

Genetic Diversity of the ORF5 Gene of Porcine Reproductive and Respiratory Syndrome Virus Isolates in Southwest China from 2007 to 2009

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Abstract

To gain insight into the molecular epidemiology and possible mechanisms of genetic variation of porcine reproductive and respiratory syndrome (PRRS) in Yunnan Province of China, the ORF5 gene of 32 PRRSV isolates from clinical samples collected from 2007 to 2009 were sequenced and analyzed. Nucleotide and amino acid analyses were carried out on 32 isolates and representative strains of the North American genotype, European genotype and two representative Chinese isolates. Results revealed that these isolates share 86.9–99.0% nucleotide and 87.5–98.0% amino acid identity with VR-2332 the prototypical North American PRRSV, 61.7–62.9% and 54.3–57.8% with Lelystad virus (LV) the representative strain of European genotype, 91.2–95.4% and 90.0–94.5% with CH-1a that was isolated in mainland China in 1996, 88.1–99.3% and 85.5–99.0% with JX-A1 the representative strain of High pathogenic PRRSV in China, and 86.2–99.8% and 85.5–100.0% between isolated strains of different years, respectively. Phylogenetic analysis revealed that all 32 PRRSV isolates belonged to the North American genotype and were further divided into two different subgenotypes. Subgenotype 1 comprised twenty two Yunnan isolates which divided into two branches. Subgenotype 2 comprised ten isolates which closely related to the RespPRRS vaccine and its parent strain VR-2332. The functional domains of GP5 such as the signal peptide, ectodomain, transmembrane regions and endodomain were identified and some motifs in GP5 with known functions, such as primary neutralizing epitope (PNE) and decoy epitope were also further analyzed. Our study shown the great genetic diversity of PRRSV in southwest China, rendering the guide for control and prevention of this disease.

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Introduction

Porcine reproductive and respiratory syndrome (PRRS) is the most economically significant disease of swine worldwide. The disease mainly causes premature delivery, miscarriage, stillbirth, mummified fetuses, severe pneumonia, edema and conjunctivitis in pigs. Typical PRRS is also called “blue ear” disease due to a representative symptom of the infected piglets [1]. In addition, coinfection and secondary infection causes significantly higher mortality rate [2].

PRRS first emerged in late 1987 in the United States and three years later in Europe. Two genotypes are recognized for Porcine reproductive and respiratory syndrome virus (PRRSV), the North American genotype and the European genotype, as represented by the prototypes VR-2332 and Lelystad virus (LV), respectively [3]. In mainland China, the North American genotype was first reported in 1996 and it has spread throughout the country with considerable genetic variation [4].

PRRSV belongs to the family Arteriviridae in the order Nidovirales, a family of positive-sense, single stranded linear RNA viruses. PRRSV is an enveloped arterivirus, 50–60 nm in size. The genome is about 15 kb in length which contains nine ORFs. The 3' end of the genome encodes four membrane-associated glycoproteins (GP2, GP3, GP4 and GP5, encoded by

subgenomic mRNAs 2a, 3, 4 and 5), two unglycosylated membrane proteins (E and M, encoded by subgenomic mRNAs 2b and 6) and a nucleocapsid protein [5,6]. The 5' untranslated region (5' UTR) includes stem loop 2 which is a key structural element for PRRSV replication and subgenomic mRNA synthesis [7]. A recent study revealed that a 51 amino acid polypeptide referred to as ORF5a was encoded by an alternative ORF of the subgenomic mRNA encoding GP5, and a similar ORF was present as an alternative reading frame in all PRRSV subgenomic RNA5 genes and in all other arteriviruses [8].

The GP5 protein is the primary envelope protein, a glycoprotein of approximately 200 amino acids with an apparent molecular mass of 26 kDa. There are 2–4 glycosylation sites, a 31 amino acid signal peptide and 6 antigenic determinants which induce neutralizing antibodies included in GP5. The presence of a major neutralization epitope in the N-terminal ectodomain implied that GP5 is involved in receptor recognition [9].

As the main candidate protein for development of subunits vaccine, also due to its immunological significance and polymorphic nature, GP5 has been the target for analysis of genetic diversity of PRRSV [10]. In the present study, ORF5 gene of 32 PRRSV isolates from clinical samples in Yunnan (southwest China) during the period of 2007–2009 were sequenced and

analyzed to better understand the molecular epidemiology of PRRS.

Materials and Methods

1. Sample collection

A total of 810 clinical samples including lung, blood and semen were sampled from different swine herds that experienced outbreaks of severe reproductive failure in pregnant sows and respiratory problems in sucking and post-weaning piglets in Yunnan Province, southwestern China from 2007 to 2009.

Sampling procedures were approved by the Animal Care and Use Committee of Yunnan Province, China, which does not issue a number to any animal study. Our sampling processes were assisted by local authorities and veterinarians. Blood was sampled in each PRRS suspect cases, boar semen was collected with an artificial vagina and lung samples were collected during necropsy. Pieces of the tissues from pigs were homogenized for RNA extraction and virus isolation, remaining samples were kept at -70°C .

2. RNA Extraction and Reverse Transcription

Total RNA was extracted from the tissue homogenates using Qiagen RNeasy Mini kit. The extracted RNA was dissolved in RNase-free pure water and used immediately for reverse transcription in a final 20 μl reaction volume. Reaction mixtures consisted of 6 μl RNA solution, 0.5 μl 10 mM Y4 reverse primer, 0.5 μl (40 U) RNAase inhibitor (Toyobo Co. Japan), 4 μl 5 \times MMLV Buffer and 1 μl 10 mM dNTPs. Before 1 μl (100 U) MMLV (Takara Co. Dalian, China) was added, the mix was incubated at 70°C for 5 min and then placed on ice for 5 min. Reverse transcription was conducted at 42°C for 1 hour, with a final termination at 70°C for 15 min.

3. Primer design and RT-PCR

Based upon multiple sequence alignment, a set of PRRSV primers Y3 and Y4 were designed for RT-PCR assay. The sense primer Y3 was 5'-cca tgt tgg aga aat gct tg-3' and the reverse primer Y4 was 5'-cgg cgg cga ctc acc ttg ag-3'. The cycling conditions were 95°C for 5 min, followed by 30 cycles of 94°C for 40 sec, 56°C for 40 sec, 72°C for 60 sec, and a final extension at 72°C for 10 min. This yielded a 708 bp PCR product containing complete ORF5 gene of PRRSV.

4. Cloning, sequencing and phylogenetic analysis of PCR products

The PCR products were purified using PCR purification kit (TaKaRa Co. Dalian, China) and cloned into the pMD18-T vector (TaKaRa Co. Dalian, China). The plasmid was then used to transform *E. Coli* DH5 α . The plasmids were extracted and the inserts sequenced at Sangon Biological Engineering Company (Shanghai, China). The 708 nt consensus sequences were aligned using DNAMAN (version 6.0, Lynnon Co.). Genetic distances between PRRSV sequences were calculated with the DNASTar software (Hitachi, version 7.1, Madison WI, USA). Phylogenetic and molecular evolutionary genetic analyses were conducted using the neighbor-joining method with MEGA (Version 4, Tamura, Dudley, Nei, and Kumar 2007). A total of 29 North American genotypes and 1 European genotype were used as references in the analysis (Table 1).

5. Amino acid analysis of GP5

To get a better understanding of the genetic relationship and evolution of PRRSV in Yunnan from 2007 to 2009, phylogenetic analysis was carried out based on the sequences of the ORF5 gene

Table 1. Representative PRRSV Strains used in the phylogenetic and sequence analyses.

NO.	Name	Area	Year	Accession No.
1	LV	Netherlands	1993	M96262
2	VR-2332	USA	1995	U87392
3	Clone20	China:Hubei	2003	FJ899592
4	4139	USA:MN	2005	EU756000
5	RespPRRS	USA	2005	AF066183
6	SCQ	China:Sichuan	2006	DQ379479
7	BHA	China:Guangxi	2006	EF104600
8	CC-1	China:Jilin	2006	EF153486
9	HB-3(cz)	China:Hebei	2006	EU478435
10	5554	Canada:MB	2006	EU756566
11	JSyx	China:Jiangsu	2006	EU939312
12	JX-A1*	China:Jiangxi	2006	EF112445
13	GZZB	China:Guizhou	2007	EU140611
14	JX3	China:Jiangxi	2006	EU213137
15	Hainan-2	China:Hainan	2007	EF398052
16	GDB11	China:Guangdong	2007	GU980181
17	pJX148	China:Jiangxi	2007	EF488048
18	HBAN1	China:Hubei	2008	EU371943
19	4034-2-V-2008	South Korea	2008	FJ972733
20	JN-H5	China: Shandong	2008	HM016158
21	FJ07B	China:Fujian	2008	GU358694
22	CQJ-1	China: Chongqing	2008	FJ919335
23	AHXS4	China:Hubei	2008	EU399857
24	JX5	China:Jiangxi	2006	EU213139
25	GZJL	China: Guizhou	2009	FJ947000
26	BZHM	China:Shandong	2009	GU977226
27	BB0907	China:Guangxi	2009	HQ315835
28	5112HCM	Viet Nam:Ho Chi Minh	2010	HQ700878
29	Ch-1a#	China:Beijing	1996	AY032626
30	WUH1	HuBei	2007	EU187484

*JX-A1 is the representative strain of HP-PRRSV in China since 2006;
#Ch-1a is the first PRRSV isolate in mainland China in 1996.
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of Yunnan Province isolated PRRSV strains, together with some North American genotype isolates from China and other countries. In particular, some known functional domains of GP5 such as the signal peptide, ectodomain, transmembrane regions and endodomain were identified, some motifs in GP5 like primary neutralizing epitope (PNE) and decoy epitope were also analyzed according to a previous report [11].

Results

1. RT-PCR survey of clinical samples

PRRSV was detected in 51 (10 strains in 2007, 25 strains in 2008 and 16 strains in 2009) out of 810 clinical samples by RT-PCR, the total positive rate was 6.3% (51/810).

2. Sequence and phylogenetic analyses of ORF5

51 strains sequences were designated in order of isolation from YN07-00 to YN09-50. A further blast-search using the public database

Table 2. Information of 32 PRRSV isolated in Yunnan Province, China.

No.	Isolate	Isolation year	Genebank accession No.	No.	Isolate	Isolation year	Genebank accession No.
1	yn07-0	2007	JF920761	17	yn08-20	2008	JF920771
2	YN-1	2007	FJ151490	18	yn08-23	2008	JF920774
3	YN-2	2007	FJ361883	19	yn08-24	2008	JF920775
4	YN-3	2007	FJ361884	20	yn08-28	2008	JF920779
5	YN-4	2007	FJ361885	21	yn08-29	2008	JF920780
6	YN-5	2007	FJ361886	22	yn08-31	2008	JF920782
7	YN-6	2007	FJ361887	23	yn09-35	2009	JF920786
8	YN-8	2007	FJ361889	24	yn09-37	2009	JF920788
9	YN-10	2008	FJ361891	25	yn09-38	2009	JF920789
10	yn08-11	2008	JF920762	26	yn09-39	2009	JF920790
11	yn08-12	2008	JF920763	27	yn09-40	2009	JF920791
12	yn08-13	2008	JF920764	28	yn09-42	2009	JF920793
13	yn08-14	2008	JF920765	29	yn09-43	2009	JF920794
14	yn08-17	2008	JF920768	30	yn09-44	2009	JF920795
15	yn08-18	2008	JF920769	31	yn09-47	2009	JF920798
16	yn08-19	2008	JF920770	32	yn09-50	2009	JF920801

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in GenBank of the amplification fragment found 19 sequences (No. 7, 9, 15, 16, 21, 22, 25, 26, 27, 30, 32, 33, 34, 36, 41, 45, 46, 48, 49) shared identical sequences and were removed, so that finally 32 Yunnan strains were identified in this study. These sequences for GP5 genes of individual isolates were deposited in GenBank (Table 2).

Further sequence analysis identified all 32 isolated strains belonging to North American genotype. Compared with representative strain VR-2332 for the North American genotype, LV for the European genotype, CH-1a and JX-A1 for Chinese isolates, nucleotide homologies ranged from 86.9–99.0%, 61.7–62.9%, 91.2–95.4% and 88.1–99.3%, respectively, and 86.2–99.8% between different years isolated strains (Table 3). A phylogenetic tree was constructed using the neighbor-joining method based on sequences of 32 isolates of PRRSV from Yunnan Province and 29 references PRRSV sequences from China and other countries. As shown in Figure 1, all 32 PRRSV isolates were divided into two different subgenotypes. Subgenotype 1 comprised twenty two Yunnan isolates which divided into two branches. One branch include 13 strains which all the strains isolated from Yunnan and the other include nine Yunnan isolate, along with JX-

A1 the representative strain of HP-PRRSV in China since 2006 and other related PRRSV. Subgenotype 2 comprised ten isolates that were highly homologous to each other and closely related to the RespPRRS vaccine and its parent strain VR-2332.

3. Amino acid analysis of the GP5

To investigate the amino acid difference among the two subgenotype isolates, the GP5 amino acid sequences of 32 PRRSV isolates were aligned, together with some North American genotype isolates from China and other countries. Multiple alignments of GP5 sequences of Chinese PRRSV isolates indicated that all 32 isolates encode a GP5 protein of 200 amino acid residues, but that substitutions were extensive (Fig. 2).

Pairwise comparison revealed 85.5–100.0% deduced amino acid similarity between the 32 Yunnan PRRSV isolates and shared 87.5–98.0% amino acid identity with the prototypical North American PRRSV VR-2332, 54.3–57.8% with strain LV the European type, 85.5–99.0% with JX-A1, and 90.0–94.5% with CH-1a (Table 3).

Compared with those in subgenotypes 2, Yunnan PRRSV strains in subgenotypes 1 were found to be highly variable in the

Table 3. Analysis of nucleotide and amino acid identity of ORF5 among 32 PRRSV isolates and with represent PRRSV isolates LV, VR-2332, RespPRRSV, Ch-1a, and highly pathogenic strain JX-A1.

Isolation Years(No.)		2007	2008	2009	LV	VR-2332	RespPRRS	JX-A1	Ch-1a
2007(9)	nt	86.9–99.8	86.2–99.8	86.2–99.8	61.7–62.9	87.6–99.0	87.2–99.3	88.4–98.8	91.5–94.9
	aa	86.5–100.0	85.5–99.5	86.0–99.5	55.3–57.3	88.0–98.0	87.0–99.0	86.0–97.5	90.5–92.5
2008(13)	nt	-	86.2–99.8	86.2–99.8	61.7–62.9	86.9–99.0	87.2–99.3	88.1–99.3	91.2–95.4
	aa	-	85.5–99.5	86.0–100.0	54.3–57.8	87.5–98.0	86.5–99.0	85.5–99.0	90.0–93.5
2009(10)	nt	-	-	94.5–99.8	61.9–62.2	86.9–89.4	87.1–89.1	93.7–99.2	92.9–95.2
	aa	-	-	94.0–99.5	56.3–57.3	87.5–89.0	86.5–88.0	93.0–99.0	92.5–94.5

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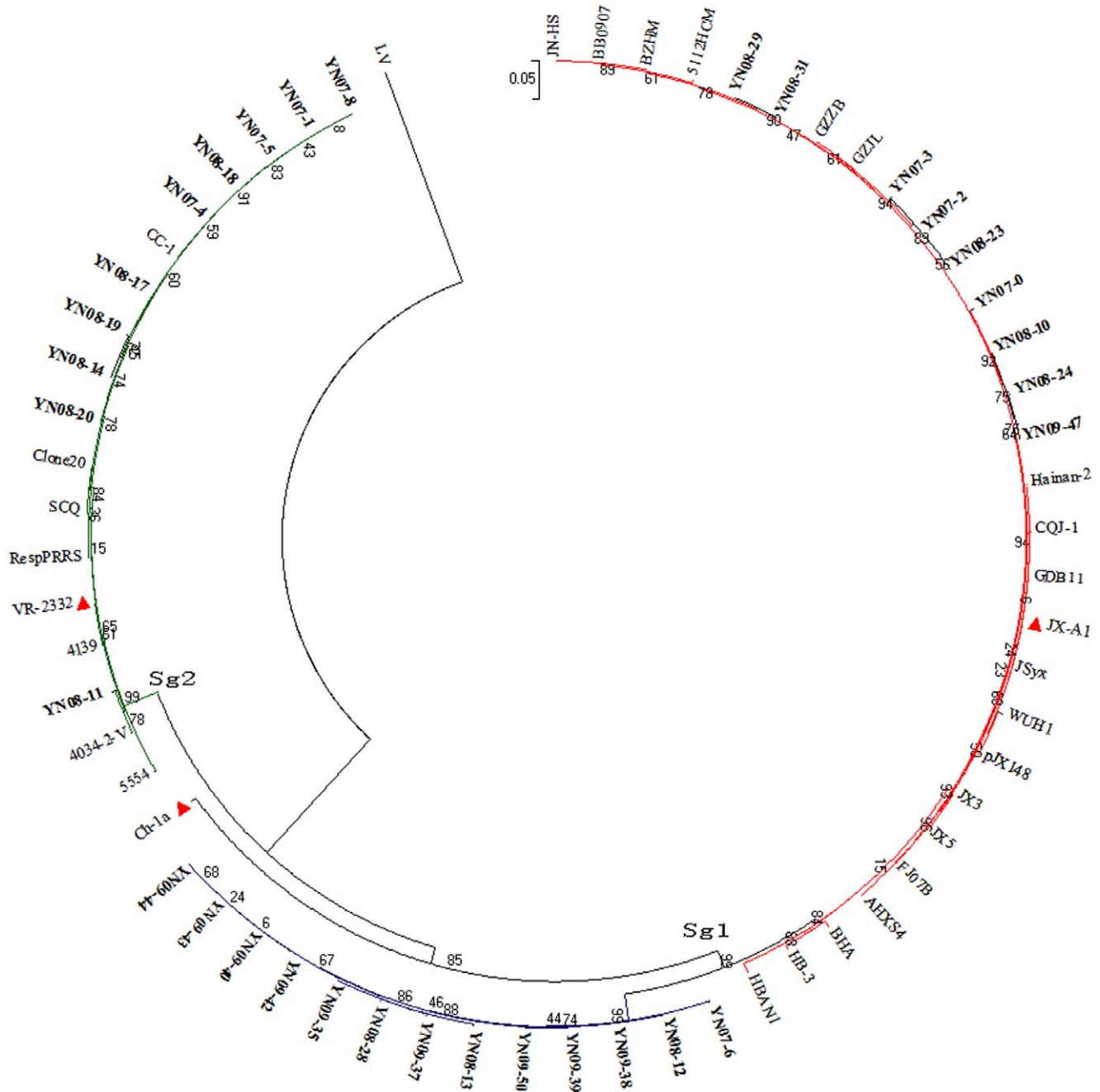


Figure 1. Phylogenetic relationship of PRRSVs. A neighbor-joining tree was constructed based on sequences of the ORF5 gene of PRRSV, with bootstrap values calculated from 1,000 replicates. Subgroups are marked with different colors and the strains isolated in this study are indicated in bold.

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primary neutralizing epitope (PNE) and contained an amino acid mutation (F39/L39→I39). To differentiate these isolates of different subgenotypes, the unique or concurrent amino acid mutations were observed. Three (L39→F39/I39, N58→S58/Q58, K59→H59; and G9→C9, V94→A94, V185→A185) and two (R13→Q13, R151→G151) unique mutations observed in subgenotype 1 and subgenotype 2, respectively. Other representative concurrent amino acid mutations (S16→F16, C24→Y24, A29→V29, S66→T66, A92→G92, 101F→101Y, 102V→102Y, T121→I121, A137→S137, I161→V161, R164→G164, I189→L189, P200→L200) were also found in two subgenotypes.

Discussion

PRRS has become widespread since its initial outbreak in mainland China at the end of 1995, the infection rate of Chinese swine herds has risen to 90% [12]. An increasing number of field PRRSV strains have been isolated from different regions of China. However, all described PRRS viruses in China, including the HP-PRRSV isolates almost simultaneously emerged both in China and Vietnam in 2006, are of the North American genotype [13]. Recently, there was report that European genotype PRRSV isolates were detected in swine herds both with and without

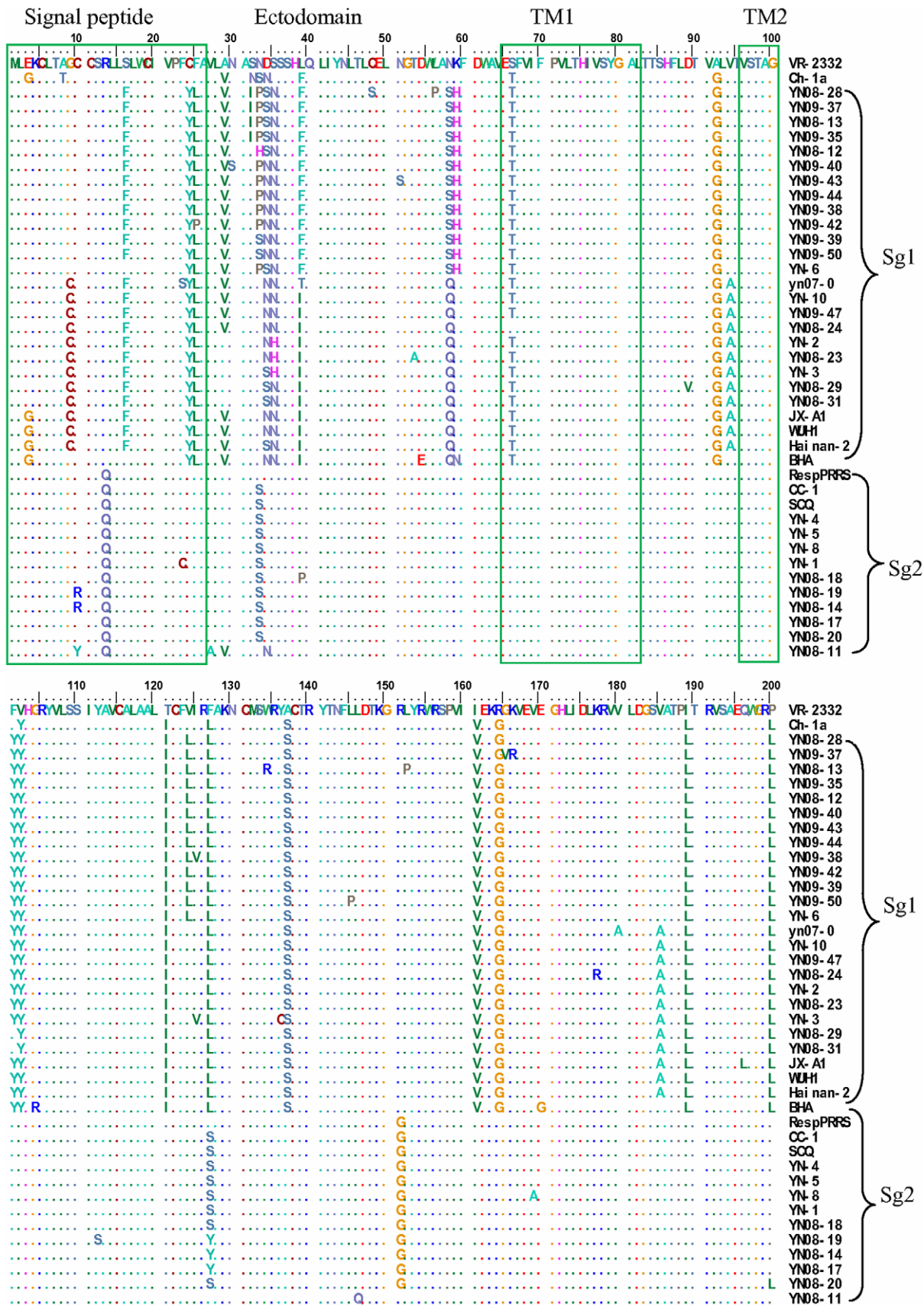


Figure 2. Analysis and comparison of amino acid mutations in ORF5 gene of 32 Chinese isolates and 9 reference strains. Dots (.) indicate the same amino acids as in VR2332 and substitutions are indicated by the amino acid letter codes. The functional domains indicated by color boxes are reported according to Zhou et al. [11]. doi:10.1371/journal.pone.0033756.g002

clinical symptoms in China. Further sequence analysis revealed that they shared 87.0–91.5% and 58.0–58.2% identity with the European and North American genotype prototypic strains LV and VR-2332, respectively [12]. The coexistence of both genotypes PRRSV in Chinese swine herds provides a suitable environment for developing new viral biological characteristics. Meanwhile, it complicates the prevention and control of PRRSV in China.

ORF5 gene has been considered to be one of the least conserved ORFs of PRRSV with respective sequence variability of up to 17%, which lead to a high degree of genetic polymorphism under constant selective pressure even within the same genotype, and the emergence of acute PRRS or highly variable PRRSV. The amino acid substitutions of the GP5 protein between the PRRSV isolates and representatives are mostly observed in the heterogenic signal sequence and the two hyper variable regions [14,15]. Studies based on ORF5 gene sequences of Chinese isolates have revealed extensive genetic diversity, even in highly pathogenic PRRSV that emerged in 2006 [11,16].

GP5 of PRRSV is associated with virus neutralization. Three B cell epitopes, two T cell epitopes exists in the North American genotype, and at least two of these epitopes are neutralization epitopes. The PNE of both North American and European PRRSV strains are located in the middle of the GP5 ectodomain, which previously identified as S37H(F/L)QLIYN or S37H(F/L)QLIYN LTLCELNG. The PNE plays an important role in viral neutralization, and the residues H38L39/F39 in this domain are considered to be the critical amino acids of the neutralizing epitope [17,18]. In this study, analysis of PNE found that the key antibody combined site (L/F)₃₉ were mutation to I39 (isolates YN-2, YN-3, YN08-23, YN08-24, YN08-29, YN08-31, YN09-47), T39 (YN07-0) or P39 (YN-1), also amino acid mutations C48→S48 (YN08-28) and N51→S51 (YN09-43) observed in Yunnan isolates. These mutations likely lead to the failure of immunization in Yunnan Province. Nevertheless, further studies are necessary to provide additional evidence. In consideration of that the immunogenicity and specificity of the linear neutralization epitopes are not only determined by the amino acid sequence, but also by conformational effects that are probably induced by amino acid changes upstream or downstream of the epitope [19].

The epitope comprised of A27/V27LVN near the PNE is the main antibody recognition site for epitope A. This epitope may also act as a decoy, which elicit most of the antibodies against GP5 and delay the introduction of neutralizing antibodies for at the least 3 weeks [18]. Among GP5 gene of 32 Yunnan isolates, this

decoy epitope was conserved in Subgenotype group 1, whereas nine of ten strains in Subgenotype group 2 contained one amino acid mutation at position 29 (V→A). The replacement of these four amino acids considerably reduced the recognition of this epitope.

The widely use of modified live-attenuated vaccines have reduced the incidence and severity of PRRS outbreaks in many countries. Nevertheless, live PRRSV vaccine virus exhibits an untoward tendency to spread and reversion towards virulence. The periodic identification of vaccine-like isolates poised the challenge for detection and characterization of vaccine-like isolates and field isolates of PRRSV [20–22]. Comparisons between the vaccine virus and its parental virus showed that ORF5 codon position 13, 151 and ORF6 codon position 15 play a key role in denaturing VR2332 to RespPRRSV [23,24]. When VR-2332 was attenuated to the RespPRRS/Repro vaccine strain, R13 and R151 were altered to Q13 and G151, respectively [25]. Since R13Q was included in the signal peptide, this mutation may also have an effect on transfer of GP5. It has been reported that R151G mutation has an effect on hydrophobic areas and reduces the virulence of Czech strain V502 after 152 passages that resulted in this mutation [26]. In this study, R13Q and R151G were observed in nine of ten isolates in subgenotype 2, including a middle virulent strain YN-1 that has been isolated and identified, nevertheless there is no evidence to prove that the field isolates are responsible for the reversion of the modified live PRRS vaccine.

In summary, nucleotide and amino acid sequence analysis of GP5 of 32 PRRSV strains isolated in Yunnan from 2007 to 2009 revealed the prevalence of two subgenotypes of PRRSV in this region. All strains isolated had divergence obviously from the first Chinese isolate Ch-1a, and ten isolates (subgenotype 2) were highly related to the vaccine strain. The occurrence of these vaccine strain related isolates is presumably related to the extensive use of the attenuated modified live PRRS vaccine since live PRRSV vaccines can revert to virulence. In addition, our study determined that a branch of subgenotype 1 is entirely made up of Yunnan isolates, which raising the possibility that PRRSV prevalence in this region has evolved into a separate branch.

Author Contributions

Conceived and designed the experiments: G. Yin. Performed the experiments: LG SG. Analyzed the data: WL. Wrote the paper: WL G. Yin. Sampling: G. Yang XS.

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