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The full-length glycoprotein 5 (GP5) gene and a partial nonstructural protein 2 (NSP2) gene fragment of 46 porcine reproductive and respiratory syndrome viruses (PRRSV) from pig farms in southeastern China between 2004 and 2007 were sequenced for phylogenetic analysis. All of the PRRSV isolates in this study were of the North American type, and the majority of them were clustered in subgroup II and had 84.1–89.1% amino acid sequence identity to those of subgroup I including the North American strain VR-2332. Three variable regions containing epitopes A and B in the *N*-terminal region were identified and found to be under positive selection. Several additional mutations, which were also located in the variable regions, were seen in isolates from the years 2006 and 2007 in subgroup II, as compared with those of earlier years (2004–2005) in the same group. Further analysis revealed that the majority of the subgroup II PRRSV isolates prevalent in the region since 2004 had thirteen mutation sites that distinguished them from subgroup I strains, indicating a possible introduction of a certain strain from the same source in the region or elsewhere before 2004. A 29-aa deletion in the NSP2 fragment was found in PRRSV isolates as early as in 2005, one year earlier than the virulent PRRSV with the same deletion became dominant in China. Taken together, this study shows that subgroup II PRRSV

strains with a partial deletion of *nsp2* are currently prevailing in southeastern China.

Porcine reproductive and respiratory syndrome virus (PRRSV) is the causative agent of porcine reproductive and respiratory syndrome (PRRSV) [29], characterized by severe reproductive failure in sows, respiratory disease and increased preaning mortality, as well as an influenza-like syndrome in grower-finisher pigs [3]. PRRSV, which belongs to the family *Arteriviridae*, is a small enveloped virus with an approximately 15-kb genome of positive-stranded RNA that contains eight overlapping open reading frames (ORFs). ORF1a and ORF1b encode the viral polymerase. ORFs 2, 3 and 4 encode envelope proteins, and ORFs 5, 6 and 7 code for major structural envelope (E), membrane (M) and nucleocapsid (N) protein, respectively [17, 19]. There are two genotypes of PRRSV, the North American type (NA) and the European type (EU), which share only 55–70% nucleotide identity [21]. Significant genetic variability also exists among isolates within the same genotype [9]. Although PRRSV strains identified around the world cause similar diseases in pigs, increasing data indicate that the antigenicity and pathogenicity vary substantially among different PRRSV strains [18, 25, 31, 32].

GP5, the major viral glycoprotein encoded by ORF5, is essential for virus infectivity and contains important immunological domains associated with virus neutralization [23, 26]. Due to its polymorphic characteristic [4], ORF5 has been the target for analysis of genetic diversity of PRRSV [1, 2, 5, 13]. NSP2 is a multi-domain protein of

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PRRSV [22, 30]. It also has a variable region [11] and has been used in genetic diversity studies [7, 27]. As PRRSV is highly variable in geographic terms [4, 15, 24], it is unclear how diverse the virus in southeastern China is until a recent study indicated that PRRSV strains from several Chinese regions were diverse and could be divided into two major subgroups [1]. This study was undertaken to analyse the occurrence of NSP2 deletions in PRRSV isolates from eastern China and to examine the genetic relationship between them or with those from other regions of China in an effort to find clues as to their origin.

### Sample collection

Tissue samples of lymph nodes and lungs from diseased pigs were collected between 2004 and 2007 on farms located in the neighbouring provinces of Zhejiang, Shanghai and Jiangsu in southeastern China, where there were acute or chronic outbreaks of severe reproductive problems in sows of different parities concomitant with respiratory problems in suckling and post-weaning piglets.

### RNA extraction and RT-PCR

Total RNA was extracted from homogenates of lungs and lymph nodes according to Chen et al. [6]. Primers ORF5-F (5'-GGTGGGCACKGTTTTAGCCTGTC-3') and ORF5-R (5'-GGTAATAGARAAAYGCCAAAAGCACC-3') were designed based on ORF4 and ORF6 sequences for amplification of the full-length ORF5 (from nt 13729 to 14449 of the VR2332 strain, GenBank accession no. PRU87392). The primer pairs NSP2-F (5'-GCACCAGTTCCTGCA CCGC-3') and NSP2-R (5'-AGGGAGCTGCTTGATGACACAG-3') were used to generate a 230-bp fragment for the deletion form or a 371-bp fragment for the non-deletion form of PRRSV strains (from nt 2899 to 3107 of the JXA1 strain, GenBank accession no. EF112445, and nt 2903 to 3198 of the CH-1a strain). The reverse transcription reaction contained the following components: 9 µl total RNA, 4 µl 5 × RT buffer, 0.4 mM dNTPs, 20 pmol of primer ORF5-R or NSP2-R, 5 mM dithiothreitol, 20 U RNase inhibitor (TOYOBO, Japan), and 100 U ReverTra Ace reverse transcriptase (TOYOBO, Japan), adjusted to a final volume of 20 µl with DEPC-treated ddH<sub>2</sub>O. The reaction mixtures were incubated at 42 °C for 1 h. The PCR reaction was carried out as follows: 2 µl RT product, 4 µl 5 × PCR buffer, 0.4 mM dNTPs, 20 pmol of each primer, 5U Primer STAR polymerase (TaKaRa, Japan), adjusted to a final volume of 20 µl with ddH<sub>2</sub>O. Cycling conditions included an initial denaturation at 94 °C for 5 min, followed by 30

cycles with 94 °C for 30 s, 57 °C for 30 s and 72 °C for 50 s. The final elongation step was at 72 °C for 10 min.

### Nucleotide sequencing

The PCR products amplified from PRRSV-positive samples were purified using an A-Prep DNA Gel Extraction Kit (Agen Inc., USA) and cloned into the pSIMPLE-19 vector (TaKaRa, Japan). The target fragments were sequenced on an ABI-PRISM 377 DNA sequencer.

### Bioinformatic analysis of PRRSV GP5 and NSP2 gene sequences

The GP5 genes or partial NSP2 fragments sequenced herein and those retrieved from the GenBank database (Table 1) were multiple-aligned with CLUSTAL X (version 1.83). A phylogenetic tree was constructed (MEGA version 3.1) in which the Lelystad sequence (EU genotype) served as an outgroup control. Pairwise comparison of nucleotide and amino acid sequence similarities was conducted by using MegAlign 5.03 (DNASTAR Lasergene software package). A hydrophilic profile was generated by the method of Kite and Doolittle using the DNASIS 2.5 software package. The *dN* and *dS* were calculated using the SNAP web utility (<http://hcv.lanl.gov/content/hcv-db/ SNAP/ SNAP.html>). SNAP (S non-mouse/Non-s non-mouse Analysis Program) calculates s non-mouse and non-s non-mouse substitution rates for codon-aligned nucleotide sequences based on the method of Nelsen et al. [20]. Selective pressure was measured by the rate *dN-dS*. The ratios *dN-dS* > 0, *dN-dS* = 0 and *dN-dS* < 0 mean positive selection (adaptive molecular evolution), neutral mutations and negative selection (purifying selection), respectively [14]. Variable regions were analysed according to the method of Pesente et al. [24]. *N*-linked glycosylation sites were predicted with the *N*-glycosite web utility (<http://www.hiv.lanl.gov/content/sequence/ GLYCOSITE/ glycosite.html>). Signal peptide cleavage sites in amino acid sequences were predicted with the Signal P 3.0 server web utility (<http://www.cbs.dtu.dk/ services/SignalP/>).

The PRRSV viruses isolated from southeastern China belonged to subgroup II of the North American genotype.

The 603-bp ORF5 fragments from 46 PRRSV-positive samples from 2004 to 2007 were sequenced (Table 1). Phylogenetic analysis based on ORF5 revealed that all

**1** Sequences from GenBank (No. 1-25) for comparison with those of PRRSV isolates used in this study (No. 26-71)

No.	Isolate	Region	Year	Accession no.	No.	Isolate	Region	Year	Accession no.	No.	Isolate	Region	Year	Accession no.
1	VR2332	U.S.	1995	PRU87392	26	QZ-07	Qu hou	2007	EU480719	51	QZ-05	Qu hou	2005	EU480735
2	Lel stad	Netherlands	1993	M96262	27	ZSB-07	Zhoushan	2007	EU480710	52	SX-1-05	Shao ing	2005	EU480736
3	CH-1a	Beijing	1996	AY032626	28	JX-1-07	Jia ing	2007	EU480715	53	SX-2-05	Shao ing	2005	EU480737
4	HB-1(sh)	Hebei	2002	AY150312	29	XS-1-07	Xiaoshan	2007	EU480725	54	SX-3-05	Shao ing	2005	EU480738
5	HB-2(sh)	Hebei	2002	AY262352	30	FH-07	Fenghua	2007	EU480712	55	SX-4-05	Shao ing	2005	EU480739
6	HZ-X	Zhejiang	2003	AY450301	31	SH-07	Shanghai	2007	EU480720	56	TZ-1-05	Tai hou	2005	EU480740
7	Sichuan1	Sichuan	2003	AY513611	32	WJ-1-07	Wujiang	2007	EU480722	57	TZ-2-05	Tai hou	2005	EU480741
8	YA	Henan	2004	AY633974	33	WJ-2-07	Wujiang	2007	EU480723	58	WZ-05	Wen hou	2005	EU480742
9	GD1	Guangdong	2004	AY747595	34	WJ-3-07	Wujiang	2007	EU480724	59	JX-2-05	Jia ing	2005	EU480733
10	Henan HN1	Henan	2004	AY613348	35	LA-07	Linan	2007	EU480716	60	HZ-2-06	Hang hou	2006	EU480744
11	NJ-a	Jiangsu	2004	AY737282	36	FY-07	Fu ang	2007	EU480713	61	JS-1-06	Jiangsu	2006	EU480745
12	NX06	Beijing	2007	EU097706	37	NT-07	Nantong	2007	EU480718	62	JS-2-06	Jiangsu	2006	EU480746
13	SD1	Shandong	2003	AY747596	38	FZ-07	Fu hou	2007	EU480714	63	JX-06	Jia ing	2006	EU480747
14	SD2	Shandong	2005	DQ265739	39	YH-07	Yuhang	2007	EU480755	64	JX-2-06	Jia ing	2006	EU480748
15	BJ4	Beijing	2000	AF331831	40	NB-07	Ningbo	2007	EU480717	65	JX-3-06	Jia ing g	2006	EU480749
16	JXA1	Jiang i	2006	EF112445	41	SHX-07	Shao ing	2007	EU480721	66	JX-4-06	Jia ing	2006	EU480750
17	Hainan-2	Hainan	2007	EF398052	42	CA-07	Chunan	2007	EU480711	67	JX-5-06	Jia ing	2006	EU480751
18	Lian ungang/05	Jiangsu	2007	EU148488	43	HZ-1-04	Hang hou	2004	EU480726	68	SH-2-06	Shanghai	2006	EU480752
19	Wujin/06	Jiangsu	2007	EU148494	44	HZ-2-04	Hang hou	2004	EU480727	69	TZ-06	Tai hou	2006	EU480753
20	HUB1	Hubei	2006	EF075945	45	HZ-3-04	Hang hou	2004	EU480728	70	HZ-1-06	Hang hou	2006	EU480743
21	SCQ	Sichuan	2006	DQ379479	46	HZ-4-04	Hang hou	2004	EU480729	71	JX-3-05	Jia ing	2005	EU480734
22	HEB1	Hebei	2007	EF112447	47	NB-1-04	Ningbo	2004	EU480730					
23	HN1	Henan	2003	AY457635	48	NB-3-04	Ningbo	2004	EU480731					
24	R98	Jiangsu	2006	DQ355796	49	HZ-1-05	Hang hou	2005	EU480731					
25	FJ04A	Fujian	2005	DQ246451	50	JX-1-05	Jia ing	2005	EU480732					

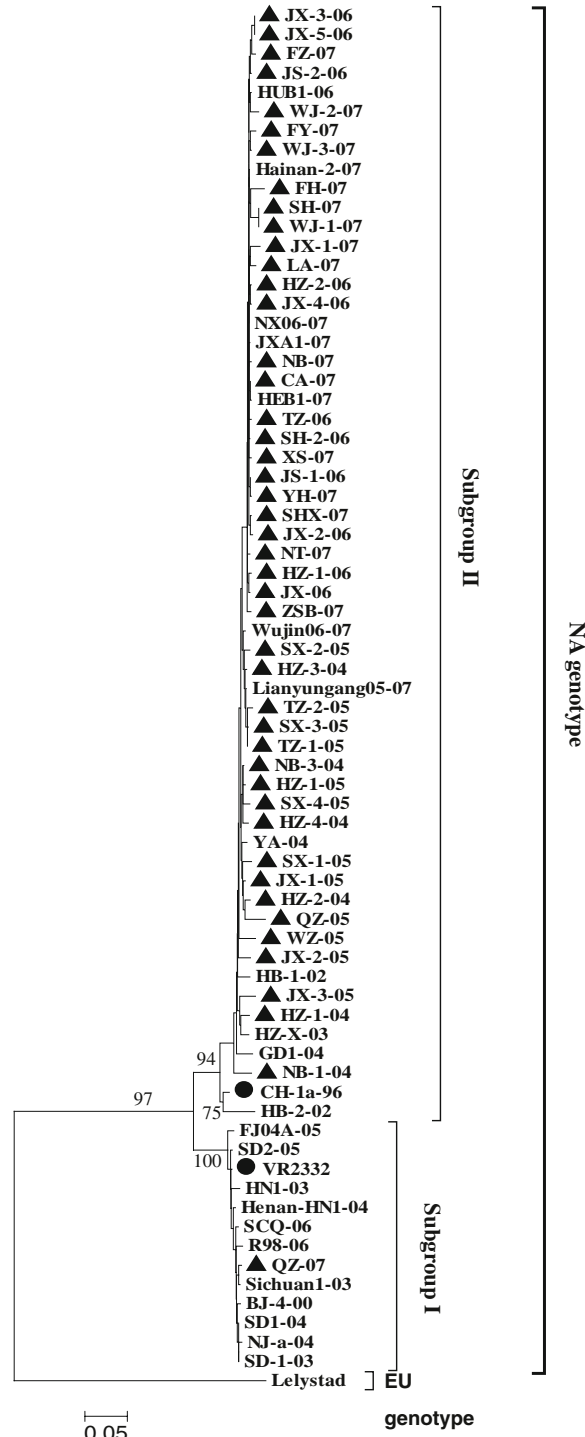
isolates in this study belonged to the NA genotype (Fig. 1a) and could be clustered into two major subgroups. Most of the isolates from southeastern China were classified into

subgroup II together with some other Chinese isolates, and only the isolate QZ-07 belonged to subgroup I, together with the prototype NA strain VR-2332. However, several

**Fig. 1** Phylogenetic analysis depicting the genetic relationship between 46 PRRSV isolates in this study (indicated by filled triangle) and other North American genotype isolates from other regions of China based on the major structural gene ORF5. The prototype American isolate VR2332 and the first Chinese isolate CH-1a are indicated by filled circle. Each isolate is named for its origin and time of isolation. The tree was constructed using the neighbor-joining algorithm based on the Kimura two-parameter distance estimation method in MEGA 3.1, and the European type isolate Lelystad was rooted as out-group. Bootstrap values representing the major branches are indicated as a percentage for 1,000 replicates. Two main subgroups of PRRSV isolates (I and II) are indicated.

Subgroup-specific substitution patterns of aa residues of GP5 of PRRSV isolates of subgroups I and II

(a)



(b)

Site	3	13	39	66	92	102	121	127	137	151	161	164	189
Subgroup I	E	Q	L	S	A	V	T	F	A	G	I	R	I
Subgroup II	G	R	I/F	T	G	Y/F/L/C	I/V	L	S	R	V	G	L

Percent nucleotide (nt) and amino acid (aa) identity of ORF5 among subgroup I, subgroup II and VR2332 strains

Subgroup	Identity level	From subgroup I (%)	From subgroup II (%)	From strain VR2332 (%)
I	nt	98.2 100	84.2 89.6	98.0 99.7
	aa	96.5 99.5	84.1 89.1	96.5 98.5
II	nt	84.2 89.6	89.6 100	88.1 90.2
	aa	84.1 89.1	90.7 99.5	85.8 89.6

earlier PRRSV isolates in China, such as CH-1a and 02-HB-2, formed a separate subset of the subgroup II isolates. The sequence identity between subgroups I and II varied from 84.2 to 89.6% (nucleotide) and 84.1–89.1% (amino acid) (Table 2). Moreover, subgroups I and II in the phylogenetic tree could be differentiated by 13 unique amino acid substitution patterns scattering in different regions around GP5 (Fig. 1b).

We found that there were 25 major nucleotide variation sites among the subgroup I and II PRRSV isolates. Nine of them were conserved between the prototype US strain VR2332, representing subgroup I isolates, and the “ancestral” Chinese strain CH-1a but were mutated at a majority of the subgroup II isolates. There were 16 sites that were conserved between CH-1a and the majority of the subgroup II isolates but were different from VR2332. Thus, the CH-1a appeared to represent an evolutionary link between subgroups I and II.

Analysis of the deduced amino acid sequences of GP5 and the partial NSP2 fragment

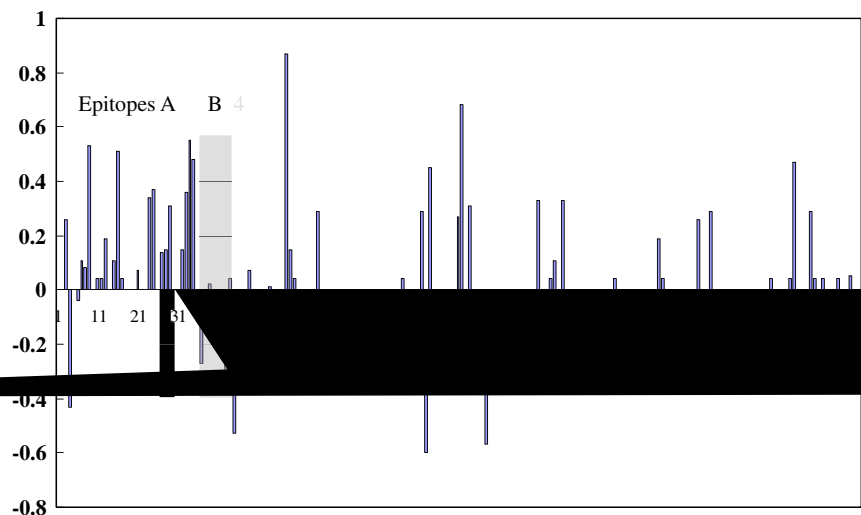
Three variable regions (VR1, VR2 and VR3) were identified in the signal sequence and putative ectodomain. The N-terminal region covering the three variable regions was

apparently under positive selection (Fig. 2). Of the 10 mapped epitopes [23], only epitope A at residues 27–30 was under positive selection (Fig. 2). In addition to 13 characteristic substitution patterns (Fig. 1b), residues 9, 16 and 185 also had substitutions in the 2006 and 2007 strains, and additional substitutions at positions 35, 49, 59 and 61 seemed to have emerged in the 2007 strains (SH-07 and WJ-1-07).

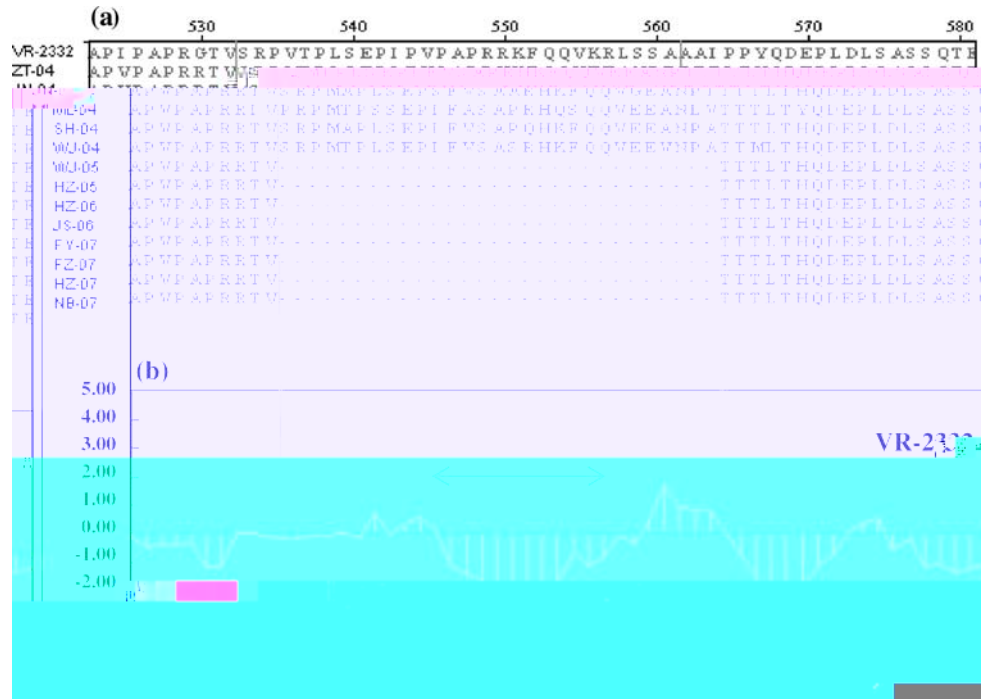
Within three potential glycosylation sites (N33, N44, and N51) in the GP5 ectodomain, the N51 site seemed to be conserved in all isolates, whereas the N33 residue was mutated in some isolates of subgroups I and II (N to S), and the N44 mutation also occurred in isolates JX-1-07 (N to K) and WZ-05 (N to S).

Analysis of the partial NSP2 sequences revealed that a 29-aa deletion of a fragment containing a major hydrophilic region had occurred from residues 533 to 561 (Fig. 3a and b). Interestingly, this deletion only existed in PRRSV isolates in and after the year 2005, including all isolates of 2006 and 2007 sequenced in the present study (Table 1), while no deletion was found in this region from isolates in 2004 (Fig. 3a).

Severe PRRSV infection has appeared in parts of China since 2006, causing huge economic losses to the swine industry, and PRRSV strains with deletion of a defined region of NSP2 were isolated from recent outbreaks [28]. However, it remains unknown if PRRSV isolates with this deletion in NSP2 were responsible for these outbreaks. We attempted to analyze the phylogenetic relationship among PRRSV isolates from the provinces of Zhejiang, Shanghai and Jiangsu in eastern China and those of other Chinese regions based on their GP5 gene sequences.



Alignment of partial NSP2 amino acid sequences from representative PRRSV isolates in this study with VR-2332 as the reference strain ( ) and hydrophobicity analysis of the region (VR2332 strain) ( ). Deletion of 29 aa residues in the top panel is shown as “-”. The arrow in the bottom panel indicates the high hydrophobic region.



The majority of the isolates sequenced in this study were clustered in subgroup II (Fig. 1a), a finding that is similar to earlier results with regard to subtyping of Chinese PRRSV isolates [1, 7]. Only one isolate, QZ-07, was in subgroup I. It remains unclear to us if this 2007 isolate was a new introduction into Quzhou, a major pig-producing area, because the 2005 isolate QZ-05 in the same region belonged to subgroup II.

Further analysis of the encoded amino acids revealed that the majority of the subgroup II PRRSV isolates prevalent in the region since 2004 had thirteen substitution patterns that made them distinct from subgroup I strains (Fig. 1b), indicating that a certain subgroup II PRRSV strain with mutations at these positions appeared to have been introduced into the region before 2004, with subsequent spread and mutation within the region. This is because it is unlikely that the PRRSV viruses in different areas in the region underwent the same mutations in the years 2004–2005 or even before. Although it remains unknown if this particular strain evolved from a subgroup I virus, we speculate that the “ancestral” Chinese PRRSV isolate CH-1a, which was isolated far back in 1996, might have acted as an evolutionary link and undergone mutational divergence into the subgroup II isolates that are dominant in southeastern China. This argument could be supported by the “linkage” pattern of the strain CH-1a: nine out of 25 major substitutions between subgroup I and II isolates in the GP5 gene were conserved between VR2332 (subgroup I) and the CH-1a strain (a subset of subgroup II), while there were also twelve codons (out of

25) that were conserved between CH-1a and other subgroup II isolates but were different from VR2332.

SNAP analysis further revealed that the VR2 region that overlaps the epitopes A and B as well as VR1 and VR2 in the N-terminal region is under positive selection (Fig. 2), probably as a result of immunological pressure due to increased vaccination against PRRSV in the past 2 years, as part of the viral strategy for immune evasion [10]. The positive selection on epitope A (Fig. 2), a decoy epitope that may diminish the immune responsiveness against an adjacent neutralizing epitope (epitope B) [16], might function in this way, although this requires further experimental verification by mutagenic approaches. We have also seen several additional mutations in isolates from the years 2006 and 2007 in subgroup II, shifting away further from historical PRRSV isolates VR2332 and those of earlier years. These sites were also located in the three VR regions, indicating that the positive selection events were still going on.

A 29-aa deletion in NSP2 (corresponding to nt 533–561 of VR2332 ORF1a) was identified in strains isolated since 2005 in this study (Fig. 3), at least 1 year earlier than the virulent PRRSV with the same deletion became dominant in China [8, 28], suggesting that these isolates were all pathogenic *in vivo* because they were from diseased pigs. Similar outbreaks of PRRSV infection in pigs with an NSP2 deletion in this particular region were also seen in Vietnam [8]. Challenge studies in SPF pigs or PRRSV-free pigs have indicated that the recently emerged PRRSV in China characterized by two discontinuous deletions in

NSP2 is the cause of the current epizootics in China [33]. Epitope mapping of PRRSV by phage display indicated that NSP2 contained a cluster of B-cell epitopes [22]. A previous study suggested the potential role of the 87-nt deletion in NSP2 in the high pathogenicity of PRRSV [28]. However, Han et al. [12] found that the NSP2 hypervariable region (324–726 nt) was dispensable for viral replication. Furthermore, the deletion mutants displayed decreased cytopathic activity and did not form visible plaques in vitro. Therefore, the role of this 29-aa deletion of NSP2 in PRRSV virulence is presumptive [28] and awaits further experimental verification.

In summary, it is apparent that subgroup II PRRSV strains with *nsp2* partial deletion have been prevailing in southeastern China. Continuing surveillance is needed from molecular epidemiological and vaccinological perspectives for better control of the disease in the region.

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