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Author(s)	Song, Mingyue; Zhang, Qiaoya; Shan, Hu; Yu, Binhui; Xiong, Yi; Li, Junwei
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Molecular Evolution of Porcine Reproductive and Respiratory Syndrome Virus in Guangxi of China from 2012 to 2015

Mingyue Song¹, Qiaoya Zhang¹, Hu Shan¹, Binhui Yu², Yi Xiong^{3,*} and Junwei Li^{1,*}

¹) College of Veterinary Medicine, Qingdao Agricultural University, Qingdao, 266109, China

²) Yijian Bioproduct Ltd, Zhuji, 311800, China

³) Center of prevention and control of animal infectious disease of Guangxi province, Nanning, 530000, China

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Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) is one of the most important pathogens for swine industry, and caused significant economic losses worldwide. To better understand the genetic diversity of PRRSVs in Southwestern China, 63 PRRSV strains were isolated in Guangxi Province from 2012–2015. GP5 genes of these PRRSV isolates were sequenced and analyzed with those of representative PRRSVs. Phylogenetic analysis of GP5 genes shows 61 isolates of those are highly homologous with JXA1 and one isolate is variant of strain CH-1a. Furthermore, NADC30-like PRRSV strains were not isolated in our study. Subsequently, five antigenic epitopes of all GP5 protein were analyzed. Results showed amino acids with high frequency of mutation are L39I, V185A, I189L, T121I, F127L, R151K, I161V, Q196L, Q196R. Among these amino acids, T121I, F127L, R151K and I161V locate in T cell epitopes. And, our results suggest further investigation should be conducted to elucidate function of these mutation sites with high frequency.

Key Words: PRRSV, GP5, evolution, phylogenetic analysis, epitopes

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is one of the most disastrous pathogens, which caused huge economic losses and continued to threat the swine industry around the world. Taxonomically, PRRSV is a member of RNA virus with positive polarity. The

size of its genome is around 15 kb which contains at least 10 open reading frames (ORFs). PRRSVs can be further divided into two genotypes: European-like or PRRSV-1, and American-like or PRRSV-2. Since the first report of mystery swine disease causing reproductive failure and respiratory disease in United States in 1987 and the etiological agent was identified as PRRSV in

*Corresponding author:

Junwei Li, College of Veterinary Medicine, Qingdao Agricultural University, Qingdao, 266109, China

E-mail: jwli@qau.edu.cn

Yi Xiong, Center of prevention and control of animal infectious disease of Guangxi province, Nanning, 530000, China

E-mail: 1054779003@qq.com

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1990s³⁾, continuous evolution of PRRSV makes it as first challenge to swine industry. Although PRRSV vaccine has been widely used around the world, the prevalence of infection by PRRSV in swine farm is still very high, and new variants of PRRSV have constantly evolved and appeared in outbreaks of swine infectious disease with increasingly divergent and virulent phenotype¹⁰⁾. In 2006, highly pathogenic (HP) PRRSV strain with mortality 20–100% in sows was reported in South China and then HP-PRRSV swept across Southeast Asia and India^{1,14)}. Since the HP-PRRSV appeared, PRRSV infection became even more serious than ever before. In 2008, genotype 2 strain NADC30 was identified in United States, the variants of NADC30 were soon isolated across China²⁵⁾. Currently, large-scale vaccination is carried out to prevent and control infection with PRRSV, however, PRRSV still remains one of the major challenges for swine industry globally and continuously causes outbreaks.

New variants of PRRSVs have appeared with increasingly pace. Intensive research suggested that mutation and genetic recombinant played an important role in the evolution of PRRSV²³⁾. On the other hand, the selective pressure enforced by the existing immunity induced by vaccination drives evolution of PRRSV at the nucleotide level as well⁶⁾. As being estimated, PRRSV has taken a higher evolutionary rate than the other RNA viruses to create new strains with ability to evade existing immunity in swine herds¹⁵⁾. Therefore, it is necessary to enforce the epidemiological surveillance and evolution analysis to adapt strategies for better prevention and control of PRRSV. Usually, vaccination is effective way to prevent and control the infectious disease. Current commercial PRRSV vaccine can induce robust neutralizing antibody against homologous virus, with limited protection against heterologous challenges. Besides, cell-mediated response is vital to clear PRRSV and kill infected cells to prevent the spread of viruses. Identification of T cell epitopes in the

antigen of PRRSV will benefit the development of effective vaccine with broad protection.

In this study, to better understand molecular epidemiology and antigenic variation in Southwestern China, ORF5 genes encoding envelope glycoprotein 5 (GP5) of 63 PRRSV isolates from clinical samples collected in the swine farms which vaccinated live-attenuated PRRSV vaccine from 2012–2015 were sequenced and analyzed, as well with several representative sequences as references. It is postulated GP5 is major neutralization antibody inducer. Studies suggested that glycosylation sites of PRRSV GP5 play important roles in PRRSV escaping, blocking or minimizing of virus-neutralizing antibody responses⁹⁾. These isolates should theoretically evolve under the pressure of existing immunity. In China, extensive vaccination was carried out to control and prevent PRRSV since the HP-PRRSV was identified in 2007 to some extent. Therefore, B cell epitopes and T cell epitopes in GP5 proteins of these PRRSV isolates were analyzed in this study.

Materials and methods

Sample collection: 494 clinical lung samples were collected from pigs reported with reproductive failure and respiratory disorder in 63 swine farms from 2012–2015 in Guangxi, China. The samples were kept in dry ice for transportation, then stored at -70°C .

cDNA synthesis, PCR and DNA sequencing: The lung tissues were homogenized for RNA extraction and total RNA was extracted from lung tissues using RNAiso Plus following the manufacture's protocol (TaKaRa). Reverse transcription PCR for amplification of ORF5 gene of PRRSV was performed as described. In brief, forward primer 5'- ATGTTGGAGAAATGCTTGACC -3' and backward primer 5'- CTAAGGACGACCCCATTC TTC -3' were designed and synthesized. Reverse transcription was conducted using 20 μl of

reaction mixture containing 11 μ l RNA extraction solution, 1 μ l 10 pmol backward primer, 4 μ l 5 \times MLV buffer, 2 μ l dNTPs (2.5 mM), 1 μ l RNase inhibitor (40 U/ μ l) and 1 μ l Reverse transcriptase XL (MLV, 100 U). The PCR was performed under the following procedure in a thermal cycler: 94°C for 4 min, followed by 30 cycles of 94°C for 15 s, 55°C for 30 s and 72°C for 10 min and holding at 12°C. The final PCR product is 603 bp in length covering the complete ORF of GP5 gene. PCR production was examined on 1.0% agarose gel electrophoresis and ligated into pMD-18T easy vector. Positive clones were indentified and sequenced (Sangon Biotech) (Sequences are available if requested).

Phylogenetic analysis and epitope analysis: 11 representative GP5 gene sequences of PRRSV were downloaded from GenBank, including GP5 gene of VR2332, the classical representative American PRRSV strain; CH-1a, the first PRRSV isolate in China in 1996; JXA-1, one of the earliest Chinese HP-PRRSV strain, and NADC30, the representative American HP-PRRSV. Nucleotides and deduced amino acids were analyzed using software DNASTAR and Clustal X 2.1. A phylogenetic tree of GP5 gene was constructed using the neighbor-joining method with computer program MEGA 6.0¹⁸⁾. Recombination events were tested by employing Recombination Detection Program¹⁴⁾. Based on published result of monoclonal antibody screening, the three B epitopes and two T cell epitopes were analyzed^{2,16)}.

Results

In this study, 63 PRRSV strains were isolated from 63 swine farms in Guangxi, China. GP5 gene of all isolates are sequenced. Then a phylogenetic tree of GP5 gene with those from representative strains was constructed, and shown in Fig. 1. Phylogenetic analysis shows most of the isolates are highly homologous with JXA1, which

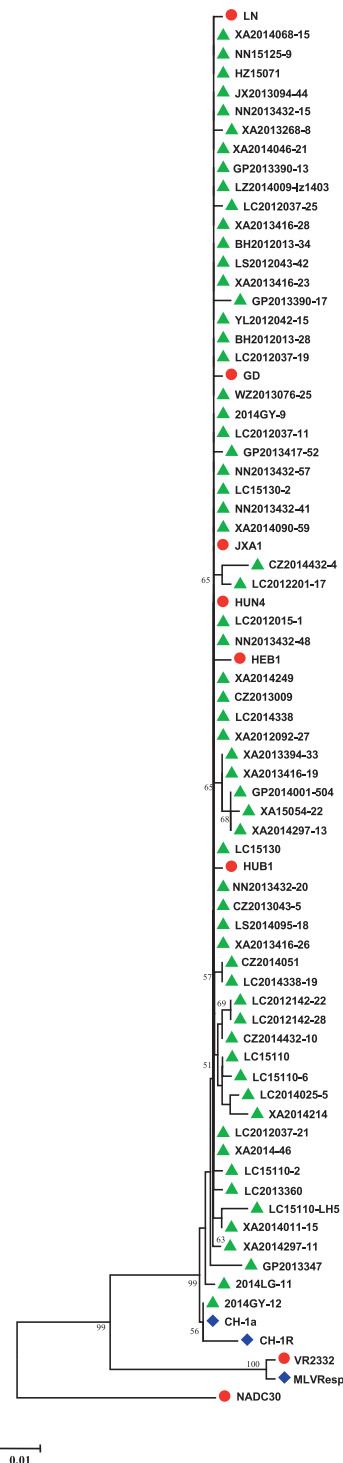


Fig. 1. Phylogenetic analysis of PRRSV isolates (indicate by filled green triangle), Chinese vaccine strains (indicated by filled blue diamond) and reference strains (indicated by filled red circle) based the OFR5 gene. The neighbor-joining tree was constructed using the kimura two-parameter in MEGA 6.0. The scale bar represent 0.01 inferred substitutions per site

Table 1. The amino acid deviation of B cell epitope

Amino acid sites	Mutation number	Mutation rate (%)	Amino acid sites	Mutation number	Mutation rate (%)
27 (V→A)	6	8.60	185 (V→A)	69	100
29 (V→A)	4	5.70	189 (I→L)	64	92.70
29 (V→D)	1	1.40	192 (R→K)	8	11.50
30 (N→S)	6	8.69	193 (V→T)	1	1.40
38 (H→Y)	6	8.69	193 (V→I)	7	10.10
38 (H→Q)	1	1.04	194 (S→Q)	1	1.40
39 (L→I)	63	91.30	195 (A→P)	1	1.40
39 (L→S)	6	8.69	196 (Q→L)	32	46.30
41 (L→S)	3	4.30	196 (Q→R)	31	44.90
184 (S→A)	1	1.40			

Table 2. The amino acid deviation of T cell epitopes

Amino acid site	Mutation Number	Mutation rate (%)	Amino acid site	Mutation number	Mutation rate (%)
117 (L→F)	5	7.40	128 (A→V)	6	8.60
118 (A→G)	1	1.40	151 (R→K)	22	31.80
120 (L→F)	1	1.40	152 (L→I)	6	8.60
121 (T→I)	69	100	161 (I→V)	63	91.30
124 (V→A)	2	2.80	163 (K→R)	1	1.40
127 (F→L)	69	100			

is the representative Chinese strain of HP-PRRSV isolated in 2006¹⁹⁾. It suggests most PRRSV isolates circulating in Guangxi in this period perhaps are variants of JXA1. However, isolate 2014GY-12 is genetically close to CH-1a. Phylogenetic analysis indicates all isolates are descendants of VR2332. However, it shows that NADC30 strain locates in another branch. Since 2012, NADC30-like PRRSVs were prevalent in several provinces of China, but in present study, NADC30-like PRRSV was not isolated in Guangxi in this period²⁵⁾. Recombination events were tested by employing Recombination Detection Program, but results show that there is no recombination detected among PRRSV isolates from Guangxi in this period.

Published results suggest GP5 is the main target by immune cells and GP5 plays a crucial role in the escape of existing immunity through mutation¹⁶⁾. Based on monoclonal antibody screening, several liner epitopes have been

identified on GP5 protein, including three B cell peptides, one primary neutralizing epitope (aa37–45), two non-neutralizing epitopes (aa 27–30, aa180–198), and two T cell peptides (aa117–131, aa149–163). Mutation of amino acids in five epitopes of 63 PRRSV isolates are analyzed by using VR2332 as standard. As showed in table 1–2, the amino acid sites with high frequency in mutation are L39I, V185A, I189L, T121I, F127L, R151K, I161V, Q196L, Q196R. In present study, 100% mutation happened on aa39 (L39I or L39S). Among these amino acids analyzed, T121I, F127L, R151K and I161V locate in T cell epitopes. Of note, function of these amino acids is still under elucidation.

Discussion

Emergence of HP-PRRSV in 2006 is the second milestone during the course of PRRSV

evolution. Infection with HP-PRRSV caused death of millions of pigs and significant economic losses for the Chinese swine industry. Since the emergence of HP-PRRSV in China, HP-PRRSV has experienced three periods of evolution in China. The first one started from 2006 and ended in 2009, in which the PRRSV isolates are highly homogeneous^{7,26)}. In the second stage from 2009–2012, there was cocirculation of multi-subgenotypes, such as JXA1-like, CH1-a-like, and VR2332-like PRRSVs²²⁾. In the third wave of epidemic of HP-PRRSV, most PRRSV isolates are NACD30-like strains which carrying the genetic marker of PRRSV mn184 strains which originally isolated in Minnesota, USA^{8,13,24)}.

Published data has demonstrated that vaccinated pigs can spread of vaccine strain to innocent animals^{4,5,20)}. Mutation and genetic recombinant play important roles in the evolution of PRRSV. There are lots of reports of recombinant events between wild PRRSVs and vaccine strain, such as Em2007 which is the result of recombination between the WUH1 strain and the highly pathogenic PRRSV vaccine strain CH-1R, and HNhx strain which is the result of recombination between the NADC30 strain and the highly pathogenic PRRSV vaccine strain circulating in China, etc^{12,21,24)}.

GP5 is the main envelope protein of PRRSV. There are five highly variable regions were indentified in GP5. Among these sites, the changes of aa32–34, aa38–39, and aa57–59 located in the N-terminal ectodomain of ORF5 significantly influenced the susceptibility of PRRSV to neutralization antibody¹¹⁾. N-linked glycosylation of PRRSV GP5 has also significant impact on its infectivity, antigenicity, and ability to induce neutralizing antibodies^{2,17)}. Ansari and colleagues mutated N34, N44, and N51, and their results showed that mutation on residue N44 did not produce infectious virus. It indicates that N44 is the most crucial amino acid residue for infectivity²⁾. In this study, mutation of N44 was not detected, but there are distinct variation in the region of aa58–61 rich of Asparagine

related to glycosylation. However, it suggested mutation in latter region does not have negative influence on the function of GP5.

Although the accurate molecular mechanism of PRRSV evolution is still not fully understood, the high degree of variability of PRRSV suggested PRRSV are constantly adapting to existing immunity and re-emerging as new variants to caused new outbreaks. Therefore, prevention and control of PRRSV is still a hard nut to the swine industry. Extensive surveillance and high effective vaccine development are needed.

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Author contributions

MY Song, QY Zhang, H Shan and BH Yu designed this study, performed sample collection and laboratory work. Y Xiong and JW Li analyzed the data and wrote the manuscript draft.

Conflict of interest

All authors declare no conflict of interest.

Research involving animal subjects

All animal samples were collected in accordance with the 2016 standards of laboratory animal in China and other related regulations in Animal Welfare Act.

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