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’-GAGGTGGGCAACTGTTTTAG-3’ and antisense primer 5’-TTCTGCTGCTTGCCGTGTT-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

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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-1a   | 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    | EU0977706   | 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    | QQ128443    | 43 | CA       | Korea    | 2006    | FJ194950    |
| 14 | HZ-X/2003 | Zhejiang | 2003    | AY450301    | 44 | PRRSV00000007823 | Canada | 2005    | EU758056    |
| 15 | 06-JX-4 | Zhejiang | 2004    | EU480753    | 45 | PRRSV00000007796 | 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    | AY353912    |
| 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    | FJ985329    | 57 | IngelvacATP | Vaccine | DQ988080    |
| 28 | Hainan-2 | Hainan  | 2007    | EF938052    | 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 | DQ867405 |
| 15 | HuN      | Hunan     | 2007 | EF517962 | 40 | 01NP1.2 Thailand | 2005 | DQ506373 |
| 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 | AF121313 | 48 | IngelvacATP Vaccine | DQ988080 |
| 24 | JZ08     | Anhui     | 2008 | FJ897566 | 49 | RespPRRSMLV Vaccine | AF066183 |
| 25 | 07BJ     | Beijing   | 2007 | FJ393459 | 50 | SP Vaccine | AF184212 |

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-1α 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-1α, ATCC VR-2332) and one NA-type isolate from subgroup 2 (01NP1 or FJ1). The results showed that PRRSV HH08 had a very high identity with NA-type isolates CH-1α 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-1α, 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
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. 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.
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 Hong Kong 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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