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Genomic analysis of a recombinant NADC30-like porcine reproductive and respiratory syndrome virus in china

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Abstract Recently, NADC30-like porcine reproductive and respiratory syndrome viruses (PRRSVs), which are genetically similar to the NADC30 strain isolated in the United States of America in 2008, have become prevalent in China. Here, a novel variant PRRSV strain named HNhx was successfully isolated on porcine alveolar macrophages from Henan province and the full-length genome sequence was determined. Phylogenetic analysis indicated that HNhx strain was classified into the NADC30-like PRRSV subgroup, in which all the strains had the unique discontinuous 131-amino acid deletion relative to that of the nonstructural protein 2 (Nsp2) of the VR2332 strain. Genetically, HNhx shared 92.9% nucleotide similarity to NADC30. Furthermore, HNhx strain contained extensive amino acid mutations in GP5. In particular, the S32H, N33D, D34N, and S36G variations resulted in that HNhx lost all the putative N-linked glycosylation sites at amino acid positions 30, 32, 33, 34, and 35. Recombination analysis revealed that HNhx was the result of recombination between the NADC30 strain and the highly pathogenic PRRSV vaccine strain circulating in China in Nsp4 (nt 5261) to Nsp9 (nt 7911). The novel genome data of HNhx will be helpful for understanding the evolution and epidemiology of PRRSV in China.

Keywords Porcine reproductive and respiratory syndrome virus · NADC30-like · Variation analysis · Recombination

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Introduction

Porcine reproductive and respiratory syndrome (PRRS) has become an epidemic disease in the global swine industry and caused enormous economic losses since its initial emergence in the United States in the late 1980s [16, 25, 32]. As the causative agent, PRRS virus (PRRSV) is a positive sense, single-stranded RNA virus. The PRRSV genome is about 15.5 kb in length and contains at least 10 open reading frames (ORFs) [5, 8, 9, 17, 26]. The first two ORFs (ORF1a and ORF1b) encode virus nonstructural proteins (Nsps), including Nsp1α, Nsp1β, Nsp2-6, Nsp7α, Nsp7β, and Nsp8-12. ORFs 2a-4 encode three membrane-associated glycoproteins: GP2, GP3, GP4, and the unglycosylated membrane protein E. ORFs 5-7 encode three major structural proteins: the envelope glycoprotein GP5, the unglycosylated membrane protein M, and the nucleocapsid protein N, respectively [7, 18, 19, 33].

Based on their genetic and antigenic characteristics, PRRSVs have been identified as two major genotypes referred to as European type (PRRSV1) and North American

type (PRRSV2) [2, 10, 21]. The representative prototypes are Lelystad virus (LV) and VR-2332, respectively [21, 32]. There are great differences in genomic nucleotide sequences and amino acid sequences of ORF between PRRSV1 and PRRSV2. In 2006, an atypical PRRS outbreak, characterized by continuous high fever, high morbidity, and high mortality in pigs of all ages, emerged in China and resulted in enormous production losses. The causative agent has been confirmed to be a highly pathogenic PRRSV variant (HP-PRRSV) with a unique 30-amino acid (30-aa) deletion in Nsp2 [29]. But it has been reported that this deletion is not related to the virulence [38]. Since then, the HP-PRRSVs gradually became the dominant strains circulating in the field. However, since 2012, novel NADC30-like PRRSV strains which showed 92.6–97.06% nucleotide similarity with NADC30 have been identified in several provinces in China [13, 15, 36, 37]. Genomic analysis showed that all the NADC30-like PRRSV isolates share a unique discontinuous deletion of 131 amino acid (131-aa) in Nsp2-coding region, which could be used as an epidemiologically genetic marker for the novel PRRSVs in China.

PRRSV is characteristic of genetically extensive variation containing the mutation and recombination. It has been described that genetic exchange exists among PRRSV strains circulating in the field [24]. In addition, although an overwhelming majority of the circulating strains were PRRSV2 strains in China, the emergence of PRRSV1 strains has been described previously [12, 13]. Thus, PRRSVs appeared to be continually expanding and evolving, leading to the reemergence of PRRSV, such as the recently emerged NADC30-like PRRSV strains [37]. The parental PRRSV strain (NADC30) was isolated in 2008 from Iowa herds experiencing outbreaks of respiratory disease and exhibited lower pathogenesis than HP-PRRSV, but similar to that of MN184 in vivo [4, 27]. Previous research data reported that some of NADC30-like PRRSV strains had undergone recombination with HP-PRRSV (JL580, HENAN-HEB), CH-1a (HNyc15), or VR2332 (HENAN-XINX) (more analysis later) [13, 15, 35, 36]. On the one hand, the combination further increased the complexity of PRRSV epidemiology. On the other hand, recombination may be associated with pathogenicity of different PRRSV strains. This will be discussed in more detail later.

In this study, we reported complete genome sequence of PRRSV strain HNhx isolated from one pig farm with clinical outbreak of PRRS in China and analyzed its genetic evolution relationship with other strains. Recombination analysis revealed that HNhx is the result of recombination between the NADC30 strain and the HP-PRRSV vaccine strain JXA1-P80 circulating in China in Nsp4 (nt 5261) to Nsp9 (nt 7911). Our study identifies a recombination event between NADC30 and PRRSV vaccine strains, which suggesting that NADC30-like PRRSV strains are prone to recombination with not only wild PRRSV strains, but also PRRSV vaccine strains. Collectively, this study revealed the genomic characteristics of the HNhx strain, and would provide valuable knowledge about recombination in PRRSV variation and evolution.

Materials and methods

The HNhx strain was isolated using porcine pulmonary alveolar macrophages from the lung tissues of diseased pigs collected from one pig farm that were experiencing high fever, reproductive and respiratory syndrome in Henan, China. It is further confirmed by immunofluorescence assay (IFA) using SDOW-17 (a monoclonal antibody against PRRSV N protein, Rural Technologies, Brookings, SD, USA) [22]. Full-length HNhx genome sequence was obtained by amplifying 6 fragments covered the full-length genome using reverse transcription-PCR (RT-PCR) with 6 special primer pairs (Table 1). The PCR products were purified using the Universal DNA Purification Kit (Tiangen, Beijing, China). A-tail was added at the 3′ end in accordance with the instructions of DNA A-Tailing Kit (TaKaRa, Dalian, China). Then, the products were subcloned into pMD®19-T Simple Vector (TaKaRa) and subsequently sequenced at Sangon Biotech, China. The 5′UTR and 3′UTR regions were obtained by rapid amplification of cDNA ends (RACE) following the manufacturer's protocol of the SMARTer® RACE 5′/3′Kit (Clontech Laboratories, Mountain View, CA, USA). The full-length genome sequence of HNhx was assembled using the DNAStar (Lasergene) software and was deposited in GenBank database under accession number KX766379. Genomic analyses were performed with the DNAMAN and DNAStar software. Phylogenetic trees were constructed

Table 1 Primers used in this paper

Primer	Sequence	Product size (bp)
N1	F-GCACTGCTTTACGGTCT	
	R-ACGCATCACATTGAGGTA	2784
N2	F-TACTCAGCTCAAGCCATCATT	
	R-CACAACACCACACGCAATC	3384
N3	F-TACTTATGCCTTCCTGCCTCG	
	R-CCATTCCAACCACAAGTTCTTCA	2970
N4	F-TTGTGCTGTATGCCGAAT	
	R-GAGAAGGTCAGAGGAATGC	4312
N5	F-CAGTGTCTATGCTTGGTT	
	R-CAGAGGAGAGCGACTAC	1612
N6	F-CACCTACACGCCAATAATG	
	R-GAGGCACAATATCAATCAGT	893
5′RACE	ACCGATCAAGAATCCCAGAC	251
3′RACE	CGCTGGAACTTGTGCCCTGT	326

using the distance-based Neighbor-Joining method in the MEGA6. Seven recombination detection methods (RDP, GENECONV, BOOTSCAN, MaxChi, Chimaera, 3Seq, and SISCAN) implemented in the Recombination Detection Program v.4.51 (RDP4) were used for identification of recombinant sequences and breakpoints. The potential recombination events would be further confirmed through similarity comparisons within a 500-bp window sliding along the genome alignment (20-bp step size) by SimPlot v3.5.1.

Results

Genome characterization of HNhx strain

The complete genomic sequence of HNhx was 15,019 nucleotides (nt) in length exclusive of the poly (A) tail. With the 5′ untranslated region (UTR) of 190 nt and 3′ UTR of 151 nt locating in the head and tail, the genome may contain 10 overlapping open reading frames distributed in the center area of genome, including ORF1a (7119 nt, 191–7309), ORF1b (4383 nt, 7297–11,679), ORF2a (11,681–12,451), ORF2b (222 nt, 11,686–11,907), ORF3 (765 nt, 12,304–13,068), ORF4 (537 nt, 12,849–13,385), ORF5a (141 nt, 13,386–13,526), ORF5 (603 nt, 13,396–13,998), ORF6 (525 nt, 13,983–14,507), and ORF7 (372 nt, 14,497–14,868).

Homology analysis

Comparative analysis showed that HNhx exhibited higher nucleotide identity (92.9%) with NADC30 strain than that with other representative strains, such as VR2332, CH-1a, HB-1(sh)/2002, JXA1 and JXA1 P80 (81.7–83.4%) (Supplementary Table 1). Similarly, Nsp1α, Nsp1β, Nsp2, Nsp9-12, and ORF3-7 all shared higher identity with NADC30 (92.7–96.8%). Nsp3 shared higher (91.6%) identity with JL580 (NADC30-like PRRSV) and ORF2 shared higher (94.4%) identity with HNjz15 ((NADC30-like PRRSV). But some Nsps including Nsp4-6, Nsp7α, Nsp7β, and Nsp8 shared higher similarity with JXA1 P80 (HP-PRRSV), and the identities were 98.0, 97.1, 97.9, 95.5, 93.9, and 96.4%, respectively (Supplementary Table 1).

Additional comparative analyses of viral aa sequences of HNhx with NADC30, VR2332, CH-1a, HB-1(sh)/2002, JXA1, and JXA1 P80 were also performed. Similar results were observed. Nsp1α, Nsp1β, Nsp2, Nsp9-12, and ORF3-7 of HNhx exhibited higher similarity with NADC30-like strains (NADC30 or JL580) than with other strains prevailing in China. Nsp4-6, Nsp7α, Nsp7β, and Nsp8 exhibited higher similarity with JXA1 or JXA1 P80.

Considering the above analysis, we speculated that HNhx strain had undergone genetic exchange with Chinese HP-PRRSV strains. Furthermore, pairwise comparisons showed that all the NADC30-like PRRSV strains isolated in China shared 88.2–95.6% complete nucleotide sequence similarity with each other, and their nucleotide similarities with NADC30 ranged from 92.2 to 97.0% (data not shown).

Phylogenetic analysis

To investigate the phylogenetic relationship of HNhx PRRSV strain with the previous PRRSV strains (Table 2), a phylogenetic tree was constructed by the

Table 2 Representative strains used in this study

Reference strains	Accession no	Origin	Reference strains	Accession no	Origin
VR-2332	AY150564.1	USA, 1995	HENXX-1	KU950372.1	China, 2014
CH-1a	AY032626.1	China, 1996	CHsx1401	KP861625.1	China, 2014
HB-1(sh)/2002	AY150312.1	China, 2002	WUH5	KU523366.1	China, 2015
RespPRRS MLV	AF066183.4	USA, 2005	WUH6	KU523367.1	China, 2015
BJ-4	AF331831.1	China, 2001	HENXC-4	KU950371.1	China, 2015
JXA1	EF112445.1	China, 2007	HNjz15	KT945017.1	China, 2015
HuN4	EU213123.1	China, 2009	HENZMD-9	KU950374.1	China, 2015
FJZ03	KP860909.1	China, 2015	JL580	KR706343.1	China, 2013
NADC30	JN654459.1	USA, 2008	HNyc15	KT945018.1	China, 2015
MN184A	DQ176019.1	USA, 2006	HENAN-XINX	KF611905.1	China, 2013
CH-1R	EU807840.1	China, 2008	HENAN-HEB	KJ143621.1	China, 2012
XW015	KF724409.1	USA, 2013	FJY04	KP860910.1	China, 2015
FJ1402	KX169191.1	China, 2014	FJW05	KP860911.1	China, 2015
JXA1 P80	FJ548853.1	China, 2008	15HEN1	KX815413.1	China, 2015
15JX1	KX815419.1	China, 2015	15SC3	KX815428.1	China, 2015
15LN3	KX815425.1	China, 2015	FJWQ16	KX758249.1	China, 2016
FJXS15	KX758250.1	China, 2015			

neighbor-joining method based on the complete nucleotide sequences (Fig. 1). Our results revealed that all of the PRRSV strains were divided into four main clusters (Cluster 1–4). Except that one recombinant strain, JL580 was independent of one branch, all the other NADC30-like PRRSVs which had been reported in China belonged to two different clusters (Cluster 1–2). And the HNhx strain was grouped into Cluster 2, together with the HENZMD-9 strain. Moreover, HNhx, JL580, and HENZMD-9 strains had more distant phylogenetic relationship with the United States isolate NADC30 strain than the other NADC30-like PRRSV isolates within Cluster 1.

Variation analyses

As the target region for the evolution and molecular epidemiology research on PRRSV, Nsp2 displays substantial genetic variations in PRRSV strains. The Nsp2-coding region of HNhx had only 68.0, 67.5, 69.8% nucleotide similarity with JXA1, CH-1a, and VR2332, respectively. But it showed moderate nucleotide similarity with NADC30 (up to 92.7%), much higher than that of with JXA1, CH-1a, and VR2332. Amino acid alignment of Nsp2 of HNhx with those of other strains showed that HNhx strain exhibited a 111-aa deletion at aa positions 323–433, 1-aa deletion at aa position 483 and 19-aa deletion at aa positions 504–522 in Nsp2 compared with VR2332, which were identical to NADC30 and MN184A. The identical deletions in Nsp2 indicated

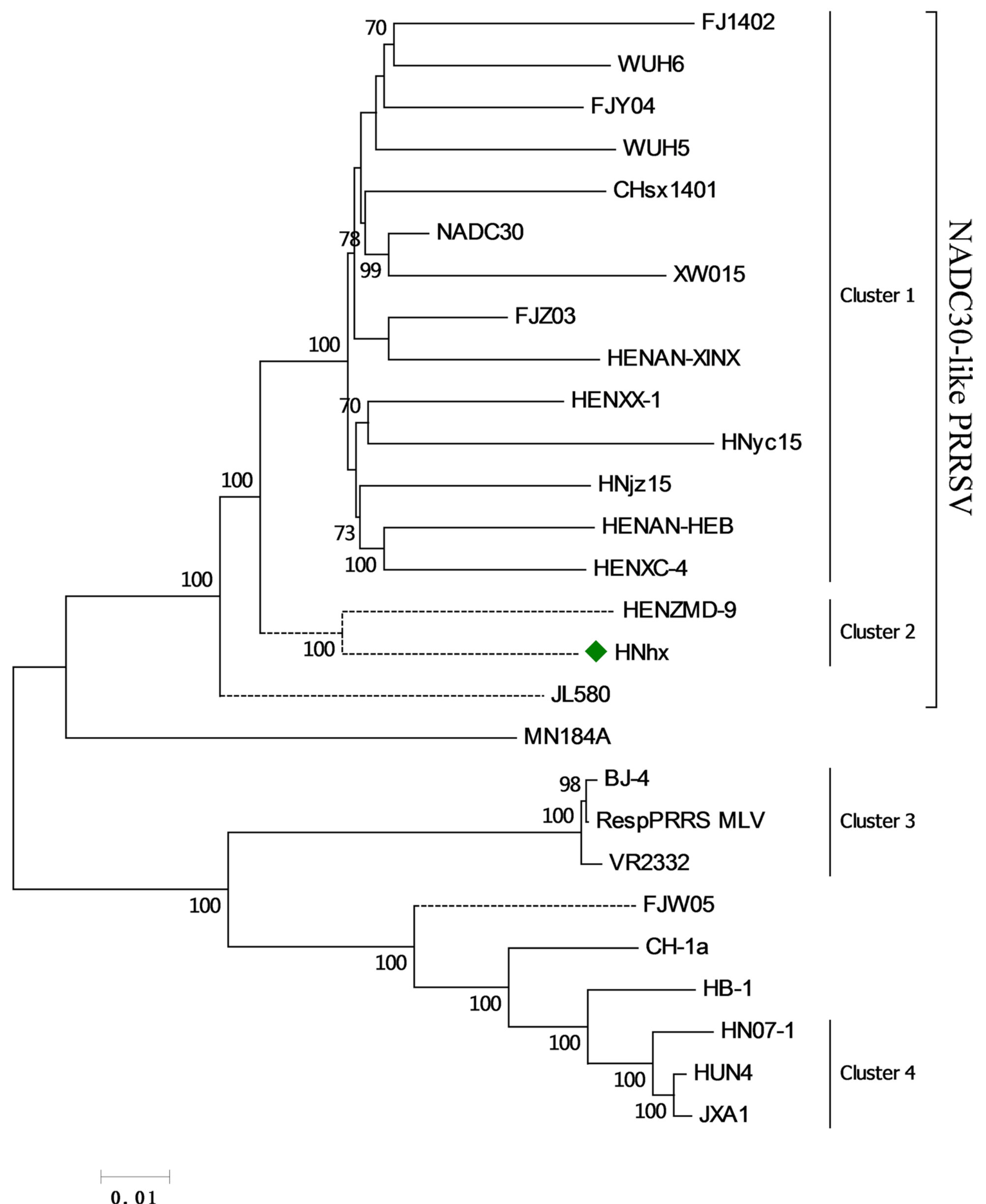

Fig. 1 Phylogenetic analysis based on the full-length genome sequences of HNhx isolate in this study and PRRSV reference strains available in GenBank. This analysis did not take into account that recombination which possibly affected the relationships shown. Filled rhombus indicates the HNhx strain from this study; the dotted lines indicate the recombinant NADC30-like PRRSV strains reported in China

that HNhx strain was closely related to the NADC30-like PRRSVs isolated in China, including JL580 and HENAN-HEB. In addition, the novel aa substitutions were found in Nsp2 of the HNhx, compared with other PRRSV strains, as listed in Table 3.

Due to the characteristic of high sequence variation and its presumed immunological significance in PRRSV infection, GP5 has always been used for analysis of genetic diversity of PRRSV. Compared with VR2332, CH-1a, HB-1(sh)/2002, JXA1, and NADC30, the amino acid identity of deduced GP5 of HNhx were 83.5, 84.5, 84.0, 83.0, and 92.5%, respectively. These results further showed that HNhx strain is highly similar to NADC30 strain. Extensive amino acid substitutions in the GP5 of HNhx PRRSV strain were found by multiple alignments, particularly in the two hypervariable regions (HVR) at aa positions 30–36 and 54–61 (Fig. 2). The S32H, N33D, D34N, and S36G substitutions in HVR1 would be resulted in HNhx strain losing all the potential N-glycosylation sites (identified by the sequence NXS/T, where X is any amino acid except proline) at the region aa 30–35 (Fig. 2, Table 4). Thus, there were only two putative N-glycosylation sites at aa 44 and 51 in GP5 of HNhx, while there are at least three putative N-glycosylation sites in GP5 of all other strains (Fig. 2, Table 4). Almost all the NADC30-like isolates (except WUH5 at aa 151) had two substitutions of R^{13} to Q^{13} and R^{151} to K^{151}. Moreover, HNhx had a unique mutation of V^{170} to A^{170}.

Recombination analysis

To determine possible recombinant events within HNhx strain, we performed recombinant detection using RDP4 software based on a set of complete genome sequences of representative PRRSV strains from different clusters in the phylogenetic tree, including NADC30, JXA1, and JXA1 P80 (Fig. 1). All the programs in RDP4 revealed that HNhx was the result of recombination between the NADC30 virus and HP-PRRSV vaccine strain circulating in China. Next, we identified two recombination breakpoints located in Nsp4 (nt 5261) and Nsp9 (nt 7911) by SimPlot v3.5.1 software (Fig. 3a). The two breakpoints separated the genome into three regions. The region between the breakpoints (minor parental region, nt 5261–7911) was closely related to HP-PRRSV vaccine strain in China (Fig. 3b), and the remaining two (major parental region, nt 1–5260 and nt 7910–15,019) exhibited higher similarity with NADC30 strain (Fig. 3c). It could be drawn that HNhx was a mosaic strain derived from NADC30 and HP-PRRSV vaccine strain in China.

Then we analyzed all reported NADC30-like strains identified in China, of which the whole genome became available on NCBI since 2013. Recombination was found in 17 NADC30-like PRRSV strains, which shows multifarious recombination pattern (Fig. 4). Twelve PRRSV strains (HENXX-1, HENZMD-9, WUH6, JL580, FJ1402, HENA0N-HEB, 15HEN1, 15JX1, 15SC3, 15LN3, FJW05, FJXS15) were indicative of recombination with HP-PRRSV. Among them, FJW05, FJXS15 could be defined as HP-PRRSV-like strain, because their major parental strain was HP-PRRSV. Three strains (CHsx1401, HNyc15, HENAN-XINX) recombined with classical PRRSV strains. Interestingly, FJWQ16 was a mosaic recombinant of three strains between NADC30, HP-PRRSV, and C-PRRSV. The breakpoints of FJWQ16 located in 6791 (Nsp7α), 8771 (Nsp9), 12,291 (ORF2), and 13,431 (ORF5), and separated the genome into five regions. The regions 1–6790, 8772–12,290, and 13,432–15,016 were closely related to NADC30; the regions 6791–8771 and 12,291–13,431 exhibited higher similarities with HP-PRRSV and C-PRRSV, respectively. Moreover, most of the breakpoints dropped into the area of Nsp2, Nsp4, and Nsp9, indicating that these regions are wobbly and easy to restructure. These results indicated that recombination events of PRRSV in China were extremely complicated.

Discussion

PRRS has been a serious threat to the swine industry since its emergence in the late 1980s. In 2006, there have been large-scale and devastating outbreaks of porcine high fever syndrome in China, caused by HP-PRRSV, which had a unique discontinuous deletion of 30 amino acids in Nsp2-coding regions. Since then, HP-PRRSVs have become the dominant strains circulating in the field [29]. However, in recent years, the widespread outbreaks of PPRS in China were associated with the novel NADC30-like PPRSV strains, which are closely related to NADC30 [37]. NADC30 strain was isolated in the United States in 2008 and introduced into China presumably by importing of breeding pigs in recent years [4, 37]. In this study, a novel PRRSV variant, named HNhx, was isolated, and its genotype and genomic characteristics were analyzed.

Comparative analysis of the nucleotide sequences showed that HNhx was closely related to NADC30 PRRSV strain, and the nucleotide identity between them was 92.9%. Pairwise comparisons showed that FJZ03 strain shared highest nucleotide identity with NADC30 strain among all the NADC30-like PRRSVs isolated in China, between which the nucleotide divergence was 3.0% and the nucleotide sequence divergence ranged from 2.5 to 12.0% within the subgroup of NADC30-like PRRSV strains located (data not shown). This highly genetic and antigenic diversity indicated that NADC30-like PRRSV strains had undergone gradual and extensive evolution in China. However, more isolates need to be isolated to further display a consummate evolutionary relationship of NADC30-like PRRSVs in China.

Table 3 Analysis of amino acid mutations and deletions in Nsp2

Strains	Amino acid sites																												
	39	196	219	242	243	289	290	297	298	324-433	455	472	474	481	483	492	504-522	533-561	569	587	617	749	763	767	827	834	906	949	1187
VR2332	K	S	P	N	K	D	V	A	A		A	G	V			P			E	A	K	S	E	E	G	A	S	H	I
CH-1a	K	P	R	N	K	N	V	A	A		A	D	V			P			E	A	N	S	E	E	G	V	S	H	I
HB-1(sh)/2002	K	P	H	N	K	N	V	A	A		A	D	V			P			E	A	S	S	G	E	G	V	S	H	I
JXA1	K	P	H	N	K	N	V	A	A		A	D	V	DEL		P		DEL	E	A	N	S	G	E	G	V	S	H	I
MN184A	K	P	P	N	K	D	V	X	A	DEL	A	G	L		DEL	L	DEL		E	A	R	S	Q	E	G	V	S	H	I
NADC30	K	S	S	N	K	D	V	V	A	DEL	A	G	F		DEL	L	DEL		E	A	R	S	R	K	G	V	T	H	I
HENAN-XINX	K	L	S	N	K	D	V	V	A	DEL	A	G	S		DEL	L	DEL		E	A	R	S	R	K	G	V	A	H	I
HENAN-HEB	K	P	H	N	K	D	V	V	A	DEL	A	G	P		DEL	L	DEL		E	A	R	S	R	K	G	V	T	H	I
JL580	K	S	S	N	K	D	V	V	A	DEL	A	G	F		DEL	L	DEL		E	A	R	S	G	E	G	V	S	H	I
CHsx1401	K	S	S	N	K	D	V	V	A	DEL	A	G	F		DEL	L	DEL		G	A	R	S	Q	K	G	I	T	H	I
FJY04	K	S	S	N	K	D	V	V	A	DEL	A	G	L		DEL	L	DEL		E	A	R	S	Q	K	G	V	T	H	I
FJZ03	K	S	S	N	K	D	V	V	A	DEL	A	G	S		DEL	L	DEL		E	A	R	S	R	K	G	V	T	H	I
FJ1402	K	P	S	N	K	D	V	E	A	DEL	A	G	S		DEL	L	DEL		E	A	R	S	R	K	G	V	S	H	I
HENXC-4	E	A	P	N	K	D	V	V	A	DEL	A	G	F		DEL	L	DEL		E	A	R	S	R	K	G	V	T	H	I
HENXX-1	K	S	S	N	K	D	V	M	A	DEL	A	G	F		DEL	L	DEL		E	A	R	S	R	N	G	V	T	H	I
HENAMD-9	K	S	S	N	K	D	V	A	A	DEL	A	G	F		DEL	L	DEL		E	A	R	S	R	K	D	V	T	H	I
HNjz15	K	S	S	N	K	D	V	V	A	DEL	A	G	S		DEL	L	DEL		E	V	R	S	Q	K	G	V	T	H	I
HNyc15	K	S	S	N	K	D	V	V	A	DEL	A	G	S		DEL	L	DEL		E	A	R	S	R	K	G	V	T	H	I
WUH5	K	S	S	N	K	D	I	V	A	DEL	A	G	F		DEL	L	DEL		D	A	R	S	R	K	G	V	T	H	I
WUH6	K	S	S	N	K	D	V	V	A	DEL	A	G	P		DEL	L	DEL		E	A	R	S	R	K	G	V	T	H	I
HNhx	G	T	L	D	R	G	F	M	V	DEL	T	S	I		DEL	Q	DEL		R	T	M	T	V	T	S	F	N	R	V

DEL amino acid deletion

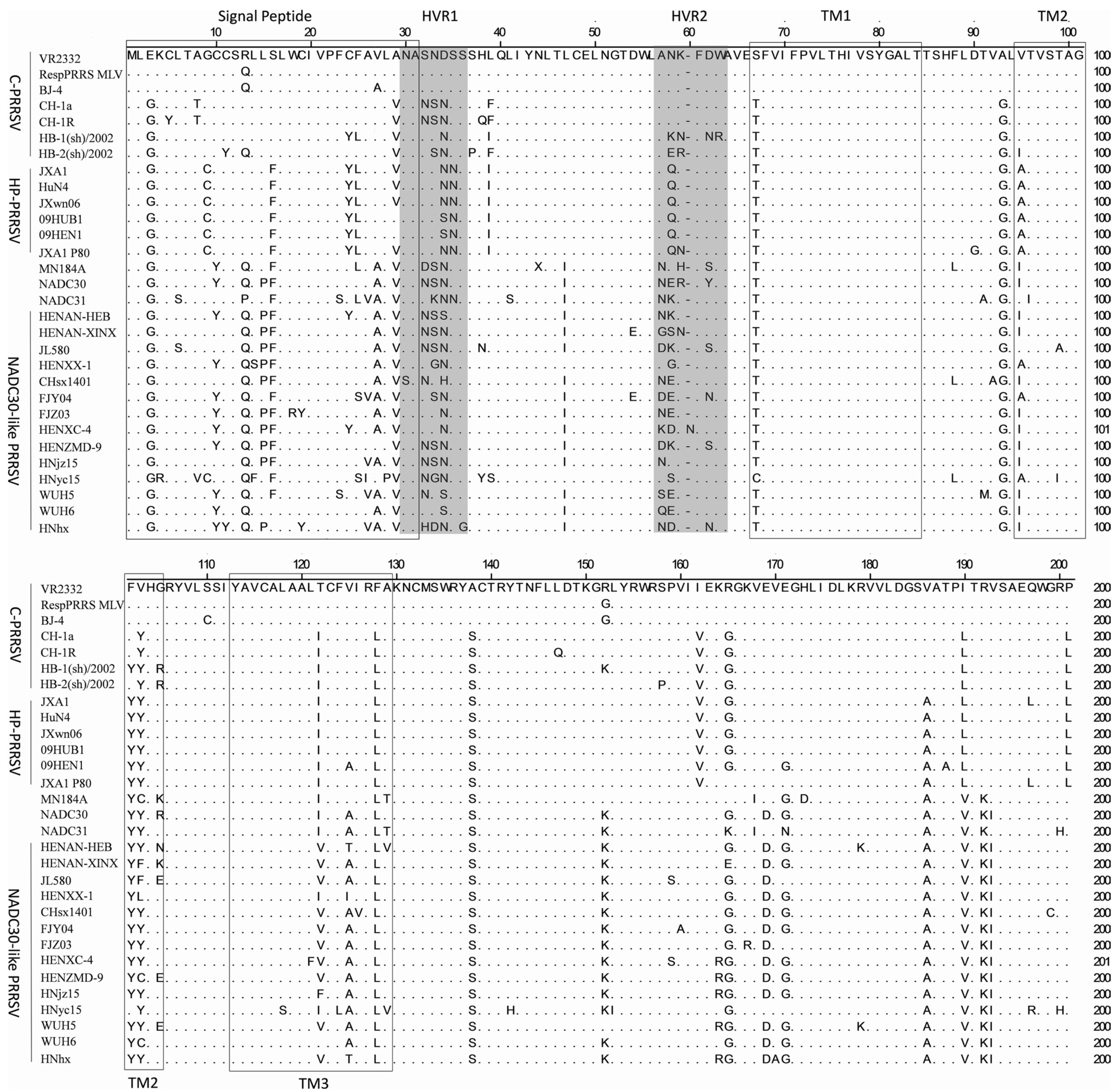

Fig. 2 Multiple alignment of GP5 amino acid sequences of PRRSV sample strain and reference strains. Gray areas indicate the two hypervariable regions (HVR) in the GP5; the boxed areas indicate the signal peptide region and three transmembrane domains (TM)

The 30-aa deletion has been used as an epidemiologically genetic marker of HP-PRRSV since 2006, although it is not related to the virulence of virus [38]. Afterwards, a great many other aa deletion and insertion in Nsp2 have been identified [12, 31]. Our results showed that the Nsp2 of NADC30-like PRRSVs contained the unique 131 discontinuous amino acid deletions relative to that of the Nsp2 of the VR2332 strain, including other NADC30-like strains previously reported. Therefore, the deletion could be used as an epidemiologically genetic marker for the NADC30-like PRRSV circulating in China. Nsp2 is crucial for viral replication and the modulation of host immunity, and Nsp2 contain the high frequency of immunogenic epitopes [6]. Nsp2 of HNhx contains many unique aa substitutions. Whether the mutations were related with the virulence needs much more research.

ORF5, encoding the major envelope protein GP5, is often selected as one of the main targets to monitor PRRSV evolution for their genetic diversities. GP5 contains the primary neutralizing epitope (PNE) of PRRSV (aa 37–45), which

Table 4 Potential N-glycosylation sites in GP5

Strains	Potential N-glycosylation sites						
	30	32	33	34	35	44	51
VR2332	✓	✓				✓	✓
CH-1a				✓		✓	✓
HB-1(sh)/2002	✓		✓	✓		✓	✓
JXA1	✓			✓	√	✓	✓
NADC30				✓		✓	✓
HENAN-HEB		✓				✓	✓
HENAN-XINX				✓		✓	✓
JL580				✓		✓	✓
HENXX-1	✓			✓		✓	✓
CHsx1401			✓			✓	✓
WUH5		✓	✓			✓	✓
WUH6	✓		✓			✓	✓
HENXC-4	✓		✓	✓		✓	✓
HNjz15				✓		✓	✓
HNyc15				✓		✓	✓
FJZ03	✓		✓	✓		✓	✓
FJY04	✓			✓		✓	✓
FJ1402	✓			✓		✓	✓
HENZMD-9				✓		✓	✓
HNhx						✓	✓

✓ distribution of amino acid positions in the potential N-glycosylation sites

is associated with virus neutralization and the H^{38} and $I^{43}Y^{44}N^{45}$ are the main recognition sites. However, HNhx and all the other NADC30-like PRRSVs isolated in China were the same as VR2332 (SHLQLIYNL) except JL580 (SNLQLIYNL) and HNyc15 (SYSQLIYNL) (Fig. 2). In the "epitope A" region (residues 27–31 containing (A/V)LVN motif) identified in previous reports, all the NADC30-like PRRSVs had changed to ALVN except HNyc15 (VPVN) and HENZMD-9 (VLVN), comparing with the classic PRRSV strain VR2332 (VLAN). However, whether the "epitope A" acts as a decoy epitope remains controversial. Recent studies demonstrated that most GP5 molecules do not contain the ''decoy epitope'' [23, 28]. Thus, the effects of the mutation is unknown. The potential N-glycosylation sites at amino acid positions 44 and 51 were conserved in all NADC30-like PRRSV strains, but the substitutions in HVR1 would lead PRRSV to have different positions and numbers of N-glycosylation sites (Fig. 2, Table 4). Interestingly, due to the loss of all the potential N-glycosylation sites in HVR1 of GP5, HNhx has fewest N-glycosylation sites (Table 4). All the NADC30-like isolates (except WUH5 at aa 151) had two substitutions of R^{13} to Q^{13} and R^{151} to K^{151}, which is reported to be related with the virulence of PRRSV [1]. Moreover, in intravirion domain, HNhx had a unique mutation (V^{170} to A^{170}) distinct from all the other strains, while in signal peptide domain (aa 1–26) domain, a unique mutation of GP5 (C^{11} to Y^{11}) in HNhx is observed. Recent data showed that traditional control strategies and current commercial PRRSV vaccines could not provide complete protection to the circulating NADC30-like PRRSVs [3]. However, further studies are warranted to determine whether these variations in the significant domain of GP5 resulted in the inability of commercial PRRSV vaccines to provide protection to the circulating NADC30-like PRRSV.

In addition to mutation, genetic recombination also plays an important role in the evolution of PRRSV. Several recent studies had demonstrated the genetic exchange between NADC30 and the classic strains circulating in China. Concretely, JL580 has been proved to be a mosaic PRRSV strain of NADC30 and HP-PRRSV with six recombination breakpoints located in Nsp2, Nsp3, Nsp7, ORF2a, and ORF4, while HNyc15 is recombined with VR2332 and CH-1a between ORF2 and ORF4. [13, 36, 37]. And other strains have multifarious recombination patterns (Fig. 4). Moreover, HNhx, isolated in this study, was indicative of recombination with PRRSV vaccine JXA1-R. We found a large proportion of recombination occurred in the nonstructural proteins coding regions. But for recombination occurred in the structural proteins coding regions, the breakpoints mainly located near the transcription-regulating sequences (TRSs), such as WUH6 (11,652) located near the TRS2, HNyc15 (12,257) and FJWQ16 (12,291) near to the TRS3, and HENXX-1 (13,923) near to TRS6. These data indicated that recombination easily occurs at sites which are similar or

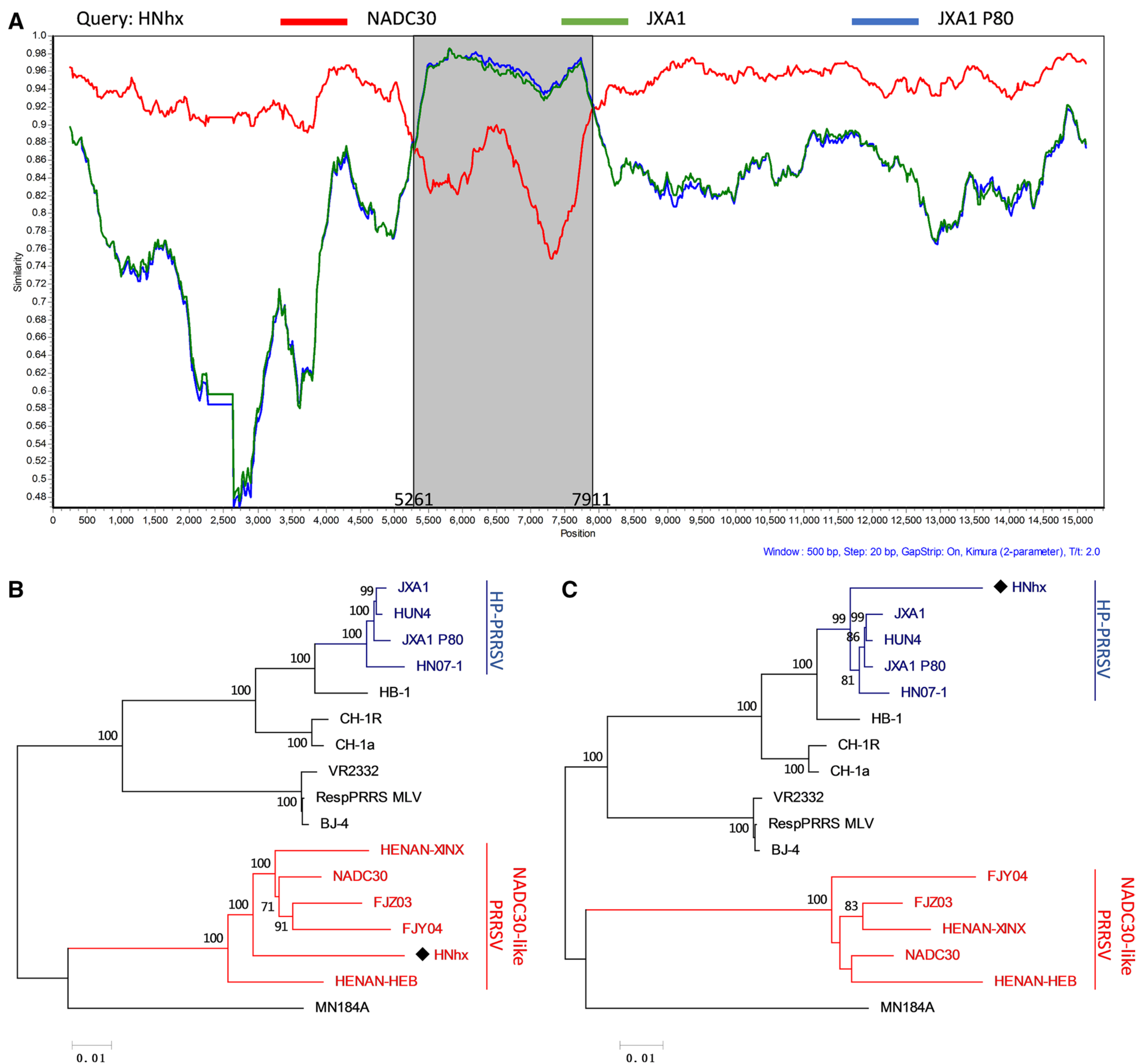

Fig. 3 Recombination analysis of PRRSV strain HNhx. **a** Genome scale similarity comparisons of HNhx (query) with NADC30 (red), JXA1 (green) and JXA1 P80 (blue). Recombination breakpoints are shown as black lines, with locations indicated at the bottom. The background color of major parental regions is white, while that of minor parental region is gray. Phylogenies of minor parental region (**b**) and major parental region (**c**) are shown below the similarity plot. The major parental NADC30-like PRRSV group is shown in red; the minor parental HP-PRRSV group is shown in blue (Color figure online)

identical to TRSs, which are consistent with previous studies [30]. Previous studies had reported that the dominant isolates in Central China had shifted from classic PRRSVs to HP-PRRSVs during 2006 to 2013 [12, 20, 34]. Recently, owing to less cross-protection of commercial vaccines [3], the NADC30-like PRRSV strains have been circulating and prevalent in Central China. Perhaps these factors give rise to the recombination between HP-PRRSVs and NADC30-like PRRSV strains. However, natural recombination is not easy to occur, and the reason why NADC30-like strain after being introduced in China is prone to recombine with PRRSV strains circulating in China remains to be clarified.

Phylogenetic analysis indicated that the recombinant strains JL580, HENZMD-9, and HNhx were far away from NADC30 strain (Fig. 1). To investigate the evolutionary process of HNhx, we conducted recombination analysis by performing the RDP4 and SimPlot v3.5.1 based on the complete genome. The recombination analysis revealed

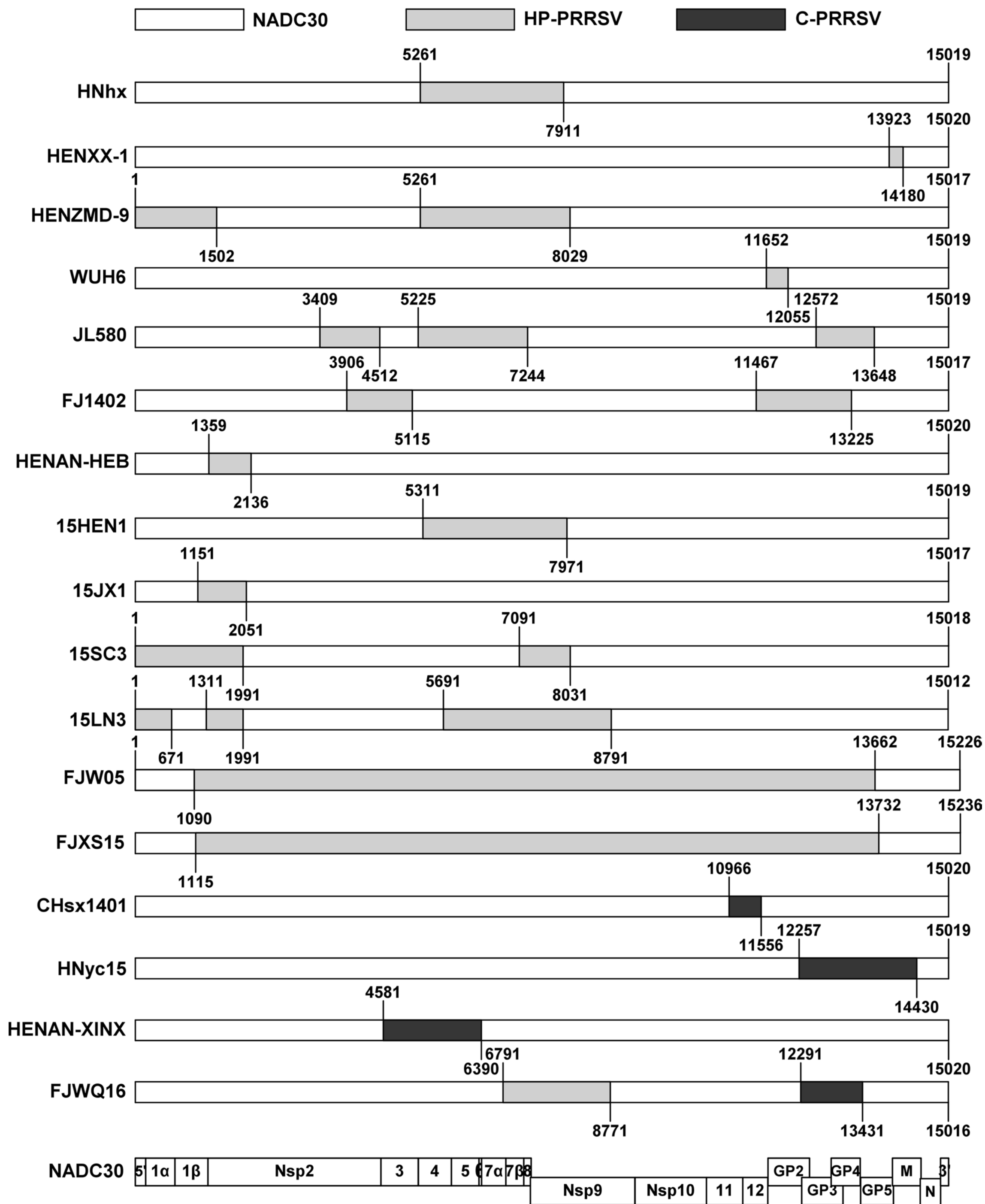

Fig. 4 Recombination analysis of the reported NADC30-like PRRSV strains in China. NADC30, HP-PRRSV (highly pathogenic PRRSV), and C-PRRSV (classical PRRSV) are represented by white, gray, and black rectangles, respectively

that HNhx might be a recombinant strain derived from the NADC30 and HP-PRRSV vaccine strain, with recombination breakpoints located in Nsp4 and Nsp9 (Fig. 3a). Recent studies demonstrated that current commercial attenuated HP-PRRSV vaccine could not provide effective protection to the NADC30-like PRRSV strains [3]. To a certain extent, commercial attenuated HP-PRRSV vaccines maybe increase the diversity of strains and accelerate the evolution of PRRSV, which will make it be a barrier for controlling PRRS outbreaks.

However, more interestingly, among NADC30-like strains reported, HNjz15 strain was less pathogenic than

the HP-PRRSV JXA1, which was identical with NADC30, a moderately virulent strain [27], while the pathogenicity of JL580 was much higher than that of NADC30 and was similar to that of the classic HP-PRRSV strains in China. Genome analysis showed that JL580 was a mosaic NADC30-like virus underwent recombination with HP-PRRSV strain 09HEN1 in the regions: Nsp2 (nt 3446) to Nsp3 (nt 4549), Nsp3 (nt 5225) to Nsp7 (nt 7244), and ORF2a (nt 12,572) to ORF4 (nt 13,648) [36]. Interestingly, the above regions excluded the Nsp9 and Nsp10-coding regions which had been demonstrated to be the virulence-determining genes for HP-PRRVs in China [14]. However, which regions of the genome associated with the virulence of PRRSV still remains controversial. An earlier reported demonstrated that major virulence determinants might locate in Nsp3-8 and ORF5 [11]. Accordingly, the differences in pathogenicity among NADC30-like PRRSV strains are not yet precisely understood. Thus, whether recombination is related to pathogenicity needs to be further elucidated. The possibility of potential reversion to virulence of recombined strains increased complexity of PRRSV prevention and the vaccines should be cautiously used. The genome analysis of PRRSV strain HNhx, characterized by recombination with HP-PRRSV vaccine strain JXA1 P80 in Nsp4 (nt 5261) to Nsp9 (nt 7911), would provide valuable help for clarifying the mechanisms for virulence change in NADC30-like PRRSVs (Fig. 3).

Conclusions

In summary, the PRRSV isolate HNhx with unique genetic variation in GP5 and Nsp2 was a novel mosaic recombinant strain between NADC30 and HP-PRRSV vaccine strain. The genomic characterization of HNhx strain enriches genomic data of PRRSV in China, which will contribute to elucidating the relationship among genotypes and phenotypes of PRRSV and provide sound evidences for recombination contributing to the variation and evolution of PRRSV.

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Author contributions Lin-jian Wang, Xin-xin Chen, and Gaiping Zhang conceived and designed the experiments. Lin-jian Wang, Bo Wan, and Zhenhua Guo performed the experiments. Bo Wan, Songlin Qiao, Rui Li, and Sha Xie contributed data analysis. Lin-jian Wang and Xin-xin Chen wrote the manuscript. Zhenhua Guo, Songlin Qiao, Rui Li, Sha Xie, Gaiping Zhang revised the manuscipt content. Gaiping Zhang, Songlin Qiao, and Xin-xin Chen approved final version of manuscript.

Compliance with ethical standards

Conflicts of interest All authors state that they have no conflicts of interest. This article does not contain any studies with animals performed by any of the authors. Informed consent was obtained from all individual participants included in the study.

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