Genetic characterization of a novel recombined porcine reproductive and respiratory syndrome virus 2 among Nadc30-like, Jxa1-like and TJ-like strains

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Funding information
The National Key Research and Development Program, Grant/Award Number: 2018YFD0500802: The Science and Technology Program of Sichuan province, Grant/Award Number: 2018JY0369; The Sichuan Veterinary Medicine and Drug Innovation Group of the China Agricultural Research System, Grant/Award Number: CARS-SVDIP

Abstract
Porcine reproductive and respiratory syndrome (PRRS) is one of the most economically devastating viral diseases in the global pig industry, including China. Recently, we successfully isolated a porcine reproductive and respiratory syndrome virus (PRRSV) from lung tissue and peripheral blood of piglets at a farm from Dujiangyan in Sichuan, China, and named it the DJY-19 strain. The full-length genome sequence of DJY-19 shared 86.8%–94.1% nucleotide similarity with NADC30-like and NADC30 PRRSV strains. We compared the open reading frame (ORF) 5 gene of DJY-19 with 34 PRRSV strains from Genbank. Phylogenetic analysis showed that DJY-19 clustered with NADC30 strains, characterized by a predicted 131-amino-acid deletion in the nonstructural protein (NSP) 2. The results of homology analysis showed that the homology between DJY-19 and NADC30 (JN654459.1) strains was the highest (95.9%), whereas homology with other domestic strains was lower (80.9%–92.6%). Furthermore, we identified four recombination breakpoints in the DJY-19 genome; they separated the DJY-19 genome into four regions. The 8106–9128 nucleotide (nt) region of DIY-19 was highly similar to the TJ strain, and the 12106–12580 nt region of DIY-19 was highly similar to the JXA1-R strain. Our findings demonstrate that DJY-19 arose from the recombination of North America NADC30 strain and TJ strain and JXA1-R in China. The application of multiple attenuated vaccine strains has led to complex recombination of PRRSV strains in China. This study provides a theoretical basis for making a more reasonable PRRS virus control and prevention strategy.

Keywords
genetic evolution analysis, new recombinant porcine reproductive and respiratory syndrome virus strain, reference basis
Porcine reproductive and respiratory syndrome (PRRS) is an immunosuppressive disease. It has been characterized by respiratory symptoms and disorders in piglets and reproductive failure in sows. The causative pathogen of PRRS is porcine reproductive and respiratory syndrome virus (PRRSV). It’s an enveloped, positive-stranded RNA virus of the Arteriviridae family (Zuo et al., 2015). It has strict host and histiocytic tropism and mainly infects monocyte lines of domestic and wild pigs, especially porcine alveolar macrophages (Baron et al., 1992). The full length of PRRSV genome is approximately 15 kilobases (kb). It contains a 5′-untranslated region (UTR) and at least 10 open reading frames (ORFs) and a 3′-UTR (Hanada et al., 2005). ORF1a and ORF1b encode at least 16 non-structural proteins (NSPs). These NSPs are involved in viral replication and transcription (Snijder et al., 2001). ORF2a, ORF2b, ORF5a and ORF3–7 encode eight viral structural proteins (Belák et al., 2002). GP5 is the main viral envelope protein. It is related to virus neutralization, adsorption and cell entry. PRRSV is divided into two genotypes, including type I and type II; the ORF5 gene (which codes GP5) determines the type (Chen et al., 2006). Although the occurrence over time, genome structure and clinical symptoms for both types is similar, their nucleotide (nt) similarity is only about 60% (Thiel et al., 1993). The type II virus is prevalent in China. The probability of virus mutation producing recombinant strains is likely to increase with the use of vaccines. Recently, a recombinant PRRSV strain was found in Iowa, USA. The strain had the same gene sequence as the vaccine strain (Wang et al., 2019). The phenomenon of PRRSV recombination was not only found in type II PRRSV strains, but also in type I strains (Marton et al., 2019). PRRSV field strains recombine with vaccine strains to escape the capture of neutralizing antibodies produced after vaccination, and these recombinant and mutated strains often have strong pathogenicity, such as RFLP 1-7-4 strain found in the USA and KU-N1202, a mutated recombinant strain produced after vaccination, and these recombinant and mutated strains are likely to increase with the use of vaccines. Recently, a recombinant PRRSV strain was found in Iowa, USA. The strain had the same gene sequence as the vaccine strain (Wang et al., 2019). The phenomenon of PRRSV recombination was not only found in type II PRRSV strains, but also in type I strains (Marton et al., 2019). PRRSV field strains recombine with vaccine strains to escape the capture of neutralizing antibodies produced after vaccination, and these recombinant and mutated strains often have strong pathogenicity, such as RFLP 1-7-4 strain found in the USA and KU-N1202, a mutated recombinant strain reconstituted with VR2332, found in the Korea (Kwon et al., 2019; van Geelen et al., 2017). This kind of reorganization is more obvious in China.

The China swine industry has been impaired over the last 20 years by the far-reaching financial losses caused by PRRS (Zhou & Yang, 2010). There was an outbreak of highly pathogenic PRRSV from 2007 to 2008, with high fever, high morbidity and high mortality, and a reemergence in 2009–2010 (Tian et al., 2007). This epidemic seriously impacted the Chinese swine industry. Commercial PRRS vaccines currently in clinical use in China include eight live attenuated vaccines and two inactivated vaccines. PRRS vaccines were once well-controlled with those vaccines. In 2011–2012, different subtypes and recombinant strains of PRRSV appeared in China. In 2013, Zhou Feng found variant strains HENAN-XINX (KF611905) and HENAN-HEB (KJ143621), both of which are highly homologous to NADC30, in Henan Province, and called them NADC30-like strains (Zhou et al., 2015). Since 2014, NADC30-like strains have broken out in a large area in China, and NADC30-like strains have gradually become the dominant strains in some areas. Because the parent strains of commercial vaccines are classical PRRSV and HP-PRRSV, the vaccines have a weak effect on the prevention and control of NADC30-like PRRS. As an RNA virus, PRRSV may utilize high rates of mutation as a strategy to escape host immune surveillance (Tian, 2017). In addition, abuse of the attenuated vaccine has led to a significant variation in PRRSV strains in China. The above problems make it difficult to prevent and control PRRS. To explore the genetic evolution of the complete gene sequences of PRRSV strains isolated in Sichuan, China, we obtained the complete gene sequences of a newly isolated PRRSV strain. Subsequently, we analysed its homology, genetic relationship and recombination events with reference strains.

2 | MATERIALS AND METHODS

2.1 | Sample collection and virus culture

The PRRSV-positive material came from a swine farm in Sichuan, China, which experienced symptoms of high fever and PRRS in 2019. We collected blood serum and lung tissue and used it to inoculate Meat Animal Research Center-145 cells (Marc-145 cells). We subcultured the virus for 10 generations. After freezing and thawing and centrifuging at 4,000 rpm for 15 min, the supernatant was collected and stored at −80°C.

2.2 | RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

We extracted RNA according to the manufacturer’s instructions from the isolated strain supernatant using the Viral RNA Extraction Kit (Takara). We designed the primers online according to the sequences of the complete gene sequence of PRRSV logged by Genbank (the relevant primer information is shown in the supplementary file). We generated complementary DNA (cDNA) following the supplier’s instructions using Superscript III reverse transcriptase (Invitrogen, Waltham, MA, USA). We then used the cDNA in PCR amplification using twelve primers (A-L) specific for the viral genes as listed in Table S1. The reaction conditions were as follows: 95°C for 5 min; 30 cycles of 95°C for 30 s, 58°C for 30 s 72°C for 1 min; and a final extension at 72°C for 7 min. Furthermore, the products were Sanger sequenced in both directions by Sangon Biotech (Shanghai) Co., Ltd.

2.3 | Genetic evolution analysis

We compared the PRRSV DJY-19 (Accession No.MT075480) complete gene sequences and Nsp2 gene sequences and ORF5 gene sequences with the gene sequences from different regions in China and abroad (see Table S2). The genetic and evolutionary relationships
analysis was done using DNAStar (version 7.1) and MEGA software (version 6.06).

2.4 | Recombination analyses

The sequences of the PRRSV were identified in this study and 26 isolated sequences from Genbank for recombination screening. RDP v4.8 was used to predict the recombination sites of sequences. The recombination events have only been considered when supported by at least three of RDP, GENECONV, MAXCHI, CHIMAERA and 3SEQ methods. Then we used SIMPLOT v3.5.1 to check the recombination signals and estimate the breakpoint locations. Furthermore, the recombination events were confirmed with a neighbour-joining phylogenetic tree.

3 | RESULTS AND DISCUSSION

We named the isolated PRRSV strain DJY-19. The homology of the complete gene sequences with other strains was between 58.6% and 94.1% (Figure 1a). The ORF5 gene sequences of 34 PRRSV reference strains were obtained from the GenBank for a homology comparison and genetic evolutionary tree analysis. The results showed that the homology of the ORF5 gene with NADC30-like strains was between 84.4% and 95.9%. The ORF5 gene of DJY-19 was in the same branch as the ORF5 gene of the foreign NADC30 strain (JN654459) from the USA (95.9% homology). It was more distantly related (84.4% homology) to the TJnh1501(KX510269) strain (see Figure 1c and Table 1). Based on the results of the phylogenetic tree, the DJY-19 strain was in the same lineage as the NADC30 strain.

We used the Nsp2 gene sequences and complete gene sequences of 24 PRRSV reference strains obtained from NCBI to perform a homology comparison and genetic evolutionary tree analysis. There was a 131-amino-acid discontinuous deletion from Nsp2 (Figure 2). The homology of the Nsp2 gene with other strains was between 50.4% (LV-Netherlands-1993-M96262) and 94.2%. The Nsp2 gene of DJY-19 was in the same branch of the foreign NADC30 strain from the USA (94.2% homology). But it was more distantly related (50.4% homology) to the LV strain (Figure 1b). The homology of the Nsp2 gene with NADC30-like strains was between 85.5% and 94.2% (Table 1).

We detected all recombination events in RDP and confirmed them by SIMPLOT and phylogenetic trees. We identified two recombination breakpoints in the sequence alignment (Figure 3a). The RDP4 graph indicated that the DJY-19 was highly similar to that of TJ in the 8106–9128 nt region. This stretch contains partial Nsp9-coding regions. We also found that DJY-19 was highly familiar with JX1-R strain in the 12106–12580 nt region, which contains partial Nsp12-coding regions. The phylogenetic tree showed that the position 8106–9128 nt of DJY-19 clustered with TJ strain (Figure 3b), but the 1–8105 and 9129–15021 nt regions with reference to the NADC30 strain (Figure 3c). The DJY-19 clustered with JX1-R at position 12106–12580 nt (Figure 3d), but the 1–12105 and 12580–15021 regions with reference to the NADC30 strain (Figure 3e). In addition, we found out by asking that this farm introduced 1,000 new piglets in April 2019, and the PRRSV vaccine used for swine immunization is a TJM attenuated vaccine strain. This evidence further supports the possible recombination between the attenuated vaccine and NADC30-like PRRSV.

In 2013, two PRRSV NADC30-like strains, HENAN-XINX (KF611905) and HENAN-HEB (KJ143621), were isolated from Henan province, China. HENAN-XINX recombined with classical PRRSV, including VR-2332, HENAN-HEB recombined with HP-PRRSV, including JX1. In 2014, JL580 (KR706343) was isolated from Jilin province, China; it resulted from recombination with NADC30-like PRRSV.
| Genomic region | CH-1R        | HuN4        | JXA1-R       | NADC30       | Scnj16       | SDSU73       | TJ           | Tjih1501     | VR2332       |
|----------------|--------------|-------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|
| Complete genome nt aa | 83.1 83 | 76.3 61.3 | 83.1 83 | 94.1 86.5 | 86.5 83.2 | 83 86.8 | 84.2 69.7 | 87.6 77.5 | 82.8 62.8 |
| ORF1α nt aa | 76.3 61.3 | 87.6 61.3 | 76.4 61.9 | 86.7 61.2 | 76.6 61.2 | 87.2 95 | 89.4 94.4 | 89.4 94.4 | 89.4 94.4 |
| NSP1α nt aa | 94.1 86.7 | 95.9 86.7 | 86.9 91.1 | 89.4 94.4 | 87.2 95 | 87.6 94.4 | 89.4 94.4 | 89.4 94.4 | 89.4 94.4 |
| NSP1β nt aa | 78.2 73.9 | 78.2 73.9 | 92 90.1 | 77 74.4 | 77.8 71.9 | 78.5 74.4 | 78.3 74.4 | 80 75.9 | 78.3 74.4 |
| NSP2 nt aa | 59.5 89.5 | 60.9 89.5 | 61.2 89.5 | 85.7 91.1 | 89.4 94.4 | 89.4 94.4 | 89.4 94.4 | 89.4 94.4 | 89.4 94.4 |
| NSP3 nt aa | 82.8 91.1 | 83.5 91.1 | 94.9 97.1 | 82.5 90.7 | 84.5 92.6 | 83.3 91.7 | 83.7 91.7 | 84.2 91.7 | 84.2 91.7 |
| NSP4 nt aa | 94.9 97.1 | 94.9 97.1 | 82.5 90.7 | 84.5 92.6 | 83.3 91.7 | 83.7 91.7 | 84.2 91.7 | 84.2 91.7 | 84.2 91.7 |
| NSP5 nt aa | 88.5 91.1 | 88.5 91.1 | 94.4 97.1 | 82.5 90.7 | 84.5 92.6 | 83.3 91.7 | 83.7 91.7 | 84.2 91.7 | 84.2 91.7 |
| NSP6 nt aa | 94.9 97.1 | 94.9 97.1 | 82.5 90.7 | 84.5 92.6 | 83.3 91.7 | 83.7 91.7 | 84.2 91.7 | 84.2 91.7 | 84.2 91.7 |
| NSP7 nt aa | 88.5 91.1 | 88.5 91.1 | 94.4 97.1 | 82.5 90.7 | 84.5 92.6 | 83.3 91.7 | 83.7 91.7 | 84.2 91.7 | 84.2 91.7 |
| NSP8 nt aa | 88.5 91.1 | 88.5 91.1 | 94.4 97.1 | 82.5 90.7 | 84.5 92.6 | 83.3 91.7 | 83.7 91.7 | 84.2 91.7 | 84.2 91.7 |
| NSP9 nt aa | 94.9 97.1 | 94.9 97.1 | 82.5 90.7 | 84.5 92.6 | 83.3 91.7 | 83.7 91.7 | 84.2 91.7 | 84.2 91.7 | 84.2 91.7 |
| NSP10 nt aa | 94.9 97.1 | 94.9 97.1 | 82.5 90.7 | 84.5 92.6 | 83.3 91.7 | 83.7 91.7 | 84.2 91.7 | 84.2 91.7 | 84.2 91.7 |
| NSP11 nt aa | 94.9 97.1 | 94.9 97.1 | 82.5 90.7 | 84.5 92.6 | 83.3 91.7 | 83.7 91.7 | 84.2 91.7 | 84.2 91.7 | 84.2 91.7 |
| NSP12 nt aa | 94.9 97.1 | 94.9 97.1 | 82.5 90.7 | 84.5 92.6 | 83.3 91.7 | 83.7 91.7 | 84.2 91.7 | 84.2 91.7 | 84.2 91.7 |
| GP2 nt aa | 88.2 89.1 | 88.2 89.1 | 88.6 89.1 | 92.1 90.3 | 87.9 88.3 | 88.8 89.1 | 88.7 89.1 | 87.7 87.5 | 87.7 87.5 |
| GP3 nt aa | 81.2 80.8 | 81.2 80.8 | 81.2 80.8 | 81.2 80.8 | 81.2 80.8 | 81.2 80.8 | 81.2 80.8 | 81.2 80.8 | 81.2 80.8 |
| GP4 nt aa | 88.2 87.9 | 88.2 87.9 | 88.7 87.7 | 91.8 88.3 | 87.7 87.2 | 85.5 87.7 | 85.7 87.7 | 87.9 87.2 | 87.9 87.2 |
| GP5 nt aa | 85.4 84.6 | 85.4 84.6 | 85.4 84.6 | 85.4 84.6 | 85.4 84.6 | 85.4 84.6 | 85.4 84.6 | 85.4 84.6 | 85.4 84.6 |
| M nt aa | 87.2 87.7 | 87.2 87.7 | 87.2 87.7 | 87.2 87.7 | 87.2 87.7 | 87.2 87.7 | 87.2 87.7 | 87.2 87.7 | 87.2 87.7 |
| N nt aa | 89.5 89.5 | 89.5 89.5 | 89.5 89.5 | 89.5 89.5 | 89.5 89.5 | 89.5 89.5 | 89.5 89.5 | 89.5 89.5 | 89.5 89.5 |
| 8106–9128 nt nt aa | 95.8 97.6 | 95.8 97.6 | 97.6 97.6 | 97.6 97.6 | 97.6 97.6 | 97.6 97.6 | 97.6 97.6 | 97.6 97.6 | 97.6 97.6 |
| 12106–12580 nt nt aa | 96.1 98.9 | 96.1 98.9 | 98.9 98.9 | 98.9 98.9 | 98.9 98.9 | 98.9 98.9 | 98.9 98.9 | 98.9 98.9 | 98.9 98.9 |
with HP-PRRSV, such as 09NEN1. The Chsx1401 (KP861625) and HNyc15 (KT945018) strains reported in 2015 recombined
with VR-2332/CH1a. Researchers performed an epidemiological
survey on PRRSV in 16 provinces of China. Recombination anal-
yses showed that nine of 28 isolates and one isolate from other
laboratories were potentially complicated recombinants between
the vaccine JXA1-R-like strains and the predominant circulating
strains (Zhao et al., 2007). FJXS15 (KX758250) reported in 2016
recombined with JXA1-R. XJzx1-2015 (KX689233) was reported
in 2016 recombined with QYYZ (JQ308798). In 2018, Zhou et al.
reported that five NADC30 PRRSV recombinant strains (SCnj16,
SCcd16, SCn17, SCcd17 and SCya17) were isolated from Sichuan
in China, and all of them recombined with JXA1-like, VR-2332-like
and QYYZ-like in single or multiple fragments. The results showed
that the variation in PRRSV prevalent in Sichuan was significant
(Zhou, Kang, et al., 2017; Zhou, Yang, et al., 2017; Zhou, Kang,
Xie, et al., 2018). The hotspots for recombination of NADC30-like PRRSV with other virus strains
occurred in both non-structural protein regions, including nsp1a,
nsp2–9 and nsp11, and structural protein regions, such as ORF2-7
(Zhao et al., 2007). In this study, the NADC30-like PRRSV DJY-19
strain was isolated from a vaccinated pig farm in the Sichuan prov-
ince. Recombination analysis showed that the DJY-19 strain was
a recombinant PRRSV. Furthermore, the DJY-19 8106–9128 nt
gene fragment was closely related to TJ PRRSV, and 12106–12580
nt gene fragment was closely related to JXA1-R PRRSV. The re-
remaining fragments were similar to NADC30 PRRSV. We found that the recombinant segment (nt 8106–9128)
of DJY-19 displayed high homology with JXA1-R, TJ, HuN4 and TJnh1501
(approximately 98.9%). These data indicate that the DJY-19 is a re-
combinant MLV-evolved virus. Therefore, the efficacy and safety of HP-PRRSV MLV vaccines deserve attention. Sichuan is a big pig
raising Province in China. PRRSV MLV vaccines were widely used
and there were abuse cases of MLV. The recombination of the
PRRSV has caused great pressure to prevent and control PRRSV
at the present time.

At present, many scholars have done a lot of research on tradi-
tional PRRSV MLV vaccines against recombinant wild strains. Due to
immune evasion strategies and the antigenic heterogeneity of the
virus, current commercial PRRSV vaccines (killed-virus and modi-
fied-live vaccines) are of unsatisfactory efficacy, especially against
heterologous infection (Hu & Zhang, 2014; Pileri & Mateu, 2016).
The protective effect of the vaccine on the field strains was evalua-
ted by the indexes of viraemia, time of high fever and virus elimina-
tion. Previous studies have shown that the autogenous inactivated
PRRSV vaccines can enhance the neutralization antibody level in
pigs after immunizing pigs with the same type of PRRSV commer-
cial vaccines (Geldhof et al., 2013). Piglets were vaccinated with
typeI PRRSV MLV vaccine and challenged with typeII field PRRSV
strain. The results showed that non-neutralizing antibodies induced
by vaccination prior to challenge might play a key role in protecting
against field PRRSV infection, especially in the early time course (Ko
et al., 2016). The duration of viraemia and fever was reduced after
the pigs immunized with typeII PRRSV MLV vaccines and infected
non-mutated typeII field PRRSV (Lager et al., 2014). The protective
effect of commercial vaccines on the mutant field PRRSV strains
was very poor. The results show that some commercial MLV vac-
cines (JXA1-R, TJM-F92, VR2332 and R98) can only provide partial
protective efficacy in the vaccinated piglets upon virus (HNjz15,
CHsx1401, FJ203, FJ1402) challenges (Lager et al., 2014; Zhou, Kang, et al., 2017). In summary, the emergence of new PRRSV recombinant strains exerts great pressure on the prevention and control of PRRSV.

**FIGURE 3** SimPlots for all putative recombinants analysed in this study. (a) We calculated recombination analysis with Simplot 3.5.1 software. We used the complete DJY-19 genome as the query sequence. Recombination breakpoints are shown with black dotted lines, and the locations are shown at the bottom. (b) Phylogenetic analysis based on the 8106–9128 nucleotide (nt) region. (c) Phylogenetic analysis based on the 1–8105 and 9129–15021 nt regions. (d) Phylogenetic analysis based on the 12106–12580 nt region. (e) Phylogenetic analysis based on the 1–12105 and 12580–15021 nt regions.
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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.