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The full-length gl coprotein 5 (GP5) gene and a partial nonstructural protein 2 (NSP2) gene fragment of 46 porcine reproductive and respirator s ndrome viruses (PRRSV) from pig farms in southeastern China bet een 2004 and 2007 ere sequenced for ph logenetic anal sis. All of the PRRSV isolates in this stud ere of the North American t pe, and the majorit of them ere clustered in subgroup II and had 84.1 89.1% amino acid sequence identit 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 ere identi ed and found to be under positive selection. Several additional mutations, hich ere also located in the variable regions, ere seen in isolates from the ears 2006 and 2007 in subgroup II, as compared ith those of earlier ears (2004 2005) in the same group. Further anal sis revealed that the majorit 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 else here ell before 2004. A 29-aa deletion in the NSP2 fragment as found in PRRSV isolates as earl as in 2005, one ear earlier than the virulent PRRSV ith the same deletion became dominant in China. Taken together, this stud sho s that subgroup II PRRSV strains ith a partial deletion of nsp2 are currentl prevailing in southeastern China.

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Porcine reproductive and respirator s ndrome virus (PRRSV) is the causative agent of porcine reproductive and respirator s ndrome (PRRSV) [29], characteri ed b severe reproductive failure in so s, respirator disease and increased pre eaning mortalit , as ell as an in uen a-like s ndrome in gro er- nisher pigs [3]. PRRSV, hich belongs to the famil *Arterividae*, is a small enveloped virus ith an appro imatel 15-kb genome of positive-stranded RNA that contains eight overlapping open reading frames (ORFs). ORF1a and ORF1b encode the viral pol merase. 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, respectivel [17, 19]. There are t o genot pes of PRRSV, the North American t pe (NA) and the European t pe (EU), hich share onl 55 70% nucleotide identit [21]. Signi cant genetic variabilit also e ists among isolates ithin the same genot pe [9]. Although PRRSV strains identi ed around the orld cause similar diseases in pigs, increasing data indicate that the antigenicit and pathogenicit var substantiall among different PRRSV strains [18, 25, 31, 32].

GP5, the major viral gl coprotein encoded b ORF5, is essential for virus infectivit and contains important immunological domains associated ith virus neutrali a-tion [23, 26]. Due to its pol morphic characteristic [4], ORF5 has been the target for anal sis of genetic diversit of PRRSV [1, 2, 5, 13]. NSP2 is a multi-domain protein of

PRRSV [22, 30]. It also has a variable region [11] and has been used in genetic diversit studies [7, 27]. As PRRSV is highl variable in geographic terms [4, 15, 24], it as unclear ho diverse the virus in southeastern China is until a recent stud indicated that PRRSV strains from several Chinese regions ere diverse and could be divided into t o major subgroups [1]. This stud as undertaken to anal se the occurrence of NSP2 deletions in PRRSV isolates from eastern China and to e amine the genetic relationship bet een them or ith those from other regions of China in an effort to nd clues as to their origin.

Sample collection

Tissue samples of l mph nodes and lungs from diseased pigs ere collected bet een 2004 and 2007 on farms located in the neighbouring provinces of Zhejiang, Shanghai and Jiangsu in southeastern China, here there ere acute or chronic outbreaks of severe reproductive problems in so s of different parities concomitant ith respirator problems in suckling and post eaning piglets.

RNA e traction and RT-PCR

Total RNA as e tracted from homogenates of lungs and l mph nodes according to Chen et al. [6]. Primers ORF5-F (5′-GGTGGGCACKGTTTTAGCCTGTC-3′) and ORF5-R (5′-GGTAATAGARAAYGCCAAAAGCACC-3′) ere 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′-AGGGAGCTGCTTGATGA CACAG-3′) ere 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 stain, GenBank accession no. EF112445, and nt 2903 to 3198 of the CH-1a stain). The reverse transcription reaction contained the follo ing 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 nal volume of 20 µl ith DEPC-treated ddH_2O. The reaction mi tures ere incubated at 42 C for 1 h. The PCR reaction as carried out as follo s: 2 µl RT product, 4 µl 5 × PCR buffer, 0.4 mM dNTPs, 20 pmol of each primer, 5U Primer STAR pol merase (TaKaRa, Japan), adjusted to a nal volume of 20 µl ith ddH_2O. C cling conditions included an initial denaturation at 94 C for 5 min, follo ed b 30 c cles ith 94 C for 30 s, 57 C for 30 s and 72 C for 50 s. The nal elongation step as at 72 C for 10 min.

Nucleotide sequencing

The PCR products ampli ed from PRRSV-positive samples ere puri ed using an A Prep DNA Gel E traction Kit (A gen Inc., USA) and cloned into the pSIMPLE-19 vector (TaKaRa, Japan). The target fragments ere sequenced on an ABI-PRISM 377 DNA sequencer.

Bioinformatic anal sis 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) ere multiple-aligned ith CLUSTAL X (version 1.83). A ph logenetic tree as constructed (MEGA version 3.1) in hich the Lel stad sequence (EU genot pe) served as an outgroup control. Pair ise comparison of nucleotide and amino acid sequence similarities as conducted b using MegAlign 5.03 (DNASTAR Lasergene soft are package). A h drophilicit pro le as generated b the method of K te and Doolittle using the DNASIS 2.5 soft are package. The dN and dS ere calculated using the SNAP eb utilit (http://hcv.lanl.gov/content/hcv-db/SNAP/SNAP.html). SNAP (S non mous/Non-s non - mous Anal sis Program) calculates s non mous and non-s non mous substitution rates for codon-aligned nucleotide sequences based on the method of Nelsen et al. [20]. Selective pressure as measured b the rate dN-dS. The ratios $d\mathrm{N}\text{-}d\mathrm{S} > 0$, $d\mathrm{N} - d\mathrm{S} = 0$ and $d\mathrm{N} - d\mathrm{S} < 0$ mean positive selection (adaptive molecular evolution), neutral mutations and negative selection (purif ing selection), respectivel [14]. Variable regions ere anal sed according to the method of Pesente et al. [24]. *N*-linked gl cos lation sites ere predicted ith the *N*-gl coside eb utilit (http:// .hiv.lanl.gov/content/sequence/GLYCOSITE/gl cosite.html). Signal peptide cleavage sites in amino acid sequences ere predicted ith the Signal P 3.0 server eb utilit (http:// .cbs.dtu.dk/services/SignalP/).

The PRRS viruses isolated from southeastern China belonged to subgroup II of the North American genot pe

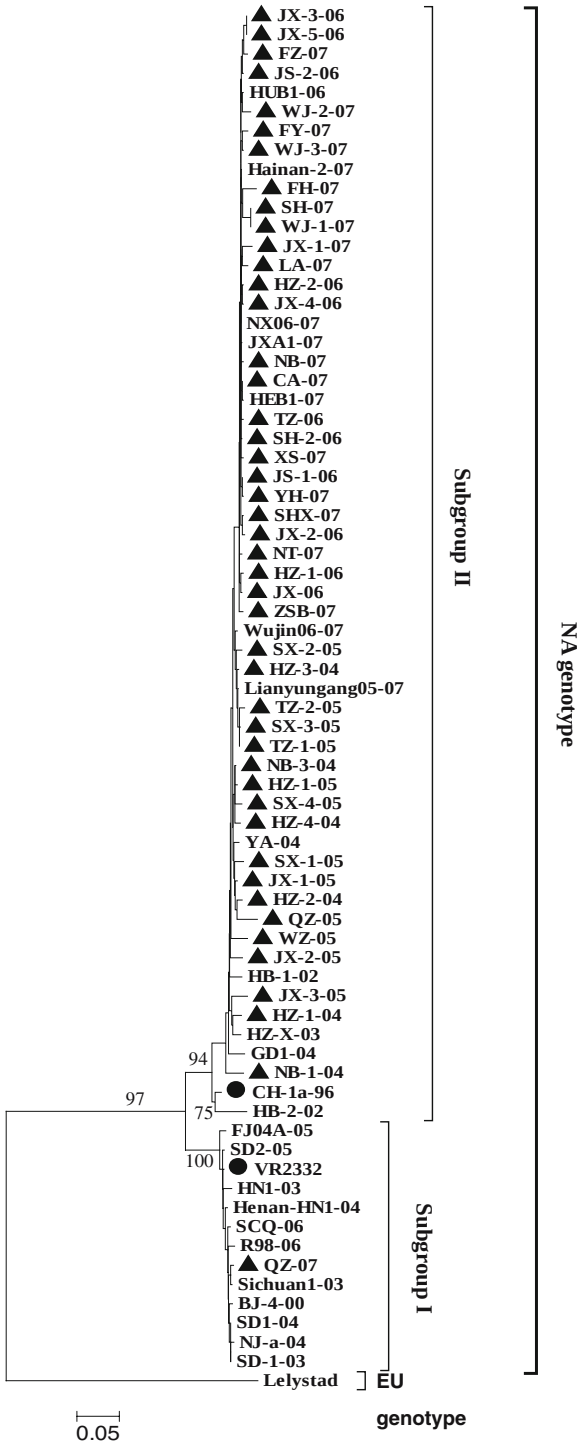
The 603-bp ORF5 fragments from 46 PRRSV-positive samples from 2004 to 2007 ere sequenced (Table 1). Ph logenetic anal sis based on ORF5 revealed that all

Sequences from GenBank (No. 1 25) for comparison ith those of PRRSV isolates used in this stud (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 stud belonged to the NA genot pe (Fig. 1a) and could be clustered into t o major subgroups. Most of the isolates from southeastern China ere classi ed into subgroup II together ith some other Chinese isolates, and onl the isolate QZ-07 belonged to subgroup I, together ith the protot pe NA strain VR-2332. Ho ever, several

(a)



Ph logenetic anal sis depicting the genetic relationship bet een 46 PRRSV isolates in this stud (indicated b *filled triangle*) and other North American genot pe isolates from other regions of China based on the major structural gene ORF5. The protot pe American isolate VR2332 and the rst Chinese isolate CH-1a are indicated b *filled circle*. Each isolate is named for its origin and time of isolation. The tree as constructed using the neighbor-joining algorithm based on the Kimura t o-parameter distance estimation method in MEGA 3.1, and the European t pe isolate Lel stad as rooted as out-group. Bootstrap values representing the major branches are indicated as a percentage for 1,000 replicates. T o main subgroups of PRRSV isolates (I and II) are indicated.

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

(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) identit of ORF5 among subgroup I, subgroup II and VR2332 strains

Subgroup	Identit 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 identit bet een 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 ph logenetic tree could be differentiated b 13 unique amino acid substitution patterns scattering in different regions around GP5 (Fig. 1b).

We found that there ere 25 major nucleotide variation sites among the subgroup I and II PRRSV isolates. Nine of them ere conserved bet een the protot pe US strain VR2332, representing subgroup I isolates, and the "ancestral" Chinese strain CH-1a but ere mutated a a in the majorit of the subgroup II isolates. There ere t elve sites that ere conserved bet een CH-1a and the majorit of the subgroup II isolates but ere different from VR2332. Thus, the CH-1a appeared to represent an evolutionar link bet een subgroups I and II.

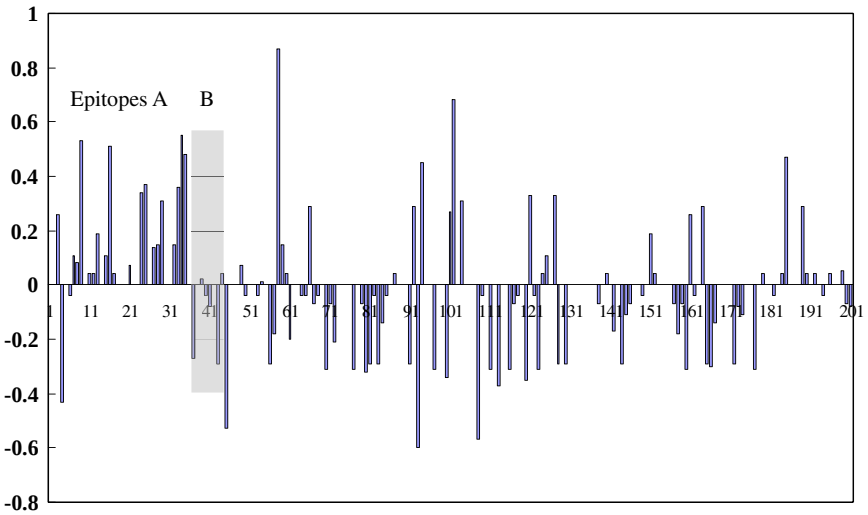
Anal sis of the deduced amino acid sequences of GP5 and the partial NSP2 fragment

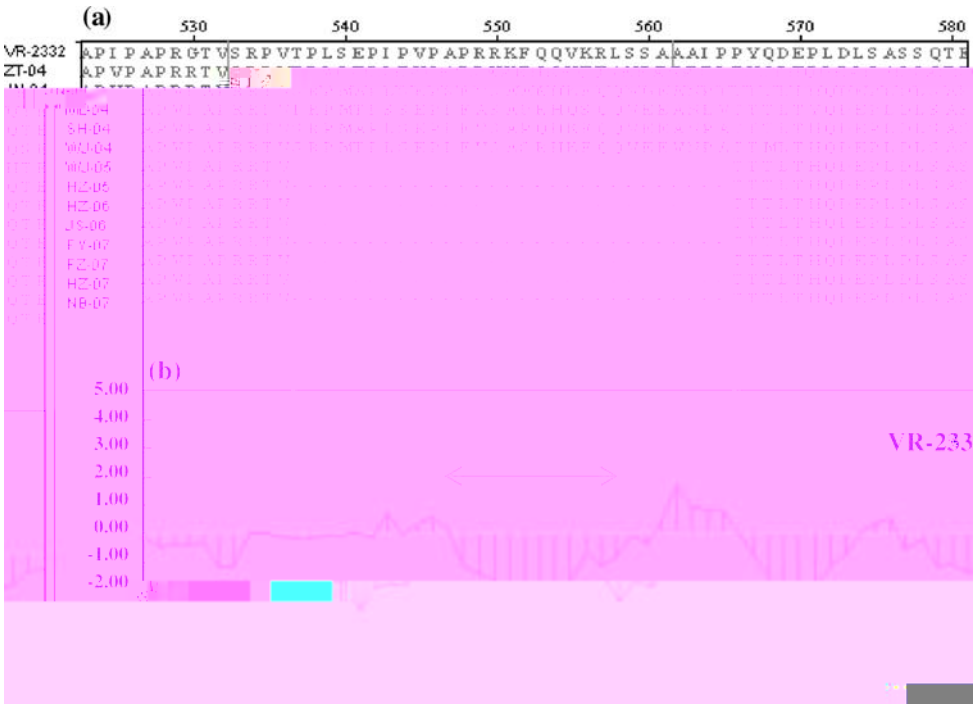
Three variable regions (VR1, VR2 and VR3) ere identi- ed in the signal sequence and putative ectodomain. The N-terminal region covering the three variable regions as apparentl under positive selection (Fig. 2). Of the t o mapped epitopes [23], onl epitope A at residues 27 30 as under positive selection (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 gl cos lation sites (N33, N44, and N51) in the GP5 ectodomain, the N51 site seemed to be conserved in all isolates, hereas the N33 reside as 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).

Anal sis of the partial NSP2 sequences revealed that a 29-aa deletion of a fragment containing a major h dro- philic region had occurred from residues 533 to 561 (Fig. 3a and b). Interestingl , this deletion onl e isted in PRRSV isolates in and after the ear 2005, including all isolates of 2006 and 2007 sequenced in the present stud (Table 1), hile no deletion as 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 s ine industr , and PRRSV strains ith deletion of a de ned region of NSP2 ere isolated from recent outbreaks [28]. Ho ever, it remains unkno n if PRRSV isolates ith this deletion in NSP2 ere responsible for these outbreaks. We attempted to anal se the ph logenetic 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 stud ith VR-2332 as the reference strain ( ) and h drophobicit anal sis of the region (VR2332 strain) ( ). Deletion of 29 aa residues in the top panel is sho n as " ". The arro in the bottom panel indicates the high-h drophilicit region

The majorit of the isolates sequenced in this stud ere clustered in subgroup II (Fig. 1a), a nding that is similar to earlier results ith regard to subt ping of Chinese PRRSV isolates [1, 7]. Onl one isolate, QZ-07, as in subgroup I. It remains unclear to us if this 2007 isolate as a ne introduction into Qu hou, a major pig-producing area, because the 2005 isolate QZ-05 in the same region belonged to subgroup II.

Further anal sis of the encoded amino acids revealed that the majorit 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 PPRSV strain ith mutations at these positions appeared to have been introduced into the region before 2004, ith subsequent spread and mutation ithin the region. This is because it as unlikel that the PRRS viruses in different areas in the region under ent the same mutations in the ears 2004 2005 or even before. Although it remains unkno n if this particular strain evolved from a subgroup I virus, e speculate that the "ancestral" Chinese PRRSV isolate CH-1a, hich as isolated far back in 1996, might have acted as an evolutionar link and undergone mutational divergence into the subgroup II isolates that are dominant in southeastern China. This argument could be supported b the "linkage" pattern of the strain CH-1a: nine out of 25 major substitutions bet een subgroup I and II isolates in the GP5 gene ere conserved bet een VR2332 (subgroup I) and the CH-1a strain (a subset of subgroup II), hile there ere also t elve codons (out of 25) that ere conserved bet een CH-1a and other subgroup II isolates but ere different from VR2332.

SNAP anal sis further revealed that the VR2 region that overlaps the epitopes A and B as ell as VR1 and VR2 in the N-terminal region is under positive selection (Fig. 2), probabl as a result of immunological pressure due to increased vaccination against PRRSV in the past 2 ears, as part of the viral strateg for immune evasion [10]. The positive selection on epitope A (Fig. 2), a deco epitope that ma diminish the immune responsiveness against an adjacent neutrali ing epitope (epitope B) [16], might function in this a , although this requires further e perimental veri cation b mutagenic approaches. We have also seen several additional mutations in isolates from the ears 2006 and 2007 in subgroup II, shifting a a further from historical PRRSV isolates VR2332 and those of earlier ears. These sites ere also located in the three VR regions, indicating that the positive selection events ere still going on.

A 29-aa deletion in NSP2 (corresponding to nt 533 561 of VR2332 ORF1a) as identi ed in strains isolated since 2005 in this stud (Fig. 3), at least 1 ear earlier than the virulent PRRSV ith the same deletion became dominant in China [8, 28], suggesting that these isolates ere all pathogenic in vivo because the ere from diseased pigs. Similar outbreaks of PRRSV infection in pigs ith an NSP2 deletion in this particular region ere also seen in Vietnam [8]. Challenge studies in SPF pigs or PRRSV-free pigs have indicated that the recentl emerged PRRSV in China characteri ed b t o discontiguous deletions in

NSP2 is the cause of the current epi ootics in China [33]. Epitope mapping of PRRSV b phage displa indicated that NSP2 contained a cluster of B-cell epitopes [22]. A previous stud suggested the potential role of the 87-nt deletion in NSP2 in the high pathogenicit of PRRSV [28]. Ho ever, Han et al. [12] found that the NSP2 h pervariable region (324 726 nt) as dispensable for viral replication. Furthermore, the deletion mutants displa ed decreased c tol tic activit 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 a aits further e perimental veri cation.

In summar , it is apparent that subgroup II PRRSV strains ith 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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