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Phylogenetic characterization of genes encoding for glycoprotein 5 and membrane protein of PRRSV isolate HH08

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A porcine reproductive and respiratory syndrome virus (PRRSV) was obtained from clinic samples. Genes 5 and 6 encoding for the viral glycoprotein 5 and a membrane protein of the PRRSV designated as HH08 were amplified by reverse transcription-PCR. These sequences were compared with reference sequences derived from different geographical locations. The results indicated that the virus belongs to the North American type rather than European. Comparative analyses of the genetic diversity between the PRRSV isolate HH08 and other Chinese as well as foreign reference strains of PRRSV were discussed based on the sequence comparison and the topology of phylogenetic trees constructed in this study.

Keywords: GP5, M protein, phylogenetic analysis, PRRSV

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is the causative agent of PRRS. This disease is an emerging swine disease that was originally recognized in North America in 1987 and in Europe in 1990 [8,18]. Since the first PRRSV was isolated in Europe in 1990, the disease became one of the most economically important diseases in most pig-producing countries [5,13].

PRRSV is an enveloped single-stranded positive sense RNA virus belonging to the family of *Arteriviridae*, order *Nidovirales* [2]. The approximately 15 kb viral genome encompasses nine identified open reading frames (ORFs). ORFs 1a and 1b encode viral replicase polyproteins, and ORFs 2a, 2b, 3 through 7 encode the viral structural proteins, glycoprotein (GP)2, envelope (E), GP3, GP4, GP5, membrane (M) protein as well nucleocapsid (N) protein, respectively [16]. The North American type (NA-type) and the European type (EU-type) have been identified as the two viral genotypes of PRRSV and both genotypes share only 55～70% homologous identity at the nucleotide level [14].

The major GP5 is functionally important in terms of its role in virus neutralization [6]. At the same time, it has been the target for the genetic analysis of PRRSV due to its polymorphic characteristics [1,3,4,9]. PRRSV M protein and GP5 are incorporated in virions mainly as a disulfide-linked heterodimer or as a disulfide-linked multimer with an approximate molecular weight of, respectively, 40 and 87 kDa [11,12]. GP5 and M proteins are considered very important in the arousal of humoral and cellular immune responses against PRRSV infection and may be excellent candidate proteins in the bioengineering of vaccine [5,7,19].

Increasing amounts of evidence show that PRRSVs isolated from different geographic locations share discrepant molecular characteristics [10,13,14]. Based on the sequences of GP5 and M protein, we comparatively analyzed the genetic diversity between a local PRRSV designated as HH08 isolated from northeastern China and other reference isolates from various regions around the world.

Materials and Methods

Sample origin and cDNA amplification

Samples (lymph nodes and lung) of clinical diseased pigs from a small farm in Heilongjiang Province, northeastern China were collected. Viral RNA was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Viral cDNA was synthesized using Oligo dT primer according to the manufacturer's instructions (TaKaRa, China). Sense primer 5′-GAGGTGGGCAACTGTTTTA G-3′ and antisense primer 5′-TTCTGCTGCTTGCCGTT GTT-3′ were used for amplifying a fragment covering the ORF5 and ORF6 genes of PRRSV. The PCR profile included 95°C for 2 min and then 30 cycles of 94°C for 1 min, 55.9°C for 30 sec, 72°C for 90 sec, followed by a final extension at 72°C for 10 min. The PCR products purified using PCR purification kit (Nanjing Keygen Biotech, China) were subjected to DNA sequencing directly.

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Sequence retrieval

The achieved GP5 sequence of the PRRSV isolate HH08 were compared with 32 China-derived and 26 foreign PRRSVs published in GenBank. The M sequence of PRRSV isolate HH08 were compared with 27 China-derived isolates and 22 foreign strains. Several PRRSV vaccine strains such as MLVRepPRRS and CH-1a were included. The information regarding the isolate name, origin, isolating time as well as GenBank accession number is provided in Tables 1 and 2.

Sequence comparison and phylogenetic tree analysis

Sequence homologous comparison was performed using the Lasergene software package V5.0 (DNAStar, USA). The phylogenetic trees were generated using the sequence alignment based on the genes encoding GP5 and M protein from the above-mentioned PRRSV isolates by the Lasergene software package V5.0 [17].

Results

Homologous identity among the PRRSVs

The sequencing reports of ORFs 5 and 6 indicated that both were composed of 603 and 525 nucleotides (nt), respectively. The lengths of both ORFs differed between NA and EU types; for example, the lengths of ORFs 5 and 6 of most EU-type PRRSVs are 606 nt and 522 nt, respectively. The lengths of both ORFs of most NA-types of PRRSVs are 603 and 525 nt, respectively. The sequence comparison showed that the ORF5 gene of PRRSV HH08 had 88.9～99.2% and 87.4～98.5% homologous identity with that of isolates from Mainland China at nucleotide and amino acid levels, respectively. It shared the highest identity with isolate CH-1a at the nucleotide (99.2%) and amino acid (98.5%) levels. In addition, it had 58.1～95.6% and 55.8～94.5% homologous identity with the selected

Table 1. Information on the open reading frame (ORF)5 of porcine reproductive and respiratory syndrome viruses (PRRSVs) used in this study

No	Isolate	Origin	Year	Accession no	No	Isolate	Origin	Year	Accession no
1	HH08	Heilongjiang	2008	GQ184821	31	04-HZ-1	Hangzhou	2004	EU480726
2	CH-1α	Beijing	1996	AY032626	32	HKEU16	Hongkong	2007	EU076704
3	CC-1	Jilin	2006	EF153486	33	Jiangxi-3	Jiangxi	2007	EU200961
4	Henan-HN1	Henan	2004	AY613348	34	ATCC VR-2332	USA	1990	U89392
5	NX06	Beijing	2007	EU097706	35	Lelystad	Netherlands	1991	M96262
6	BJ4	Beijing	2000	AF331831	36	Neb-1	USA	2008	EU755263
7	HEB1	Hebei	2006	EF112447	37	PRRSV2000000831	USA	2008	EU759973
8	HB-2(sh)	Hebei	2002	AY262352	38	SD-01-07	USA	2001	AY395079
9	SD-1	Shandong	2004	AY747596	39	SD-02-10	USA	2002	AY395081
10	SD-2	Shandong	2005	Dq265739	40	SD-02-11	USA	2002	AY395078
11	NJ-α	Jiangsu	2004	AY737282	41	SD-03-12	USA	2003	AY395074
12	R98	Jiangsu	2006	DQ355796	42	KNU-07	Korea	2007	FJ349261
13	QM070731	Anhui	2009	GQ128443	43	CA	Korea	2006	FJ194950
14	HZ-X/2003	Zhejiang	2003	AY450301	44	PRRSV0000007823	Canada	2005	EU758056
15	06-JX-4	Zhejiang	2004	EU480753	45	PRRSV0000007796	Canada	2005	EU758032
16	06-JX-5	Zhejiang	2004	EU480754	46	GK	Russia	2008	EU071251
17	GS2004	Gansu	2004	EU880443	47	ND-3	Russia	2007	EU071249
18	FJ04A	Fujian	2005	DQ246451	48	01NP1	Thailand	2000	AY297112
19	HuN	Hunan	2007	EF517962	49	00CS1	Thailand	2000	AY297111
20	HUB1	Hubei	2006	EF075945	50	28639	Denmark	1998	AY035912
21	GD-1	Guangdong	2004	AY747595	51	32-10	Denmark	1992	AY035913
22	GZJL	Guizhou	2009	FJ947000	52	obu-1	Belarus	2005	DQ324676
23	CYB-1	Chongqing	2009	FJ919342	53	Spain28	Spain	2003	DQ345755
24	SCQ	Sichuan	2006	DQ379479	54	PIADC-PRRS	Philippines	2008	FJ641194
25	Sichuan1	Sichuan	2003	AY513611	55	ciad010	Mexico	2005	DQ250080
26	YN10	Yunnan	2008	FJ361891	56	MLVRepPRRS	Vaccine		AF159149
27	SX2009	Shanxi	2009	FJ895329	57	IngelvacATP	Vaccine		DQ988080
28	Hainan-2	Hainan	2007	EF398052	58	RespPRRSMLV	Vaccine		AF066183
29	06-SH-2	Shanghai	2006	EU480749	59	SP	Vaccine		AF184212
30	MD-001	Taiwan	1999	AF121131					

Table 2. Information on the ORF6 of PRRSVs used in this study

No	Isolate	Origin	Year	Accession no	No	Isolate	Origin	Year	Accession no
1	HH08	Heilongjiang	2008	GQ184822	26	R98	Jiangsu	2006	DQ355796
2	CH-1α	Beijing	1996	AY032626	27	FJ1	Fujian	2005	AY881994
3	CC-1	Jilin	2006	EF153486	28	HKEU16	Hongkong	1999	EU076704
4	HeN-2	Henan	2008	FJ556871	29	ATCC VR-2332	USA	1990	U89392
5	NX06	Beijing	2007	EU097706	30	Lelystad	Netherlands	1991	M96262
6	BJ-4	Beijing	2000	AF331831	31	SD-01-07	USA	2001	AY395079
7	HEB1	Hebei	2006	EF112447	32	SD-02-10	USA	2002	AY395081
8	HB-2(sh)	Hebei	2002	AY262352	33	SD-01-08	USA	2001	AY395080
9	SD-ZQ	Shandong	2007	EU086604	34	16138	USA	1996	EF523346
10	SY0608	Jiangsu	2007	EU144079	35	MN184B	USA	2005	DQ176020
11	HS08	Anhui	2008	FJ897567	36	8981	USA	2004	AY569974
12	NB/04	Zhejiang	2004	FJ536165	37	PA8	Canada	2002	AF176348
13	FJ04A	Fujian	2005	DQ246451	38	IAF96-557	Canada	1996	U75443
14	Jiangxi-3	Jiangxi	2007	EU200961	39	01CB1	Thailand	2006	DQ864705
15	HuN	Hunan	2007	EF517962	40	01NP1.2	Thailand	2005	DQ056373
16	HuB1	Hunan	2006	EF075945	41	111/92	Denmark	1992	AY035944
17	GD	Guangdong	2007	EU825724	42	7571	Denmark	1996	AY035943
18	GZZB	Guizhou	2007	EU190975	43	CA	Korea	2006	FJ194950
19	CBB-1-F3	Chongqing	2008	FJ889129	44	KNU-07	Korea	2007	FJ349261
20	SCQ	Sichuan	2006	DQ379480	45	EDRD-1	Japan	1992	AB288356
21	GS2004	Gansu	2004	EU880443	46	Kitasato93-1	Japan	1999	AB023782
22	SX2009	Shanxi	2009	FJ895329	47	MLVRepPRRS	Vaccine		AF159149
23	MD-001	Taiwan	1999	AF121131	48	IngelvacATP	Vaccine		DQ988080
24	JZ08	Anhui	2008	FJ897566	49	RespPRRSMLV	Vaccine		AF066183
25	07BJ	Beijing	2007	FJ393459	50	SP	Vaccine		AF184212

foreign PRRSVs at nucleotide and amino acid levels, respectively. At the nucleotide level, it shared 89.6%, 91.2%, 91.2% and 92.2% sequence identity and 93.5%, 89.4%, 89.4% and 93.5% sequence identity at the amino acid level with vaccines IngelvacATP, MLVRepPRRS, RespPRRSMLV and SP, respectively.

The sequence comparison showed that the ORF6 gene of PRRSV HH08 had 69.7～100% nucleotide and 81.4～100% amino acid homologous identity with isolates from Mainland China. It shared 100% identity with isolate CH-1a. With selected foreign PRRSVs, it had 64～98.9% and 77.9～97.1% homologous identity at the nucleotide and amino acid levels, respectively. It shared 99.3%, 97.8%, 97.8% and 96%, as well as 96.6%, 97.1%, 97.1% and 96% sequence identity with vaccines IngelvacATP, MLVRepPRRS, RespPRRSMLV and SP at nucleotide and amino acid levels, respectively.

Phylogenetic analysis

Based on the ORF5 and ORF6 gene sequences, corresponding phylogenetic trees were independently constructed. As shown in Fig. 1, all the PRRSV isolates from Mainland China were NA-type, and PRRSV HH08, CH-1a and HB-2(sh) were located in the same clade. These isolates and above-mentioned PRRSV vaccines were included in the same group.

Multiple sequence alignment

Based on the analysis of the phylogenetic trees, the sequences of PRRSV HH08 ORFs 5 and 6 were compared with two EU-type isolates (HKEU16, Lelystad), two NA-type isolates from subgroup 1 (CH-1a, ATCC VR-2332) and one NA-type from subgroup 2 (01NP1 or FJ1). The results showed that PRRSV HH08 had very high identity with NA-type isolates CH-1a and ATCC VR-2332. There were several point mutations in the ORF5 gene (Fig. 2). As far as the ORF6 gene encoding the M protein is concerned, PRRSVs CH-1a, HH08, VR-2332 as well as FJ1 shared highly conserved sequences. More interestingly, the EU-type isolates HKEU16 and Lelystad also had higher homologous identity in ORF6 than ORF5 (data not shown). The results indicated that the PRRSV M gene is highly conserved.

Discussion

Since the appearance of the first PRRSV Chinese isolate in 1996 [1], many local isolates have been found in different

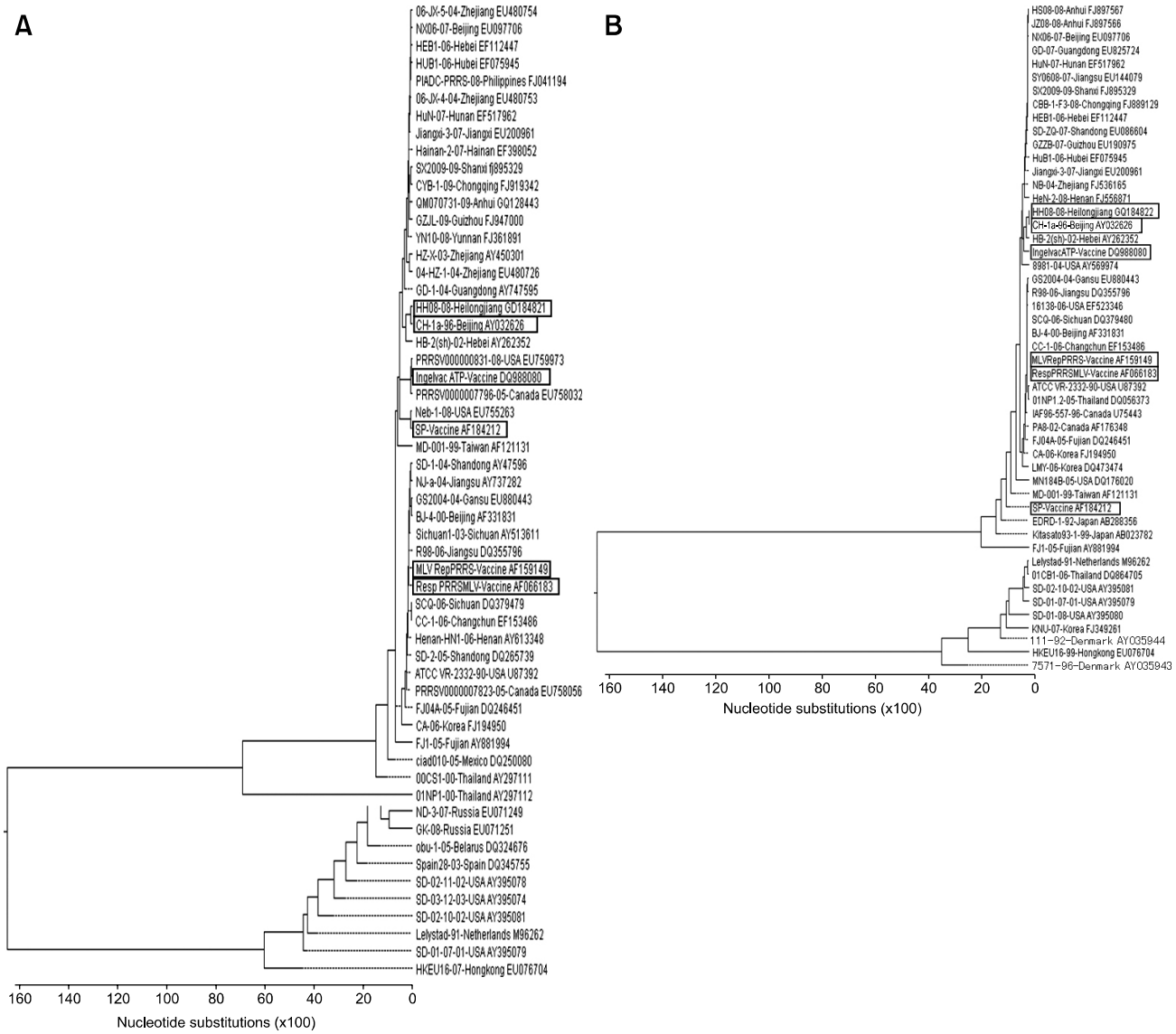

Fig. 1. Phylogenetic tree construction. Based on the open reading frame (ORF)5 and ORF6 gene sequences, the corresponding phylogenetic trees for ORF5 (A) and ORF6 (B) genes were constructed. The isolate name, isolating year, origin place as well as GenBank accession number are indicated. The virus isolated in this study and the vaccine strains are framed. The bootstrap value is 10,000.

geographic locations in China. The outbreak of PRRS often causes enormous economic losses in the pig-producing industry. Analysis of PRRSV origin and evolution is one of the important references for effective vaccine design and use. In this study, we isolated a local PRRSV from the northeastern region of China. The virus was isolated from clinical samples of several diseased pigs characterized by severe respiratory disease, high fever and flu-like syndrome from a small pig farm. The pigs were not inoculated with any PRRSV vaccines, although some of neighboring pig farms used commercially available vaccines such as MLV RespPRRS/Repro vaccine or killed CH-1a vaccine. Some efforts have been made to isolate more viruses, but no more PRRSV isolates were identified in the clinical samples. Our sequencing and subsequent sequence alignment showed that the new isolate shared the highest homologous identity with PRRSV vaccines CH-1a and VR 2332. However, since CH-1a is a killed vaccine strain used in China, HH08 might be a mutant of VR 2332 related PRRSVs. More information is needed to analyze the origin and phylogeny of this virus in the future. Factors such as the introduction of animals infected by PRRSV, use of vaccines or cross-infection from nearby regions may also be responsible for the appearance of newly emerging PRRSVs.

The topology of the phylogenetic trees indicated that all the PRRSV isolates from Mainland China were NA-type.

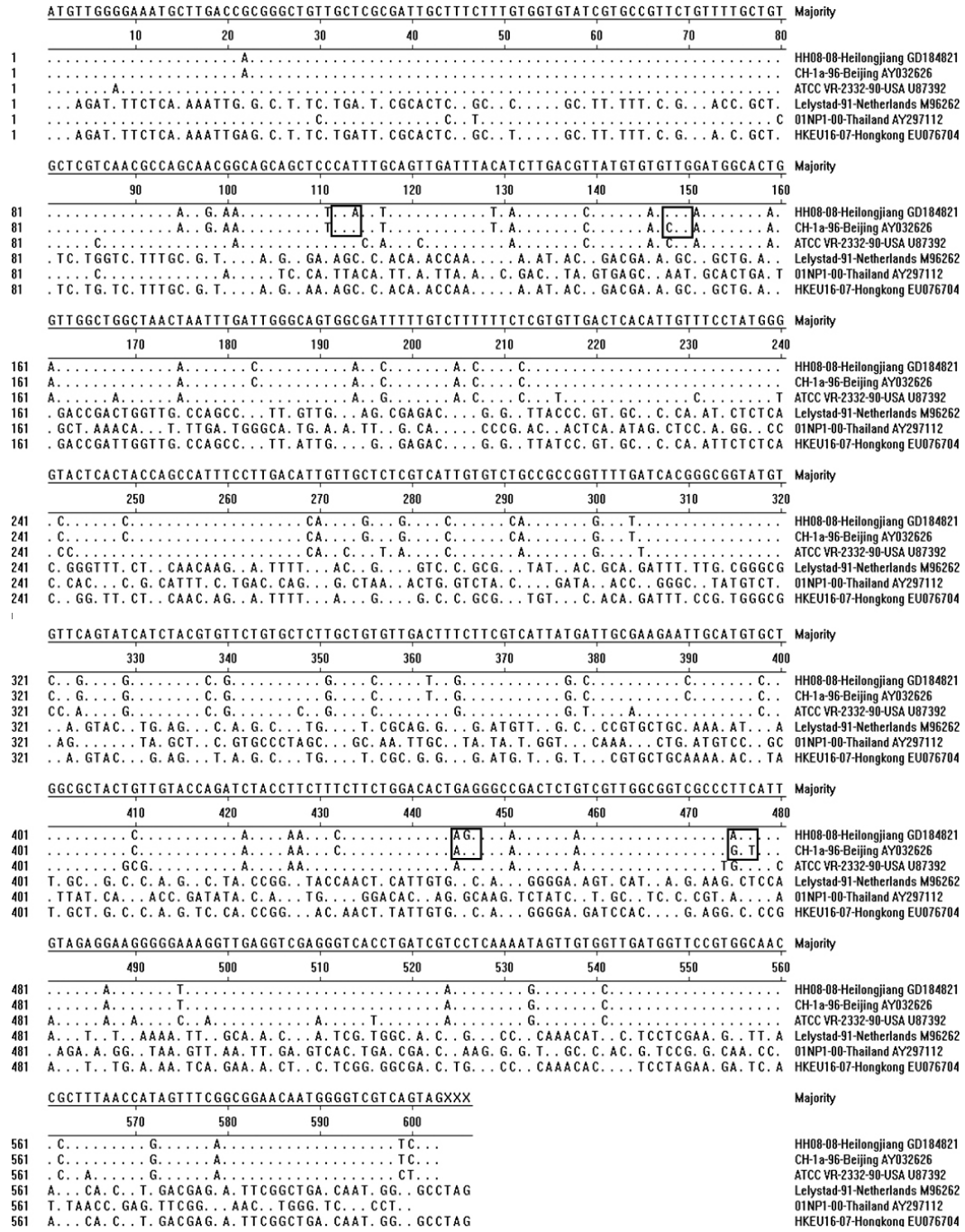

Fig. 2. Multiple sequence alignment of the ORF5 gene. The ORF5 gene of porcine reproductive and respiratory syndrome virus (PRRSV) isolate HH08 was compared with representative ORF5 genes of different PRRSV subgroups based on the phylogenetic tree analysis. The framed parts are point mutations in the GP5 gene between HH08 and CH-1a, and a total of three amino acid mutations are identified. There is a silence mutation in nucleotide position 148.

These isolates and the above-mentioned PRRSV vaccines were included in the same group. Although most Chinese PRRSV isolates have been isolated from different geographic locations, they were closely related as shown in the phylogenetic trees, with the exception of a Hongkong isolate, HKEU16, which was classified as EU-type and located in the other clade of the phylogenetic trees (Fig. 1). PRRS was initially confirmed in China in 1996, with the NA-type PRRSV spreading widely across China. Since then, the PRRSV Chinese isolates CH-1a and VR2332 were widely

used as vaccines. However, most of the latest emerging isolates have had high identity with the vaccines, indicating the fact that the currently used inactivated and the live attenuated vaccines in China appears to be ineffective against highly pathogenic PRRSV infections. A recent report regarding the sub-genotypes of PRRSV in China pointed out that NA-type PRRSVs were further divided into six sub-genotypes [21]. The HH08 isolate was shown to have a very high homologous identity with CH-1a, which places it into sub-genotype V, and this isolate is distinct from some sub-genotype I 2008 viruses isolated in the same location. At the same time, the existence of different virus genotypes has complicated the epidemic situations, and co-infection of the existed PRRSVs with other pathogens might be related to the appearance of highly pathogenic PRRSVs [10,20].

Multiple sequence alignments showed that PRRSV HH08 shared very high identity with NA-type isolates. However, there were several point mutations in the ORF5 gene. Although it is unclear why the diseased pigs showed PRRS syndromes, the sporadic point mutations in the gene encoding for GP5 may be important for viral genetic diversity, tropism and virulence. It was reported that residues H38L39 were the critical amino acids of the neutralizing epitope [6,15]. The residue H38 in the CH-1a isolate was changed into residue Q in HH08. In addition, there were two other mutations in residues K149 and V159 in CH-1a which were replaced by R149 and I149 in HH08. The importance regarding these mutations among the ORF5 genes is currently under investigation. In contrast, there was no mutation in the ORF6 genes encoding the M proteins of PRRSV isolates CH-1a and HH08, indicating that the M gene was highly conserved between these two PRRS-viruses. Interestingly, most Asian PRRSV isolates, including all isolates from Mainland China as well as several vaccines, were found to be NA-type. So far, we have no direct evidence showing that NA-type PRRSV, including the HH08 isolated in East Asian countries such as Korea, Japan and China, were from a common ancestor. However, most of them share a high homologous identity with some vaccine strains [1]. Moreover, we cannot exclude the possibility that multiple-infection and other unidentified pathogens among the diseased pigs might lead to the development of PRRS-like syndrome.

Sequence comparison and phylogenetic tree analysis showed that there was a possibility of shedding PRRSV vaccine strains in the field via unidentified routes, highlighting the importance of continuous surveillance for PRRS as well as the development of novel PRRSV vaccines. It would be meaningful to investigate whether there is any possibility of virulence recovery from the vaccines. At the same time, genetic and evolutionary analysis of full-length genomes of more PRRSV isolates may be important to delineate the degree of homology among PRRSVs and for effective vaccine design in the future.

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