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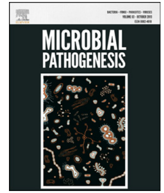

Emerging of two new subgenotypes of porcine reproductive and respiratory syndrome viruses in Southeast China

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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) is one of the leading swine pathogens and causes major economic loss to the global swine industry. In this study, a total of 49 PRRSV isolates were collected from different swine herds in seven provinces in Southeast China from 2014 to 2015. All the ORF5 genes and some Nsp2 genes were sequenced. Phylogenetic analysis showed that all the isolates belonged to the North America genotype. Among them, five isolates formed a new subgenotype IV derived from highly pathogenic PRRSV (HP-PRRSV). Six isolates formed subgenotype III, which were closely related to the NADC30 strain in the US. These isolates formed 13 putative *N*-linked glycosylation site (NGS) patterns based on N30, 33, 34, 35, 44 and 51. There were fewer NGSs of isolates in subgenotype IV than in subgenotype III. This indicates that the two new subgenotypes of PRRSV strains with different NGS patterns were spreading in those regions of China. The genetic diversity should be considered for the control and prevention of this disease.

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1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) causes major economic losses in the global swine industry, and is characterized by premature delivery, miscarriage, stillbirth, mummified fetuses, interstitial pneumonia, edema and conjunctivitis [1–3]. The causative virus is porcine reproductive and respiratory syndrome virus (PRRSV), a small enveloped, positive-sense, single-stranded RNA virus belonging to the Arteriviridae family [4–6].

PRRSV can be divided into two distinct genotypes, the North American (NA) type 1 genotype and the European (EU) type 2 genotype. The representative prototypes are VR-2332 and Lelystad virus (LV), respectively [1,7]. The genome of PRRSV is ~15 kb in length and contains $\geq$10 open reading frames (ORFs), including two large ORFs (ORF1a and 1b) and several smaller ORFs (ORF2–7), capped at the 5′ end and polyadenylated at the 3′ end. ORF1a and 1b encode 14 small nonstructural proteins, among which, Nsp2 is the most variable [8–13]. ORF2–7 encode eight structural proteins of the virus, including the major envelope glycoprotein GP5 (ORF5) and the recently discovered ORF5a protein (ORF5a) [8]. ORF5 and Nsp2 are considered to be the most variable genes of PRRSV within each genotype [14–16]. GP5 is a major envelope protein, comprising ~200 amino acids. It has an amino acid signal peptide, glycosylation sites, and antigenic determinants that can induce neutralizing antibodies [17]. As a result of its immunological significance and polymorphic nature, GP5 has been used for analysis of genetic diversity of PRRSV [18]. Nsp2 is a region shows the highest diversity in the viral genome, including insertions and various deletions [19,20]. In particular, in the Chinese HP-PRRSV isolates, a discontinuous deletion of 90 bases was reported as an epidemiological genetic marker [21,22].

PRRS was first reported in mainland China at the end of 1995 and HP-PRRSV isolates emerged in 2006 [18,23]. Since the initial outbreak, an increasing number of field PRRSV strains have been isolated from different regions of China, which were related to viral recombination and mutation [16,24–26]. To investigate the epidemiological and evolutionary characteristics of PRRSV in Southeast China during 2014–2015, we collected 564 clinical lung samples from piglets in different regions. The ORF5 genes and some of the Nsp2 genes of 49 PRRSV isolates were sequenced and

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analyzed. We found that four subgenotypes of PRRSV, including two new subgenotypes, had spread widely in Southeast China.

2. Materials and methods

2.1. Sample collection

A total of 564 clinical lung samples were collected from piglets from different swine herds that experienced high fever, reproductive and respiratory syndrome in seven provinces in Southeast China from 2014 to 2015. A portion of each sample was homogenized for RNA extraction and stored at −70 °C.

2.2. RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from tissue homogenates using the TRIzol reagent (Life Technologies, New York, USA). cDNA was constructed by M-MLV reverse transcriptase (Promega, Madison, WI, USA) with 7 μl RNA. Two microliters of cDNA was used as a template in the subsequent PCR. The complete ORF5 nucleotide sequences were amplified with GP5-F (5′-ATGTTGGGGAAGTGCTTGACCGCGT-3′) and GP5-R (5′-CTAGAGACGACCCCATTGCTCCGCT-3′). A pair of primers, Nsp2-F (5′-GGCAGTCATAAGTGGTACGGTGC-3′) and Nsp2-R (5′-GCAACAATGCCAAGCCTAAGC-3′), were used to amplify the Nsp2 genes. The cycling conditions were 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 3 min, and a final extension at 72 °C for 5 min.

2.3. Cloning, sequencing and phylogenetic analysis of PCR products

The PCR products were purified using a PCR purification kit (DingGuo, Beijing, China) and cloned into the pMD19-T vector (TaKaRa, Dalian, China). The plasmid was then used to transform *Escherichia coli* DH5α. The inserts were sequenced at Genscript Biological Engineering Company (Jiangsu, China). Phylogenetic and molecular evolutionary genetic analyses were conducted using the neighbor-joining method with MEGA version 6.0 [27]. Bootstrap confidence value was calculated using 500 replicates. A total of 19 North American genotypes and two European genotypes were used as references in the analysis (Table 1).

Table 1
Representative PRRSV strains used in the phylogenetic analysis.

No.	Name	Isolated country	Year	Accession no.
1	Lelystad virus	Netherlands	1993	M96262
2	VR2332	USA	1995	U87392
3	CH-1a	China	1996	AY032626
4	HB-1(sh)/2002	China	2004	AY150312
5	EuroPRRSV	USA	2004	AY366525
6	RespPRRS MLV	USA	2005	AF066183
7	JXA1	China	2006	EF112445
8	Prime Pac	USA	2006	DQ779791
9	MN184C	USA	2008	EF488739
10	SY0608	China	2009	EU144079
11	HN-HW	China	2010	FJ797690
12	NADC30	USA	2012	JN654459
13	10-10GX-1	China	2012	JQ663558
14	GXZCH32-2006	China	2012	JX046265
15	P-ZZ-081107	China	2012	GQ466016
16	GXCHZ01-2004	China	2012	JX046262
17	AH/JXXT/2012	China	2014	KM377850
18	ZJ/HZhf/2012	China	2014	KM377860
19	XJu-1	China	2014	KF815525
20	JL580	China	2015	KR706343
21	HLJ58	China	2015	KR706344

2.4. Amino acid analysis of GP5

To obtain a better understanding of the evolution of PRRSV in Southeast China in the past 2 years, the deduced GP5 amino acid sequences were aligned with some reference strains using BioEdit version 7.0.9.0 (http://www.mbio.ncsu.edu/BioEdit/page2.html). Two important epitopes of GP5, the decoy epitope (epitope A) and the primary neutralizing epitope (PNE, epitope B), were analyzed. The potential NGSs were also analyzed using NetNGlyc version 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/). Some known functional domains such as the signal peptide, ectodomain, transmembrane regions and endodomain were identified according to previous report [28].

3. Results

3.1. RT-PCR survey of clinical samples

The 564 clinical samples were collected from different pig farms in Southeast China between 2014 and 2015. Of these samples, 343 (60.82%) were positive for PRRSV detection by RT-PCR. The ORF5 sequences of 49 PRRSV-positive strains were amplified and sequenced for further analysis. Strain names, areas and accession numbers are summarized in Table 2.

3.2. Sequence and phylogenetic analysis of ORF5 of PRRSV isolates

The ORF5 sequences of 49 PRRSV isolates were compared with PRRSV strains JXA1, VR2332 and NADC30 (Table 3). To understand the relationship between field isolates and strains that had been reported (Table 1), a phylogenetic tree was constructed based on the ORF5 gene sequences and assessed through bootstrap analysis using the neighbor-joining method with 500 replications (Fig. 1). The 49 strains were all clustered within the North American genotype and can be further divided into subgenotypes I–IV. Only two strains were clustered into subgenotype II with the representative strain VR2332 and its vaccine strain Resp PRRS. There were six isolates closely related to NADC30 strain in the US and they formed a separate cluster subgenotype III, together with the recently reported strains HLJ58 and JL580 [29]. Five isolates from different provinces formed a new separate cluster subgenotype IV.

3.3. Amino acid analysis of GP5

To understand better the extent of the genetic diversity, the amino acid sequences obtained from the ORF5 gene sequences of the 49 PRRSV strains were aligned with their representative strains. The GP5 amino acid sequence for each strain consisted of 200 residues with no insertions or deletions (Fig. 2). The hypervariable region observed among the isolates included the signal peptide and the ectodomain. Epitope A, known as residues $A^{27}/(V)L^{28}V^{29}N^{30}$, was highly varied among different subgenotypes. Eight of 36 isolates in subgenotype I and two isolates in subgenotype II had a substitution of A^{29} to V^{29}. All the isolates in subgenotype IV and HZ1503 in subgenotype III had a substitution of S^{30} to N^{30}. Epitope B, residues 37–44, was conserved among different subgenotypes, except IV.

Potential NGSs were also observed at six different positions: N30, N33, N34, N35, N44 and N51 (Table 4). The positions and numbers of NGSs within a subgenotype exhibited variable patterns. The 49 PRRSV isolates formed 13 putative NGS patterns. There were fewer NGSs in subgenotype IV than in subgenotype III. NGS variations were located mainly between aa 30–35 near or within hypervariable region 1 (aa 32–34). The N44 and N51 sites were relatively conserved except for a few isolates. N33 appeared rarely

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