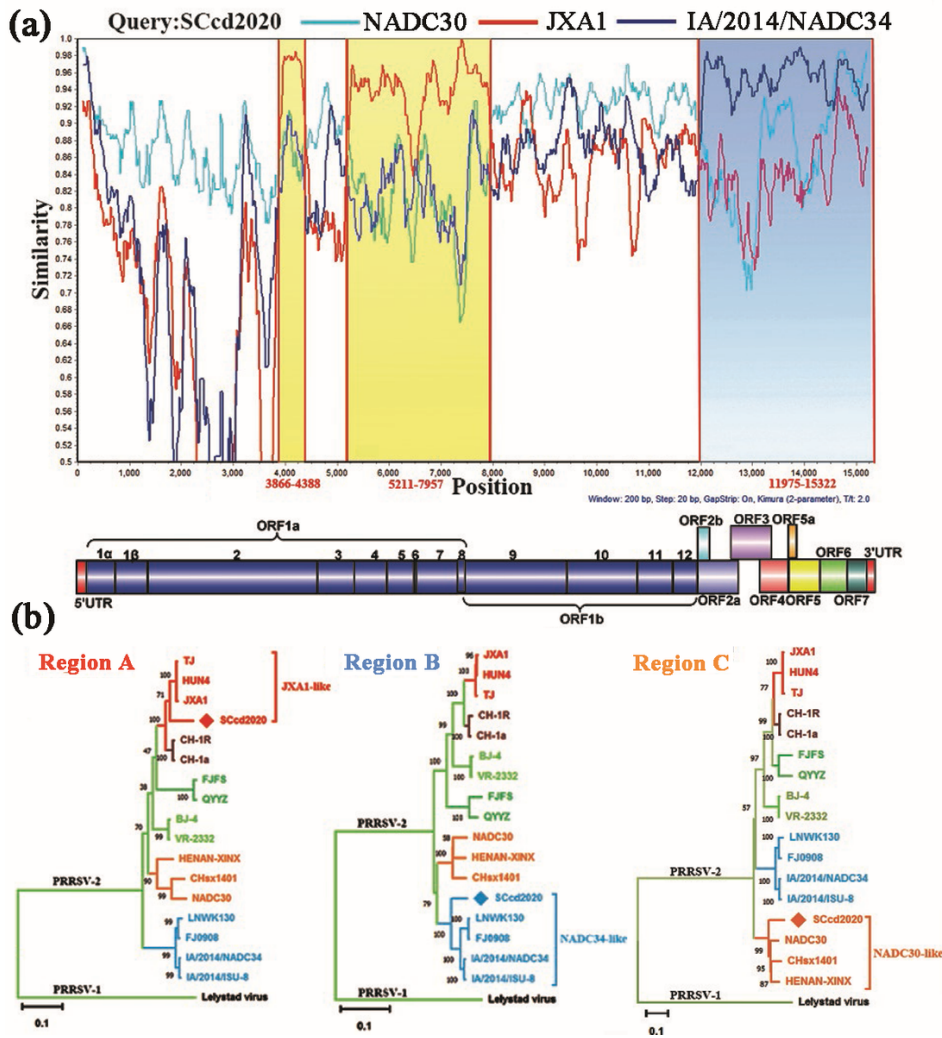
Figure 4

Genome recombination analysis of SCcd2020 isolate. The y-axis indicates the percentage similarity between the query sequence (SCcd2020) and three representative sequences. Genome scale similarity comparisons of SCcd2020 (query) with NADC30 (Cyan), JXA1 (red), and IA/2014/NADC34 (blue). The supposed recombination regions are shown with two yellow shadows and a blue shadow. The recombination breakpoints are marked at the bottom with nucleotide sites and viral genome structure referenced to VR-2332. Phylogenetic trees based on each recombinant fragment (Regions A–C) of SCcd2020 was constructed to confirm the accuracy of recombination events. Region A indicates the nucleotide sequences of yellow shadow, region B indicates the nucleotide sequences of blue shadow, and the region C indicates the nucleotide sequences of white regions. The SCcd2020 isolate in this study is labeled with "diamond".
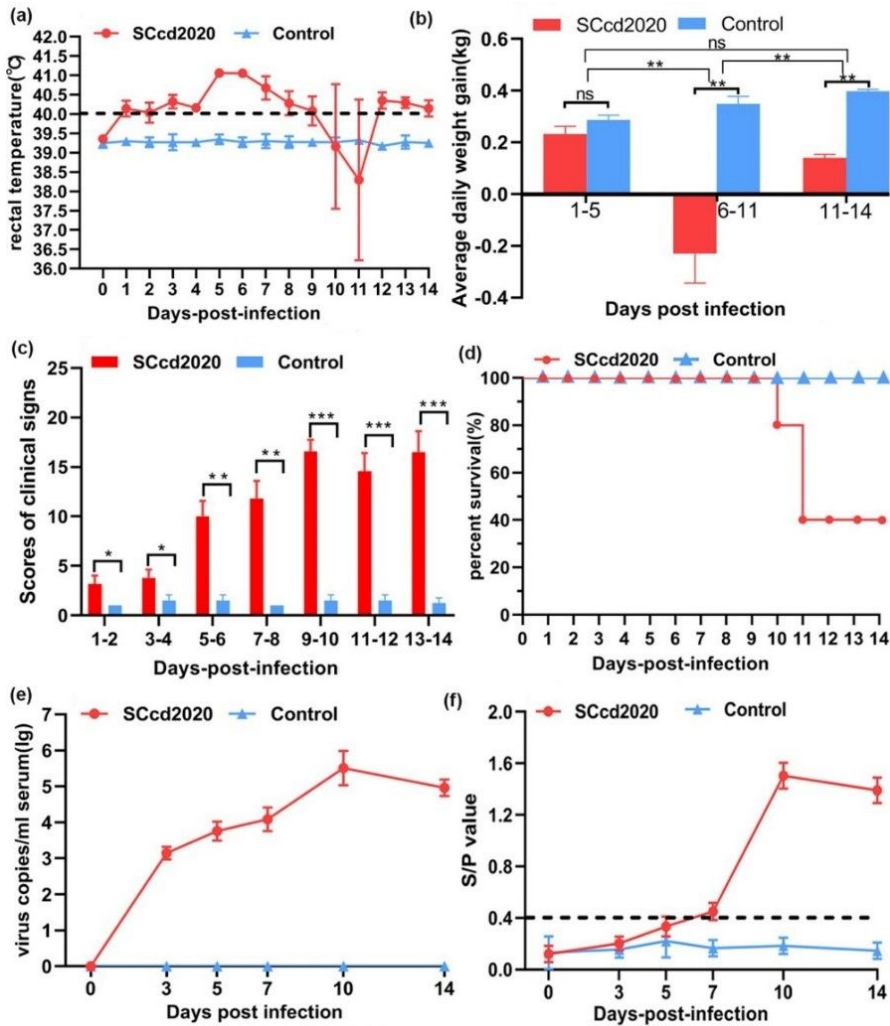
Figure 5

The rectal temperature, weight gain, clinical sign scores, survival rate, and viremia and antibody levels of pigs in the animal experiment. (a) Rectal temperatures of pigs inoculated with SCcd2020 and uninfected RPMI-1640 medium. The clinical fever cut-off value was set at 40.0 °C. (b) Average daily weight gain of the inoculated pigs during the challenge experiment. (c) The scores of clinical signs during every two days of the the challenge study. (d) The survival and mortality curves of the inoculated pigs. (e) The PRRSV copy numbers in serum of inoculated 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 SCcd2020- and RPMI-1640 medium-inoculated groups (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).
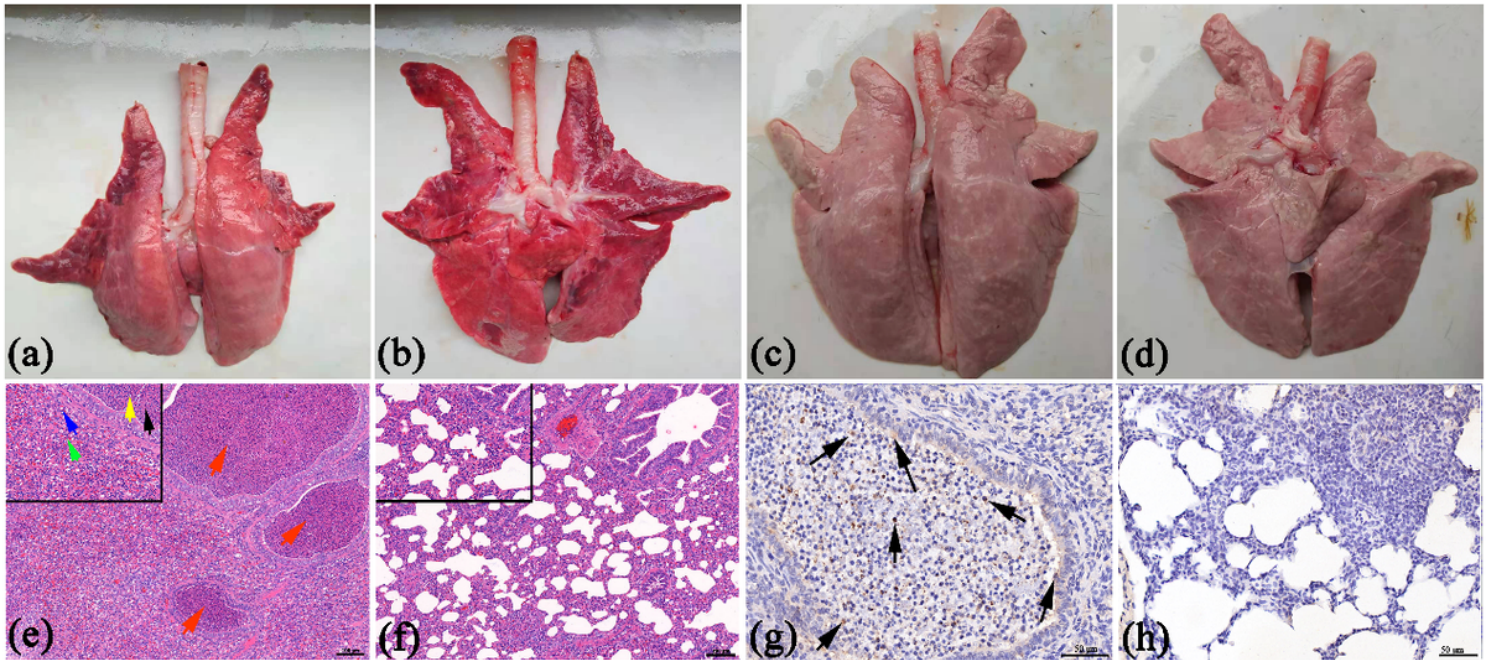
Figure 6

Gross and microscopic lung lesions observation of the inoculated piglets. (a, b) Severe hemorrhagic pneumonia in dorsal and ventral sides of lung with pulmonary consolidation and edema were observed in SCcd2020-inoculated pigs. (c, d) No gross lung changes were observed in the RPMI-1640-inoculated pigs. (e) Interstitial pneumonia characterized by marked thickening of the alveolar septa, hyperplasia and necrosis of alveolar epithelium, and severe inflammation characterized by infiltrating neutrophils and lymphocytes. Fibrous tissue hyperplasia and a small amount of red blood cells can be observed in the lung stroma. (f) No obvious microscopic pulmonary lesions were observed in the RPMI-1640-inoculated pigs. Original magnification, 200× and 400×. (g) The virus antigen was mainly distributed in a cytoplasm of macrophages in the bronchioles and within the alveolar wall cells. (h) No positive staining was detected in the control group. Original magnification, 400×.

Supplementary Files

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