Genetic analysis of porcine productive and respiratory syndrome virus between 2013 and 2014 in Southern parts of China: identification of several novel strains with amino acid deletions or insertions in nsp2

Shaofeng Hong†, Ying Wei†, Siyuan Lin, Jiabing Huang, Wei He, Jing Yao, Ying Chen, Ouyang Kang, Weijian Huang* and Zuzhang Wei*

Abstract

Background: Porcine respiratory and reproductive syndrome virus (PRRSV) is one of the most economically significant pathogens in the Chinese swine industry. ORF5 and nsp2 are highly variable regions of the PRRSV genome. Therefore, nsp2 and GP5 are often selected for investigation of variations and phylogenetic analyses for their genetic diversities. Knowledge of the molecular evolution of PRRSV field strains may contribute to the control of PRRS in China.

Results: The results of multiple sequence alignments of GP5 showed that there is 84.5–100% aa identity among the 56 strains in this study. These strains shared 84.5–99.0% aa identity with the prototypical type 2 PRRSV VR-2332 and 56.6–59.2% with strain LV, prototypical type 1 PRRSV. Phylogenetic analysis showed there is considerable diversity among PRRSV ORF5 and the existence of two lineages (5 and 8). Most of the strains were classified into lineage 8 with multiple sub-lineages (3, 4 and 6). Moreover, PRRSV strains with 5 novel patterns of deletions or insertions in the nsp2 region were found.

Conclusions: Phylogenetic analysis based on ORF5 sequences indicated the diversity of PRRSV in southern parts of China and the strains with 30 aa deletion in nsp2 are dominant in the porcine population. Also, new PRRSV strains with different patterns of deletions or insertions in nsp2 are emerging. The data presented here constitute a useful basis for further epidemiological studies regarding the heterogeneity of PRRSV strains in China and provide a basis for the prevention of PRRS in southern parts of China.

Keywords: PRRSV, Genetic analysis, GP5, nsp2, Deletion

Background

Porcine reproductive and respiratory syndrome virus (PRRSV) is acknowledged as one of the most economically important diseases for the swine industry worldwide [1]. PRRSV, the etiological agent of porcine reproductive and respiratory syndrome (PRRS), is a single-stranded, enveloped, RNA virus. The PRRSV genome consists of approximately 15.4 kb and contains a 5′-untranslated region (UTR), open reading frames (ORFs), a 3′-UTR and a 3′-poly(A) tail. The 5′ two-thirds of the genome encodes polyproteins that are processed by viral protease to 14 nonstructural proteins (npsps) [2]. The 3′ one-third region of genome encodes the structural proteins that are translated from a 3′-5′ co-terminal, nested set of subgenomic mRNAs. In addition to the three major structural proteins, GP5, M and N, the genome of

* Correspondence: weijianhuang-1@163.com; huangweijian-1@163.com; zuzhangwei@gxu.edu.cn
† Shaofeng Hong and Ying Wei contributed equally to this work.
Laboratory of Animal infectious Diseases and Molecular Immunology, College of Animal Science and Technology, Guangxi University, Nanning 530005, People’s Republic of China

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PRRSV encodes minor structural proteins, GP2, 2b, GP3, GP4 and ORF5a [3–5].

It has been shown that PRRSV is continuously evolving through point mutations and genome recombination, which can lead to some new emerging antigenic variant strains [6]. According to the genetic diversity, PRRSV has been classified as two separate species: type 1 (European) PRRSV and type 2 (North American) PRRSV. The two genotypes share about 60% identity at the nucleotide level [7]. GP5 is highly variable and contains important immunological domains associated with viral neutralization [8]. Nsp2 is the most variable region of PRRSV genome and substitutions, deletions and insertions have been observed in the nsp2 coding region [9, 10]. Therefore, nsp2 and GP5 are often selected for investigation of variations and phylogenetic analyses for their genetic diversities. The genetically extensive variation of PRRSV is likely to pose a major obstacle for the effective control of the most economically significant disease that affects the swine industry [11].

PRRS outbreaks were documented in an intensive pig farm in China at the end of 1995, and it has become one of the most important swine diseases in the Chinese swine industry. In 2006, a large outbreak of porcine high fever syndrome (PHFSD), caused by a highly pathogenic PRRSV (HP-PRRSV), emerged in China and affected over 20 million pigs with about 400,000 fatal cases [12]. The disease was subsequently confirmed in southeastern Asian countries including Vietnam, Laos and the Philippines, and this has caused disastrous damage to the swine industry [13, 14]. Whole-genome analysis of the isolated viruses revealed that these PRRSV strains could be grouped into genotype 2 and were highly homologous to a Chinese isolate HB-1 (96.5% nucleotide identity) [15]. It was furthermore observed that these emerging strains contained a single amino acid deletion at position 481 and a 29-amino acid deletion from position 532 to 560 in nsp2 [12, 16]. The study with regards to the origin of the HP-PRRSV did not find recombination or large fragment replacement, which suggests that all HP-PRRSVs originated from the same Chinese ancestor by gradual evolution [15].

Guangxi is one of the biggest breeding regions in the southern parts of China. The genotype prevalence of PRRSV in South China is not currently known. The aim of this study is to investigate the genetic variation of PRRSV using strains isolated in 2013–2014 from pigs that exhibited symptoms of the disease.

Results
Prevalence of PRRSV in Guangxi Province, China from 2013 to 2014

Of the 475 filed samples collected from clinically diseased pigs found between 2013 and 2014 in Guangxi Province of China, 133 samples (28%) were positive for PRRSV, as determined by specific PCR. These results indicated that PRRSV was widely distributed among swine populations in the southern parts of China.

Sequence analyses of the ORF5 gene and nsp2 hypervariable regions of PRRSV

One hundred and thirty-three PRRSV positive samples were used for ORF5 gene and nsp2 HVR amplification. Fifty-six ORF5 sequences and 35 nsp2 HVR sequences were selected for sequencing and analysis (Table 1). To investigate the amino acid difference among PRRSV strains, the GP5 amino acid sequences of 56 PRRSV strains were aligned, together with some North American genotype strains and those from China and other countries. The results showed that all 56 strains encoded a GP5 protein of 200 amino acid residues, but substitutions were extensive (Fig. 1). Sequence alignments revealed that there was 84.5–100% amino acid identity between the 56 Guangxi PRRSV strains and shared 84.5–99.0% amino acid identity with the prototypical type 2 PRRSV VR-2332, 87.0–99.0% with JX-A1, 89.5–92.5% with CH-1a and 56.6–59.2% with prototypical type 1 PRRSV strain LV (Additional file 1: Table S1).

To investigate variation in the deduced amino acid sequences of ORF5 gene products, the amino acid sequences of 56 PRRSV strains including some representative strains were aligned. As shown in Fig. 1, critical amino acid variations in some motifs and regions such as the peptide signal, HRV, the decoy epitope (DCE), the primary neutralizing epitope (PNE) and T epitopes were found in GP5 of these strains. Decay epitopes of VR-2332 is 27VLAX30 and of JXA1 is 27VLVN30. Strains GXLB1403, GXGL1305a/b and GXNN1310b/c contained the same aa in their decoy epitopes (27VLAN30) as those of VR2332. Specific substitution at position 27 (27V→27A) was found in strain GXWZ1401b, while strains GXGG1305a, GXGG1306 and GXBH1311b contained variations at positions 28 (28L→28P) and strains GXBS1401a, GXWZ1301a and GXYL1308b have a specific substitution at position 30 (30N→30D) in the decoy epitope compared to those of VR2332 and JXA1. The other strains have the same aa at the decoy epitope as those of JXA1. Great diversities in HRV1 and HRV2 were found at amino acid positions 32–37 and 57–62, respectively. Specifically, substitutions (S32→N32, S32→G32, N34→S34, N35→G35 and S36→G36) in HRV1 of some strains, resulted in loss or gain of the N-linked glycosylation site at specific position in GP5 (Table 2). Amino acid substitutions in the primary neutralizing epitope were also found. Strains GXLB1403, GXGL1305a/b and GXNN1310b/c contained the same aa in the primary neutralizing epitope (37SHLQILNYLN35) as those of VR2332. All PRRSV strains in sub-genotypes
## Table 1 Geographic origin and amplified sequence size of target genes from clinical samples in this study

| No | Name of strain | Collection date | Area   | ORF5(bp)/accession number | NSP2(bp)/accession number |
|----|----------------|-----------------|--------|---------------------------|---------------------------|
| 1  | GXBH1310b      | 2013.10         | Baihai | 603/MG604994              | NA                        |
| 2  | GXBH1311a      | 2013.11         | Baihai | 603/MG604995              | NA                        |
| 3  | GXBH1311b      | 2013.11         | Baihai | 603/MG605048              | NA                        |
| 4  | GXBH1404       | 2014.04         | Baihai | 603/MG605047              | 1322/MG604959             |
| 5  | GXBS1310       | 2013.10         | Baise  | 603/MG605046              | 1682/MG604960             |
| 6  | GXBS1401a      | 2014.01         | Baise  | 603/MG605045              | 1679/MG604961             |
| 7  | GXBS1410a      | 2014.10         | Baise  | 603/MG605043              | 1682/MG604962             |
| 8  | GXBS1410b      | 2014.10         | Baise  | 603/MG605042              | 1682/MG604963             |
| 9  | GXBS1410c      | 2014.10         | Baise  | 603/MG605041              | 1682/MG604964             |
| 10 | GXGS1304       | 2013.04         | Guigang| 603/MG605040              | NA                        |
| 11 | GXGS1305a      | 2013.05         | Guigang| 603/MG605039              | NA                        |
| 12 | GXGS1305b      | 2013.05         | Guigang| 603/MG605038              | NA                        |
| 13 | GXGS1306       | 2013.06         | Guigang| 603/MG605037              | 1682/MG604965             |
| 14 | GXGL1305a      | 2013.05         | Guilin | 603/MG605036              | NA                        |
| 15 | GXGL1305b      | 2013.05         | Guilin | 603/MG605035              | NA                        |
| 16 | GXHZ1401       | 2014.01         | Hezhou | 603/MG604999              | 1322/MG604966             |
| 17 | GXLB1403       | 2014.03         | Laibin | 603/MG605034              | NA                        |
| 18 | GXLZ1405       | 2014.05         | Laibin | 603/MG605033              | 1682/MG604968             |
| 19 | GXLZ1306b      | 2013.06         | Liuzhou| 603/MG605032              | 1682/MG604967             |
| 20 | GXLZ1306c      | 2013.06         | Liuzhou| 603/MG605031              | NA                        |
| 21 | GXNN1304       | 2013.04         | Nanning| 603/MG605030              | 1682/MG604969             |
| 22 | GXNN1305a      | 2013.05         | Nanning| 603/MG605029              | 1682/MG604970             |
| 23 | GXNN1305b      | 2013.05         | Nanning| 603/MG605028              | 1682/MG604971             |
| 24 | GXNN1305c      | 2013.05         | Nanning| 603/MG605027              | 1682/MG604972             |
| 25 | GXNN1305d      | 2013.05         | Nanning| 603/MG605026              | NA                        |
| 26 | GXNN1305e      | 2013.05         | Nanning| 603/MG605025              | 1682/MG604973             |
| 27 | GXNN1307       | 2013.07         | Nanning| 603/MG605024              | NA                        |
| 28 | GXNN1309a      | 2013.09         | Nanning| 603/MG605023              | NA                        |
| 29 | GXNN1310a      | 2013.10         | Nanning| 603/MG605022              | NA                        |
| 30 | GXNN1310b      | 2013.10         | Nanning| 603/MG605021              | NA                        |
| 31 | GXNN1310c      | 2013.10         | Nanning| 603/MG605020              | NA                        |
| 33 | GXNN1310f      | 2013.10         | Nanning| 603/MG605019              | NA                        |
| 34 | GXNN1312c      | 2013.12         | Nanning| 603/MG605018              | 1682/MG604974             |
| 35 | GXNN1396       | 2013.09         | Nanning| 603/MG605049              | 1626/MG604975             |
| 36 | GXNN1407a      | 2014.07         | Nanning| 603/MG604998              | 1685/MG604976             |
| 37 | GXNN1407b      | 2014.07         | Nanning| 603/MG604997              | 1682/MG604977             |
| 38 | GXNN1409       | 2014.09         | Nanning| 603/MG604996              | 1682/MG604978             |
| 39 | GXNN1410a      | 2014.10         | Nanning| 603/MG605017              | 1682/MG604979             |
| 40 | GXQZ1408       | 2014.08         | Qinzhou| 603/MG605016              | NA                        |
| 41 | GXWZ1301a      | 2013.01         | Wuzhou | 603/MG605015              | 1682/MG604981             |
| 42 | GXWZ1410a      | 2014.10         | Wuzhou | 603/MG605014              | NA                        |
| 43 | GXWZ1410b      | 2014.10         | Wuzhou | 603/MG605013              | 1682/MG604982             |
| 44 | GXWZ1410c      | 2014.10         | Wuzhou | 603/MG605012              | NA                        |
| 45 | GXYL1304       | 2013.04         | Yulin  | 603/MG605011              | 1682/MG604983             |
representative of JXA1, contained an amino acid mutation (39L → 39I) when compared with VR-2332, while strains GXLB1403 and GXYL1403a contain variations at positions 39 (39L → 39F). Substitutions (N58 → Q/G/K/R58, K59 → H/N59 and D60 → N60) in HVR2 were observed in some strains compared with VR2332. In T cell epitopes, strains GXGG1305a, GXGG1305b and GXGG1306 carried substitutions at positions 126 (I126 → T126). Some strains carried substitutions at position at 151 (R151 → K151) (Fig. 1).

Identification of several novel strains with amino acids deletions or insertions in nsp2
The nsp2 gene has the highest genetic diversity in the genomes of PRRSV field strains and also was used as an epidemiological genetic marker. To investigate the amino acid differences among PRRSV strains, a predicted 1862-bp DNA fragment containing nsp2 HVR from 35 positive samples was amplified, cloned and sequenced. As shown in Table 1, the amplified nsp2 HVR exhibited various lengths. Compared to strains VR-2332, 1 of 35 nsp2 sequences was 1722 nucleotides in length which is the same as that of VR-2332. 29 out of 35 nsp2 HV region sequences had the same length of 1682 nucleotides, containing the same 30-aa deletion as JXA1 and other HP-PRRSV strains, suggesting that strains with a 30 aa deletion in nsp2 is the dominating strain circulating in the southern parts of China.

Strains GXYL1310, GDHZ1401 and GXBH1404 had the same length of 1322 nucleotides and were found to contain the same 30-aa deletion as JXA1. They also have an extra continuous 120 aa deletion in nsp2. Strains GBS1401a, GXNN1396 and GXYL1403c contained a discontinuous 31, 49 and 123 aa deletion in their HVR, respectively, compared with strain VR-2332. We also found that one isolated strain (GXNN1407a) contained a 30 aa deletion and 1 aa insertion compared with VR-2332 and JXA1 (Fig. 2). Pairwise comparisons revealed that 86.4–100% nucleotide identity and 84.5–100% amino acid identity between the 35 Guangxi PRRSV strains and shared 65.9%–99.2% amino acid identity with the prototypical type 2 PRRSV VR-2332, 87.0–99.0% with JX-A1, 89.5–92.5% with CH-1a and 56.6–59.2% with strain LV of the European type. The data suggested that nsp2 is highly variable and novel HP-PRRSV strains with aa deletions and insertions in the nsp2 are emerging.

Phylogenetic analyses of the ORF5 gene and nsp2 hypervariable region of PRRSV
To gain a better understanding of the genetic relationship, the phylogenetic analysis based on deduced amino acid sequences of ORF5 gene products was conducted by using the 56 ORF5 sequences obtained in this study together with 39 downloaded referenced PRRSV sequences (Table 3). As shown in Fig. 3, the results showed that the PRRSV strains in this study could be divided into 2 different subgroups. Among the 56 GP5 sequences, two strains (GXBL1403 and GXYL1403a) belonged to lineage 5, as represented by VR-2332. Fifty-four strains belonged to lineage 8, with six strains being classified as sub-lineage 8.4 and three strains being classified as sub-lineage 8.6. The other 45 strains formed a large cluster being classified as sub-lineage 8.3 with the representative strains being JXA1, JXwn06 and HUN4.

Discussion
Since its emergence in China at the end of 1995, PRRSV has spread widely in all areas of China and is continuously evolving. This has led to the emergence of some new antigenic variant strains [17]. In 2006, a large outbreak of porcine high fever syndrome (PHFD), caused by a highly pathogenic form of PRRSV, emerged in China.
and Southeast Asian countries and caused major economic losses for swine farming [12, 15, 16]. In our previous study, 133 of 475 samples (28%) were positive for PRRSV, indicating that PRRSV is widely distributed among swine populations in southern parts of China. Fifty-six ORF5 sequences and 35 nsp2 HVR sequences were selected for investigation of variations and phylogenetic analyses for their genetic diversities. Sequences alignments of GP5 and nsp2 showed that there was extensive genetic variability between them (84.5–100% amino acid identity) or with the representative strain, VR-2332 (84.5–99.0% amino acid identity). GP5 based phylogenetic trees showed all these strains belonged to the type 2 PRRSV which are scattered in 2 lineages (lineages 5 and 8). But most of the strains belonged to a large cluster in sub-lineage 8.3 with the representative

| Decoy HVR1 | PSE | HVR2 |
|-----------|-----|------|
| 10        |     |      |
| 20        |     |      |
| 30        |     |      |
| 40        |     |      |

**Fig. 1** The alignment of GP5 of PRRSV. A multiple alignment of PRRSV GP5 was performed by Clustal W. The sequence of VR-2332 is shown at the top; the residues conserved with it are hidden and substitutions are indicated by the amino acid letter codes. The functional domains are shown within boxes. HRV: highly variable regions, DCE: decoy epitope, PNE: primary neutralizing epitope.
HP-PRRSV strains being JX1, JXwn06 and HUN4. This is consistent with other studies showing that the dominant PRRSV seen in Guangdong Province, which is also located in Southern China, was HP-PRRSV between 2007 and 2014 [18, 19]. The sub-lineage 8.3 PRRSV was also the predominant virus at the country-wide scale in the subsequent years since 2007 [17, 20, 21].

As a transmembrane protein, GP5 possesses two to four potential N-linked glycosylation sites that are located in a small ectodomain [22]. The N-linked glycosylation of GP5 have been shown to be involved in diverse functions such as the proper folding protein, receptor binding, virus infectivity and induction of immune response [8, 23–25]. The amino acids in the proximal region of the ectodomain of GP5 are highly variable. In this study, we showed that substitutions at each consensus sequence of N-linked glycosylation site, N-X-T/S, in GP5 of some strains, resulted in loss or gain of N-linked glycosylation sites at specific positions in GP5. The N44 and N51-linked glycosylation sites were

| Name of isolates | The number of N-glycosylation sites | The position of N-glycosylation sites |
|-----------------|------------------------------------|--------------------------------------|
| GXNN1310f, GXYL1310 | 3 | N34, N44, N51 |
| GXGG1306, GXNN1407a, GXS1401a | 3 | N35, N44, N51 |
| GXGG1305a/b, GXNN1307a, GXYL1308b, GXYL1307a, GXYL1308b | 4 | N34, N35, N44, N51 |
| GXL1403, GXYL1403a, GXL1305a | 4 | N30, N33, N44, N51 |
| GXL1305b, GXNN1301b/c/e, GXL1407b, GXS1401b | 4 | N30, N35, N44, N51 |
| GXS13010, GXS1401a/b | 5 | N30, N33, N34, N44, N51 |
| GXL1409, GXBH1310b, GXBH1311a/b, GXBH1404, GXS1410c, GXYL1304, GXL1405, GXYL1430b/d, GXL1306c/e, GXYL1304, GXS1305a/b/c/d/e, GXYL1309a, GXYL1430a, GXS1401a, GXS1312c, GXS1396, GXS1410a, GXS1304, GXYL1304, GXYL1407, GXYL1405, GXX1401 | 5 | N30, N34, N35, N44, N51 |

**Table 2** The potential N-glycosylation sites in GP5 of different strains in this study

Fig. 2 Identification of PRRSV strains with amino acid deletions or insertions in nsp2. A multiple alignment of PRRSV NSP2 HV was performed by Clustal W. The sequence of VR-2332 is shown at the top; the residues conserved with it are hidden. The deleted or inserted amino acids are labeled with boxes.
well conserved. The DCE upstream of the PNE was speculated to elicit a great abundance of the non-neutralized antibodies against GP5 and delay the production of neutralizing antibodies stimulated by PNE [8]. The alignment of GP5 showed that variations in DCE were observed and key aa substitutions in PNE were found among the strains in this study. As a result, a different number of N-glycosylation sites among the strains and the key aa variations in DCE and PNE might allow these field strains to escape neutralization by the antibodies induced by current vaccines.

The results of alignment and phylogenetic tree studies based on GP5 and HV of nsp2 also showed that a great number of emergences of PRRSV might be related to the extensive use of the attenuated modified live virus (MLV) PRRS vaccine in China. Three strains, GXYL1310, GDHZ1401 and GXBH1404, which have a specific 120 aa deletion in nsp2 were identified. It was suggested that there is the possibility that these three strains were derived directly from the widely used commercial vaccine strain, TJM, which is characterized by a 120 aa deletion in nsp2 and derived originally from the TJ strain by serial passage in MARC-145 cells of up to 92 times [26]. VR2332-derived MLV strains were also found. Two strains were clustered into this minor branch shared a high identity with MLV vaccine and its parent virus VR-2332, with amino acid similarities of 99.2 and 99.7%, respectively. Several studies showed that some prevalent PRRSV strains may be related to the reversion of commercial MLVs and the recombination between the vaccine virus and field viruses [27–31]. It has been suggested that more attention should be paid to MLV-like strains which have undergone evolutionary changes and have since circulated widely in the field.

The nsp2 of PRRSV is a highly heterogeneous protein. Remarkably, natural deletions and insertions have continued to occur in the HV of nsp2, and these have led to genome size differences among PRRSV strains [9, 32, 33]. Type 2 PRRSV with 1–150 aa deletions and 1–36 aa insertions in the nsp2 coding region has been identified in USA, China, Japan, Denmark and Thailand [9, 10, 33, 34]. In this study, most of the strains have a discontinuous 30 aa deletion, suggesting that the strain with a 30 aa deletion in nsp2 is the dominant virus prevalent in the southern parts of China. It is noted that several novel strains (GXNN1396, GXYL1403e, GXBS1401a and GXNN1407a) with additional aa deletions or insertions are also found, suggesting that strains with other types of aa deletions in nsp2 may have also been prevalent in this region. GXNN1396 has a discontinuous 30 aa deletion and a continuous 19 aa deletion at position 499–517 in nsp2 compared to VR-2332. Similar 19 aa deletions in nsp2 were also observed in a Japanese PRRSV strain, Jyc, and several USA PRRSV strains (MN184, NADC30 and NADC31), which have 19 aa deletions at position 495–513. GXYL1403e had a novel discontinuous 124 aa deletion at positions 481 and 496–619 in the nsp2-coding region in contrast to the VR2332 reference strain. Similar aa deletions at

| No. | Virus strain | Accession no. | Country | Lineage |
|-----|--------------|---------------|---------|---------|
| 1   | NADC30       | JN654459      | American | 1       |
| 2   | MN184        | EF442777      | American | 1       |
| 3   | CH-xo1401    | KP861625      | China   | 1       |
| 4   | HI1x1        | KX766379      | China   | 1       |
| 5   | PRSSV00000008569 | EU758687 | American | 2       |
| 6   | PRSSV0000008973 | EU758940 | American | 2       |
| 7   | PRSSV0000033 | DQ474791      | America | 2       |
| 8   | FJ1          | AY881994      | China   | 3       |
| 9   | GD-KP        | KU978619      | China   | 3       |
| 10  | GM2          | JN662424      | China   | 3       |
| 11  | Ibaraki08-5  | AB546113      | Japan   | 4       |
| 12  | Miyagi08-2   | AB546105      | Japan   | 4       |
| 13  | Miyagi08-3   | AB546106      | Japan   | 4       |
| 14  | VR-23332     | AY150564      | American | 5       |
| 15  | NADC-8       | AF396833      | American | 5       |
| 16  | PA8          | AH006184      | Canada  | 5       |
| 17  | NVSL-14      | AF396839      | American | 6       |
| 18  | Aichi N20    | AB175715      | Japan   | 7       |
| 19  | Neb-1        | EU755263      | American | 7       |
| 20  | PrimePac     | AF066384      | American | 7       |
| 21  | CH-1a        | AY032626      | China   | 8.1     |
| 22  | HH08         | JX679179      | China   | 8.1     |
| 23  | HBJM2        | EU399826      | China   | 8.2     |
| 24  | HBSZ         | EU399825      | China   | 8.2     |
| 25  | JX1A         | EF112445      | China   | 8.3     |
| 26  | JXwn06       | EF641008      | China   | 8.3     |
| 27  | TJ           | EU860248      | China   | 8.3     |
| 28  | GXLSN06–2012 | KC618172     | China   | 8.4     |
| 29  | JXZX2        | EU399849      | China   | 8.4     |
| 30  | AH-W01       | EU399828      | China   | 8.5     |
| 31  | HeN-2        | FJ237419      | China   | 8.5     |
| 32  | JXZX2        | HQ832215      | China   | 8.6     |
| 33  | Yunnan-08    | EU819086      | China   | 8.6     |
| 34  | HK1          | KF287132      | China   | 8.7     |
| 35  | HK4          | KF287134      | China   | 8.7     |
| 36  | JA-142       | AF396842      | American | 9       |
| 37  | SDSU73       | AY656993      | American | 9       |
| 38  | B1           | AY318777      | American | 9       |
| 39  | LV           | M96262        | Netherlands | 10     |
this region were also observed in a Chinese PRRSV strain, Em2007, which has a 68 aa deletion at position 499–566. Compared to JXA1, strains GXBS1401a and GXNN1407a have one aa deletion at position 816 and one aa insertion at position 830, respectively. One aa insertion in nsp2 was also identified in a Japanese strain, Jtg, which have one aa insertion at position 554 (Additional file 2: Table S2). The emergence of HP-PRRSV strains with 30 aa deletions in the nsp2 was once speculated to be related to its virulence. However, the following study showed that the discontinuous deletion of 30 amino acids in nsp2 was not related to the virulence of the emerging HP-PRRSV [35]. But recent studies showed that PRRSV strains with deletions in the nsp2 were more likely to be pathogenic [33, 35, 36]. Overall, the mechanism underlying spontaneous deletions in nsp2 during viral passages in vivo and their effect on viral replication and pathogenicity remains unclear.

Nsp2 is also a highly immunogenic protein. It has been shown that nsp2 contains several putative B-cell and T-cell epitopes. Antibodies against nsp2 were generated as early as 1 week after PRRSV infection [37, 38]. Most of these epitopes were found to be mapped to the HV of nsp2 which usually occur by substitutions, natural deletions and insertions. It has been shown that a natural deletion or an engineered deletion in nsp2 of PRRSV plays an important role in modulating the induction of inflammatory cytokines in vitro [36, 39, 40]. The biological and immunological characteristics of the strains with specific aa deletions in nsp2 remain topics for further studies.

Conclusions
In this study, we showed PRRSV is widely distributed among swine populations in the southern parts of China. GP5 based phylogenetic trees and sequence alignments showed that extensive genetic variability exists compared with the representative stains and the PRRSV strains with 30 amino acid deletions in nsp2 and these are dominant in the porcine population. In addition, more and more PRRSV strains with different patterns of deletions
or insertions in nsp2 are emerging. This study expands the existing knowledge of the genetic diversity and evolution of PRRSV in southern parts of China and can potentially help to better control the spread of PRRSV.

**Methods**

**Sample collection, viral RNA extraction and PRRSV detection**

Field samples ($n = 475$) (sera, lungs, lymph nodes and spleens) from clinically diseased pigs between 2013 and 2014 in different regions of Guangxi, China were submitted to the Laboratory of Animal infectious Diseases and Molecular Immunology, Guangxi University, Nanning for PRRSV testing. A summary of the samples studied is presented in Table 1. Total RNA was extracted using TRizol reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer’s instructions and then used for synthesis of cDNA with random hexamers (Fermentas, Glen Burnie, MD, USA). All the samples were screened for PRRSV by PCR using the forward and reverse primers, (5′-AAGCTGTTAAA-

CAGGGAGTGG-3′) and (5′-CCAAAGAATACCAGC

CCATCA-3′), respectively. Thermal cycling conditions were 95°C for 3 min, followed by 35 cycles of 95°C for 1 min, 59°C for 40s, 72°C for 1 min, and a final elongation step at 72°C for 10 min. Finally, the PCR products were analyzed on 1.0% agarose gel electrophoresis ultraviolet imaging. Positive samples were determined by the presence of 443 bp amplified products.

**Cells and virus**

MARC-145 cells were grown at 37°C in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). Sera or supernatants of tissue homogenates from PRRSV-positive samples were used to inoculate the MARC-145 cells for PRRSV isolation.

**Amplification of ORF5 and nsp2 hypervariable region and sequence determination**

PRRSV positive samples were used for amplification of complete ORF5 and nsp2 hypervariable regions (HVR). The forward (5′-AGGTGGGCAACCGTTTTA-3′) and reverse primers (5′-CATCACTGGCGTGAGGTAAAT-3′) were used for amplification of the complete ORF5. PCR reaction conditions were 95°C for 3 min, followed by 30 cycles of 95°C for 1 min, 59°C for 1 min, 72°C for 1.5 min, and a final elongation step at 72°C for 10 min. The forward (5′-AATGTGTGTCTTCTCGGGTGAG-3′) and reverse primers (5′-AAGCTGAAAAAACCCCAATCACC

G-3′) were used for amplification of the nsp2 HVR. PCR reaction condition was 95°C for 3 min, followed by 30 cycles of 95°C for 40 s, 57°C for 40 s, 72°C for 2 min, and a final elongation step at 72°C for 10 min. The PCR products were purified with an E.Z.N.A.TM Gel Extraction Kit (OMEGA, USA) and cloned into pBST-II vector (TIANGEN Inc., Beijing, China). Positive clones were sequenced in both directions using universal primers T7 and SP6 promoter-specific primers.

**Amino acid mutation analysis of Nsp2 HV and ORF5**

To further characterize the amino acid mutation in Nsp2 HV and GP5, differences of the amino acid sequences derived from ORF5 gene and nsp2 HVR of these strains and other representative strains from China and other countries were analyzed and aligned using the MegAlign program (version 5.01) of the DNASTAR package. (DNASTAR Inc., Madison, WI, USA).

**Phylogenetic tree analysis**

The multiple sequence alignment of the nucleotide sequences of ORF5 or nsp2 HVR were performed by using the Clustal W method in MEGA5.2. MEGA version 5.2 with the p-distance model was used to evaluate phylogenetic relationships by the neighbor-joining method with 1000 bootstrap replicates. The sequences obtained in this study were submitted to the GenBank database under the accession numbers (MG604994 - MG605049 for ORF5 and MG604959 - MG604993 for nsp2) and the reference strains from China and other countries (lineages 1 to 9) used in this study are listed in Table 2. The classification of lineages and sub-lineages was according to their description in recent studies [41, 42].

**Additional files**

**Additional file 1: Table S1.** Comparison of the GP5 sequences of the different PRRSV strains examined in this study. (DOX 14 kb)

**Additional file 2: Table S2.** The positions and sizes of aa insertions and deletions in nsp2 of PRRSV strains compared to VR2332. (DOX 26 kb)

**Abbreviations**

DCE: decoy epitope; HP: Highly pathogenic; HVR: hypervariable region; nsp: nonstructural proteins; ORF: Open reading frame; PRRS: Porcine reproductive and respiratory syndrome; PRRSV: Porcine reproductive and respiratory syndrome virus

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**Availability of data and materials**

All data were included in the manuscript as tables and figures. The sequences obtained in this study were submitted to the GenBank database under the accession numbers (MG604994 - MG605049 for ORF5 and MG604959 - MG604993 for nsp2) and the reference strains used in this study are listed in Table 2.
Authors’ contributions
HS, WY, LS and HJ conducted the experiments and analyzed the data. HW1 and YJ assisted with sample preparation and experiments. CY and KO shared ideas and discussed the research data. HW2 and WZ contributed to supervision, had the idea for the project and directed the research. All authors read and approved the final manuscript.

Competing interest
The authors declare that they have no competing interests.

Ethics approval and consent to participate
For all porcine clinical samples used in this study, written consents were obtained from farm owners and all procedures were carried out in strict accordance with the Animal Ethics Procedures and Guidelines of the People’s Republic of China. All the animal protocols in this study were approved by the Ethics Committee of Guangxi University.

Consent for publication
Not applicable.

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