Full Length Research Paper

Genomic characterization of porcine reproductive and respiratory syndrome virus TJM vaccine strain

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Abstract

Porcine reproductive and respiratory syndrome (PRRS) is an economically important disease in swine producing area. PRRSV vaccine strain TJM had been proved to have low virulence in swine. To investigate the genomic characteristics of TJM strain, the full-length nucleotide sequence was obtained and analyzed. It contains 14 960 nucleotides in length. The nucleotide variations and amino acid substitutions were randomly distributed in the genome comparing to its parental TJ strain, but mainly occurred on the nonstructural protein genes Nsp1, Nsp2, Nsp3, Nsp9 and Nsp10, as well as on the structural protein gene ORF7. The most notable variation is the 360 nucleotides deletion exhibited in the Nsp2 gene. The novel deletion in Nsp2 resulted in altered protein structure. The genomic sequence of TJM was also compared with that of two US isolates (prototypic VR-2332 strain and virulent isolate P129) and eight Chinese isolates (previous isolates CH-1a, BJ-4, HN1, HB-2(sh) 2002, MN184C, and newly highly pathogenic isolates JX143 and GDQY2). TJM and the highly pathogenic strains belong to one group on phylogenetic relationship, although TJM has a low virulence. The 120-amino acid deletion downstream the 30-amino acid deletion region is unique to the attenuated TJM strain and applicated for discriminating the vaccine TJM from other virulent PRRSV.

Keywords: Porcine reproductive, respiratory syndrome virus, genomic sequence, gene deletion.

INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) is responsible for reproductive failure in sows and gilts, as well as pneumonia in pigs of all ages (Goyal, 1993). Since it was first reported in the United States and Europe (Keffaber, 1989; Wensvoort et al., 1991), PRRS has devastated the swine industry, causing tremendous economic losses worldwide. It is now considered one of the most important diseases in countries with intensive swine industries (Meulenberg, 2000). PRRSV was associated with a high fever syndrome (HFS) since 2006 in China and eventually was determined to be the major pathogen causing HFS (Tong et al., 2007; Zhou et al., 2008). PRRSV is a positive-sense single-strand RNA

virus belonging to the family Arteriviridae, in which there are also lactate dehydrogenase-elevating virus (LDV), equine arteritis virus (EAV), and simian hemorrhagic fever virus (SHFV). The genome of PRRSV is approximately 15 kb in length and contains nine open reading frames (ORFs). The 5′ end of the genome is methyl-capped and the 3′ end polyadenylated (Poly-A). ORF1a and ORF1b constitute approximately 80% of the viral genome and code for two large polyproteins (non-structural proteins, Nsps): replicase and polymerase, which are required for virus replication (Allende et al., 1999). At least 12 Nsps are generated as a result of serial cleavages of the two polyproteins expressed from ORF 1a and ORF 1b. ORF2a, ORF2b, and ORFs 3–7 were translated into viral structural proteins GP2a, GP2b, GP3, GP4, GP5, M, and N, respectively (Snijder and Meulenberg, 1998). Recently, a new protein expressed

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by ORF5a, sized 51 aa, which overlaps the 5' end of ORF5, was discovered as potential eighth structural protein of arteriviruses (Firth et al., 2011; Johnson et al., 2011). PRRSV isolates from different geographical regions have two major genotypes represented by North American (NA) type 2prototype VR-2332 strain and European (EU) type 1 prototype Lelystad virus (LV), respectively (Murtaugh et al., 1995; Nelsen et al., 1999and Oleksiewicz et al., 1999). The two genotypes are highly divergent in spite of their structural resemblance. PRRSV genome is highly variable even within the same genotype, especially in Nsp2 and ORF5 regions due to a high rate of mutation (Forsberg, 2005; Forsberg et al., 2001) Recently, numerous reports have indicated the emergence of potentially highly pathogenic variants of PRRSV as the cause for large-scale outbreaks with high mortality in China in 2006-2008 (Li et al., 2007; Tian et al., 2007; Zhou et al., 2008). Since high pathogenic PRRSV (HP-PRRSV) caused dramatic economic loss in China, it is urgent to have an effective vaccine. PRRSV TJ strain was isolated from the serum of pigs at the acute stage of PRRSV infection in the P.R. China and identified as a highly virulent North American (NA) type 2 genotype isolate. The pigs infected PRRSV TJ strain characterized by typical clinical signs: high and continuous fever, reddened skin and blue ears. And the virus caused high morbidity and mortality. Four live attenuated NA-type PRRSV vaccines have been successfully applied in PRRS disease control, such as CH-1R deriving from the 170th passage of CH-1a, first isolated in China. However, the traditional PRRSV vaccine cannot successfully protect animal against HP-PRRSV. It is necessary to develop a new vaccine aim to HP-PRRSV. Attenuation of virus through continuous cell culture passage is a traditional way for vaccine development. After plaque-purified and propagated on Marc-145 cells, an attenuated strain was obtained from HP-PRRSV TJ strain and assigned as TJM strain. To determine the specific genomic region, nucleotide or amino acids that are responsible for attenuation of the virus, in the present study, the entire sequence of PRRSV TJM strain was obtained and analyzed in comparison with the parental as well as other PRRSV strains. Interestingly, a novel deletion was found in TJM genome. And we applied the deletion for differentiating TJM from HP-PRRSV.

MATERIALS AND METHODS

Cells and viruses

Marc -145 cells, a highly permissive clone of the African Monkey kidney cell line MA104 (Kim et al., 1993), were cultured in Eagle's Minimum Essential Medium (MEM) with 6% FBS and supplemented with 0.01% penicillin–streptomycin. PRRSV TJ strain was isolated from serum of piglets suffering from high fever syndrome (HFS) in 2006 and it was highly pathogenic to pigs by animal regression experiment(LENG et al., 2008). Sequence of TJ strain has been determined and a sequence number is assigned as EU860248. HP-PRRSV TJ strain was serially passaged on Marc-145 cells in Dulbecco's Minimal Essential Media supplemented with 6% fetal bovine serum (Gibco BRL). When approximately 80% of Marc-145 cells exhibited cytopathic effects, the cells were lysed by freeze-thawing and the released viruses were harvested by centrifugation. The virus was purified by plaque cloning and passaged on Marc -145 cells and tested by animal experiment. It was purificated by plaque cloning one time for each five passages and did three times. Animal experiments were carried out for testing the virus virulence after each plaque purification. Its 30th-passage virus was determined low-virulence, and manifested decreased pathogenicity, with no pathologic lesion and death, after injected into clinically healthy 3-week-old PRRSV-nagative pigs. For ensuring the safty to animals, the 92th-passage virus of TJ strain, rename TJM strain,is chosen as an attenuated vaccine strain. TJM strain has been used as a commercial vaccine against HP-PRRSV in China, and attained new veterinary drug registration certificate of the People's Republic of China.

RNA extractions and primers

The total RNAs were extracted from 500 µl of cell culture supernatants of PRRSV TJM, using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. The extracted RNA was then eluted in 20 µl of elution buffer and kept at –80ºC until use. In order to determine the full-length genomic sequence of the PRRSV TJM, primers were first selected based on published sequences of the North American prototype PRRSV strain VR-2332 (GenBank accession no. AY150564) and its parental virus TJ isolate (EU860248). The designations and sequences of the 14 primers used in this study are given in Table 1. These primers were designed using the Primer Premier 5 and synthesized by Invitrogen (Beijing, China).

RT-PCR and sequencing

Seven overlapping cDNA fragments spanning the entire viral genome were RT-PCR amplified by using gene-specific primer sets. Reverse transcription was conducted using 4 µl of 5×RT buffer, 1 µl of OligdT12-18 primer (500 µg/ml, Invitrogen USA), 1 µl of dNTPs (10 mM/L), 2 µl of DTT(0.1M), 40 U of RNaseOUT^TM (40U/ µl, Invit-

Table 1. Oligonucleotide primer sequences utilized to generate the full-length genome of the PRRSV TJM (not include polyA) and differentiated primers of PRRSV. Expected product size is based on the genome of PRRSV TJ strain.

Primer name	Primer sequence	Expected product (bp)	Location
P1	ATGACGTATAGGTGTTGGCTCTATGCCATGGCAAT	2831	1-35
P2	CACGTCGCGACGCGGGCACAAGTACGGGCTCACTC		2832-2866
P3	CGCGTCGCGACGTGTCCCCAAGCTGATGACACC	3622	2853-2888
P4	GGTGCTTAAGTTCATTACCACCTGTAACGGATGCC		6439-6474
P5	TGAACTTAAGCACCTATGCTTTCCTGCCCCGGATG	1156	6461-6495
P6	GGCGGCTAGCAGTTTAAACACTGCTCCTTAGTCAG		7479-7588
P7	AACTGCTAGCCGCCAGCGGCTTGACCCGCTGTGGT	2502	7603-7657
P8	CTGGAACGTTGAACCGGCACGTCCCCAAAGCCCTA		10070-10104
P9	GTTCAACGTTCCAGCAGGTACAACGCTGCAATTCC	1803	10091-10125
P10	TTCTGGCGCGCCCGAAACGCATCATTGTAATCCTC		11859-11893
P11	TTCGGGCGCGCCAGAAAGGGAAAATTTATAAAGCT	2341	11878-11912
P12	ATCAGGTGACCTTCGACCTCAACCTTACCCCCTTT		14184-14218
P13	CGAAGGTCACCTGATCGACCTCAAGAGAGTTGTGC	1131	14207-14238
P14	CGGCCGCATGGTTCTCGCC		15291-15320
Ns-U	GCGTCCTCACAGACGGAATA	1004	2971-2990
Ns-L	CGCCGAGAAGACCCAGA		3957-3974

rogen USA), 100 U of M-MLV Reverse Transcriptase (200U/ μl, Invitrogen USA), 5 μl of extracted RNA (5–50 ng), and 5.5 μl of sterile water free RNase in a total volume of 20 μl. All components were mixed and incubated at 37ºC for 50min, then 70ºC for 10min, and finally chilled on ice. Two μl of cDNA and 1 μl of specific primer (10pmol/ μl) of each in Table 1 were added to PCR reaction for amplifying each fragment. AccuPrime™ Pfx DNA Polymerase (Invitrogen USA) was used for PCR amplication. PCR reaction conditions include: denaturation at 95ºC for 5 min followed by 30 cycles of 95 ºC for 30 s, annealing at 62 ºC for 30 s, at 68 ºC for 1 min per kb, and then extension at 68 ºC for 10 min. The PCR products were identified by electrophoresis on a 1% agarose gel and purified using PureLink™ Quick Gel Extraction Kit (Invitrogen USA) according to the supplier's instructions. Those correct DNA were cloned into a pCR-Blunt vector and identified by PCR and specific restrictive enzyme digestion. Restrictive enzymes were purchased from commercial sources (New England Biolabs). At least three clones of each fragment were sequenced. Individual cDNA positive recombinant plasmids were sequenced in both directions with universal primers and additional primers selected on the basis of the obtained sequences. The sequencing reactions were completed using a PE 9600 Thermocycler and a 377 Sequencer (Perkin-Elmer) at Shanghai Invitrogen Biotechnology Company (Shanghai, China)

Analysis of nucleotide and the deduced amino acid sequences of PRRSV TJM

The reference nucleotide sequences of PRRSV strains in this study were blast-searched using the public database available in GenBank. The full-length cDNA of PRRSV TJM vaccine strain was obtained from combination of overlapped 7 fragments. The complete genome

Table 2. The number of nucleotide and amino acid changes in each gene of PRRSV TJM strain genome sequence against TJ strain.

Mutation location	number of nucleotide change	number of amino acid change
5'UTR	4	/
Nsp1	11	5
Nsp2	39	24, 120-amino acid deletion
Nsp3	13	4
Nsp4	2	1
Nsp5	4	1
Nsp6	1	0
Nsp7	5	2
Nsp8	0	0
Nsp9	15	3
Nsp10	17	4
Nsp11	2	1
Nsp12	1	0
ORF2	0	0
ORF3	0	0
ORF4	0	0
ORF5	1	1
ORF6	1	0
ORF7	4	2

nucleotide were analyzed and compared with parental virus PRRSV TJ strain and other isolates obtained from the GenBank. Nsp2 were compared among six pairs of parental/attenuated vaccines: VR-2332 and RespPRRS MLV, JA142 and Ingelvac1 ATP MLV, CH-1a and CH-1R, JXA1 and JXA1-R, HUN4 and HUN4-F112, TJ and TJM. Amino acids sequences of Nsp2 were download from GenBank. Nsp2 sequence of HUN4-F112 was cited from reference (An et al., 2010). Sequence comparison analysis software is Clustal W in software package Lasergene 7 (DNAstar Inc., USA). Phylogenetic tree was constructed using Neighbor-Joining method of the mega (molecular evolutionary genetics analysis) 5.0 software(Tamura et al., 2011). Bootstrap values above 50% are indicated on the inner branches.

RESULTS

Nucleotide and amino acid changes of PRRSV TJM genome

A two-step RT-PCR assay, using 7 pairs of primers, was conducted to amplify the entire genomic RNA of PRRSV TJM strain. Each fragment cloned into a pCR-Blunt vector was sequenced in both directions. When the sequence in both directions did not conform, samples were re-sequenced. The genome sequence of the PRRSV TJM strain was obtained from combination of the 7 individual viral specific clones. The sequence data showed that, the genomic sequence of TJM has 14, 961 nucleotides (nt) in length, excluding the poly (A) tails. The nucleotide and deduced amino acid (aa) sequences determined for the PRRSV TJM strain were compared with its parental virus, PRRSV TJ strain. The genomic organization of TJM is the same as the TJ strain, consisting of a 189-nt 5'UTR, a 14,621-nt protein-coding region containing 9 ORFs, and a 150-nt 3'UTR. Two large overlapping open reading frames, ORF1a and ORF1b with 7062 and 4385 nucleotides, respectively, follow the 5'UTR. It was found that the ORF1a of TJM was 360 nucleotides less than that of TJ (7422 nt). The lengths for the other genes were same for both strains.

Sequence analysis revealed a total of 115 nucleotides differences between TJM and TJ strains (Table 2). These mutations are located throughout the genome, except for Nsp8, ORF2, ORF3 and ORF4 and the 3'UTR. There were 4 nucleotide mutations in the 5'UTR, i.e. $UGU_{37-39} \rightarrow GUA_{37-39}$ (TJ→TJM) and $U_{60} \rightarrow A_{60}$. The nucleotide changes were found in almost all the non-structure protein genes, except Nsp8. Among these mutations, 39 nucleotide mutations were found in the Nsp2 gene. The most notable is a 360-nucleotide in-frame deletion at the genomic positions 3130-3489 of the TJ strain, resulting in deletion of 120 amino acids at positions 598–717 in the TJ Nsp2. There are 11, 13, 15 and 17 nucleotide muta-
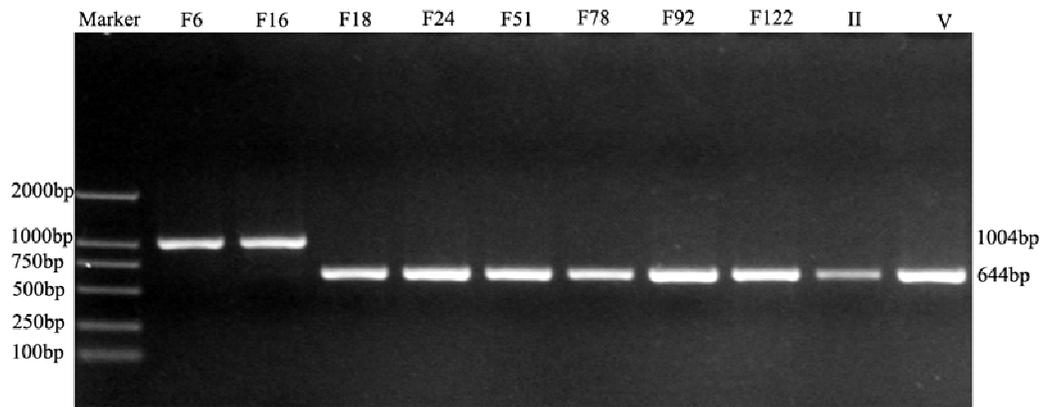
Figure 1. Stability of Nsp2 deletion during serial passage of TJ. RT-PCR for amplification of Nsp2 variable region was performed on media from infected eight passages and serum from inoculated virus F92. Marker: DL2000; different passages of TJ strain (e.g., F6, F16, F18, F24, F51, F78, F92 and F122) and the 2nd (II) and 5th (V) passages virus of TJM in pig.)

tions in Nsp1, Nsp3, Nsp9, and Nsp10, respectively. Among these mutations, many are missense mutation, which leads to the amino acid changes that could result in the changes of protein structures. Nsp1 and Nsp2 proteins have more than 50% missense mutations. Nsp1 protein identity between TJM and TJ was 98.7%. The eleven nucleotide mutations resulted in five residues changes, $Arg_{32} \rightarrow Gln_{32}$ (TJ$\rightarrow$TJM), $Val_{99} \rightarrow Met_{99}$, $Thr_{227} \rightarrow Ile_{227}$, $His_{257} \rightarrow Tyr_{257}$, $Gla_{317} \rightarrow Thr_{317}$. In addition , Nsp4-7, Nsp11 and Nsp12 also have nucleotide mutations, but less than 5 in numbers. A notable change is the $A_{5889} \rightarrow T_{5529}$ transition resulted in the change of Nsp4 protein Lys_{1963} to Asn_{1843} (TJ $\rightarrow$TJM). The site is in the carboxyl-terminal domain (extension) of 3C-like serine proteinase (3CLSP). ORF5, ORF6 and ORF7 has 1, 1 and 4 nucleotide changes, respectively. The GP5 transversion from $A_{14284} \rightarrow G_{14284}$ resulted in amino acid 196 undergoing a non-conservative change from Gln to Arg. The arginine residues at positions 13 and 151 in GP5 reported previously to be involved in the virulence (Allende et al., 2000) did not change. In general, mutation of the non-structural proteins is more than the structural proteins.

In order to determine the stability of the 120 amino acid deletion in TJM Nsp2, The primer pairs (Ns-U/L, see Table 1) stretching over the deletion were designed to amplicate the fragment including the 120 amino acid deletion. The viral RNAs were extracted from different TJ passages (e.g., F6, F16, F18, F24, F51, F78, F92 and F122) and the 2nd and 5th   passages virus of TJM in pig using Trizol reagent (Invitrogen USA). The 120 amino acid deletion was investigated by RT-PCR and sequencing. The results showed the 120 amino acid deletion always existed not only in virus-higher passage on cell culture but the virus passaging in animals (Figure1). The deletion didn't recover in susceptible animals.

Comparison of Nsp2 in several North American strains

To understand better the variable nature of Nsp2, the Nsp2 sequences of TJ and TJM were compared with other 7 strains of Northen American PRRSV reported in GenBank. In comparison with the classical strain VR-2332 (GenBank: AY150564), amino acid deletions occurred in Nsp2 of MN184C (GenBank: ABP02057) at the amino acid positions 323-433, 483, 504-522 and P129 (GenBank: AAM18557) at the amino acid positions 505510 and HB-2(sh)-2002 (GenBank: AAP57400) at the amino acid positions 470-481 and BJ-4 (GenBank: AAG49619) at the amino acid position 694. These viruses were isolated prior to 2006. High pathogenic PRRSV TJ and JX143, reported in China after 2006, have a unique noncontiguous 30-amino acid deletion in Nsp2. The deletions are located at amino acid positions 481 and 532-560. In addition, PRRSV GDQY2 had an additional 35 deletion (at the amino acid positions 470-505) in Nsp2. Howerver; the Nsp2 of PRRSV TJM strain in current study had another 120-amino acid deletion (at the amino acid positions 628-747) downstream of the 30-amino acid deletion. These deletion sites clustered in the middle of Nsp2 gene, at about amino acid positions 330-750 (Figure 2). Comparison among Nsp2 of the six pairs of parental virulent viruses and their attenuated live vaccine strains showed that TJM had more mutations in due to repetitive cell culture passage and the identity of Nsp2 of TJ/TJM pairs was the lowest (97.5%) among pair-wise sequences. Nsp2 sequences of the six pairs of
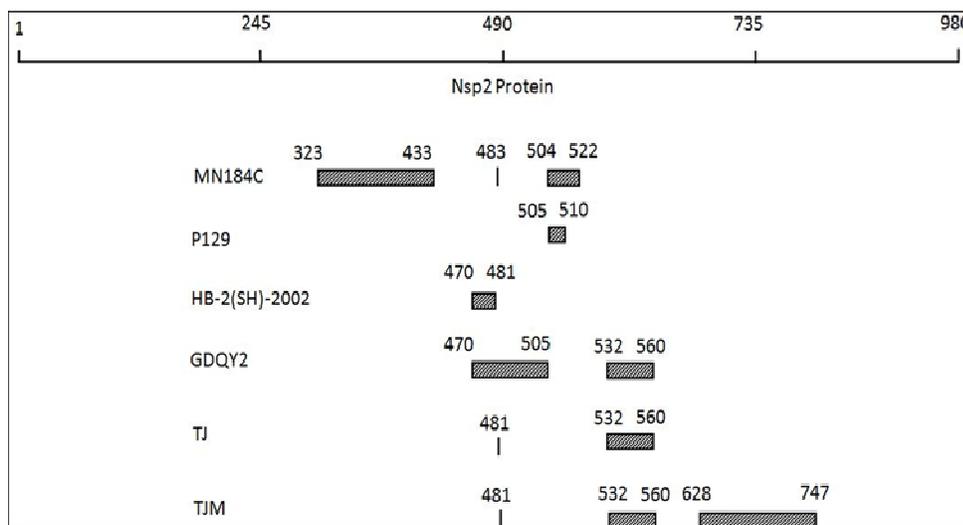
Figure 2. The deletions in the Nsp2 proteins of several PRRSV strains. The Nsp2 of PRRSV VR-2332 in amino acids size is 980. The deletions in Nsp2 gene of several typical strains were showed by black boxes. The positions of deletion were denoted with numbers. The Nsp2 gene of highly pathogenic isolate TJ has a 30-amino acid discontinuous deletion (one deletion at 481, 29 deletions at 532-560) within Nsp2 comparing to VR-2332. However, its cell adapted attenuated descendent, TJM strain, has additional 120-amino acid deletions (at amino acid 628-747) in the Nsp2, except 30-amino acid deletion. Privious strains MN184 was marked with three discontinuous deletions (111, 1, and 19 amino acids) within the Nsp2 protein. The 6-amino acid deletion appeared at the amino acid positions 505-510 of P129 Nsp2. And HB-2(sh)/2002 exhibited a 12-amino acid deletion within Nsp2; GDQY2 isolate not only had the 29-amino acid deletion and newly type 35 deletions within Nsp2, which overlapped with one deletion of the 30-amino acid discontinuous deletion.

parental /attenuated virus were clustered into the four subclades in the unrooted phylogenetic trees for the North American genotype PRRSV (Figure 3). TJ/TJM, JXA1/JXA1-R and HUN4/HUN4-F112, three pairs of parental /attenuated virus showed considerable distances from sequences reported before the HP-PRRSV epidemic, indicating that the new isolates are significantly diverged.

Predicted secondary structure of PRRSV TJM and TJ Nsp2

In order to investigate the secondary structure changes due to the unique 120-amino acid deletion of the Nsp2 of PRRSV TJM strain, the secondary structures of the Nsp2 from TJM and TJ strain were predicted by Predict protein, DNAStar and SOPMA. The result indicated the deletion lead to changes of Nsp2 protein secondary structures and three alpha helixes, five extended strands and two beta turns were absent in TJM Nsp2 protein when compared to TJ Nsp2.

Multiple alignments and phylogenetic analyses

A comparison of the full-length sequences was made between the TJM and its parental TJ strain and between TJM and other PRRSV isolates, including three American strains (VR-2332, MN184C and P129) and six Chinese isolates (CH-1a, BJ-4, HN1,HB-2(sh) 2002, JX143 and GDQY2). The TJM genomic sequence shared a much higher identity at the nucleotide level with its parental TJ isolate (99.2%) and newly isolates, JX143 (99.1%) and GDQY2 (98.6%) in China than that with privious isolates VR-2332, P129, CH-1a, BJ-4, HN1 and HB-2(sh) 2002 ranging from 89.1% to 95.1%. The identity between TJ, TJM strain and North American strains MN184C is only 84.8%.

To understand the genetic relationship of TJ, TJM with other North American type PRRSV isolates reported in GenBank, the phylogenic trees were produced by using the nucleotide sequences of the full-length genome of 27 isolates (Figure 4). Phylogenetic analyses showed that TJM and its parental virus TJ strain and the strains isolated in China after 2006 belonged to one cluster. TJM
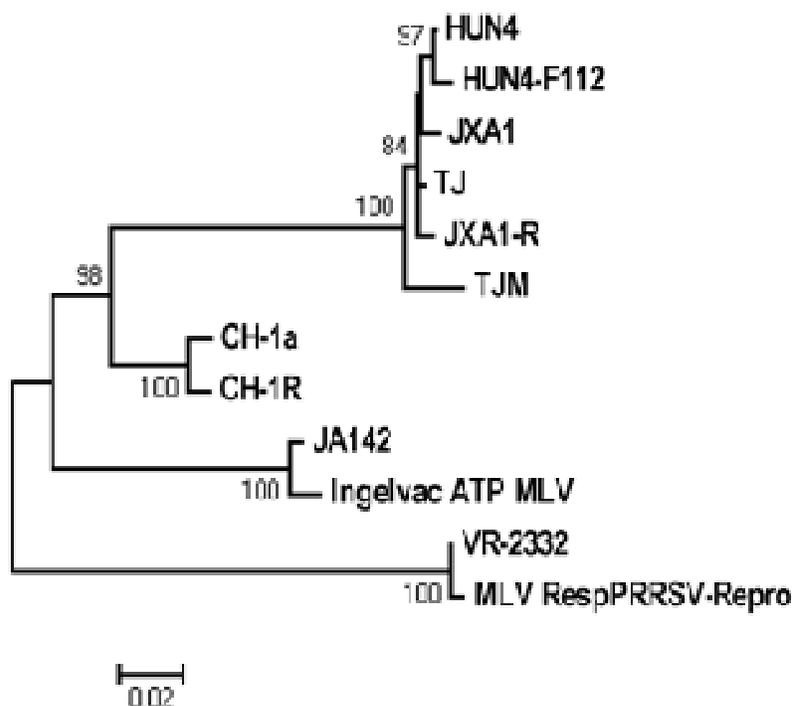
Figure 3. Phylogenetic trees of the Nsp2 amino acid sequences of six pairs of parental/attenuated virus. Unrooted trees constructed for Nsp2 sequences using the mega (molecular evolutionary genetics analysis) 5 software (version 5.05; Center for Evolutionary Functional Genomics, Tempe, AZ, USA). The phylogenetic trees show clear distinction of the HP-PRRSV parental/attenuated pairs from the viruses reported before 2006. Nsp2 amino acid sequences invoved in paper were downloaded from GenBank or from reference. (GenBank no. VR-2332, PRU8739; RespPRRS MLV, AF066183; JA142, AY424271; Ingelvac1 ATP MLV, DQ988080; CH-1a, AY032626; CH-1R, EU807840; JXA1, EF112445; JXA1-R, FJ548855; HUN4, EF63500; TJ, EU860248). Nsp2 sequence of HUN4-F112 is from reference (An et al., 2010).

exhibit a clear distinction in view of evolution from VR-2332. The CH-1R as a vaccine strain in China is closed to the first Chinese strain CH-1a (parental strain).

Differentiate the PRRSV TJM from other isolates based on 120 amino acid deletion of Nsp2

In the light of 120 amino acid deletion of TJM Nsp2, a differentiated measurement was developed. The primer pairs Ns-U/L could differentiate TJM strain from HP-PRRSV isolates. The RT-PCR results showed a 644 bp fragment was amplified from Marc-145 cell supernatants or serums infected PRRSV TJM, but 1004 bp from TJ and JX143 strains. The 120 amino acid deletion of Nsp2 was as a molecular hallmark of PRRSV TJM strain, and the method developed on the deletion could discriminate the vaccine strain TJM from HP-PRRSV. So the 120 amino acid deletion could be used as a vaccine marker

and applicating for diagnosis in the disease control measurement.

DISCUSSIONS

An unparalleled large-scale, atypical PRRS outbreak in China in 2006 was caused by a highly virulent PRRSV strain (Tian et al., 2007). It was reported that the outbreak occurred in 20 provinces, and caused the deaths of more than 20,000 pigs in a 2-week period (Zhou et al., 2008). Highly pathogenic PRRSV TJ strain was isolated in 2006 in China. Its Marc-145 cell adapted attenuated descendent, TJM strain, had been shown to be virulent in pigs with no clinical signs when vaccinated (Data about animal experiments are going to be published somewhere else by other researchers). Clues to the genetic basis of pathogenicity can be gleaned from comparison of the nucleotide sequence of a virulent
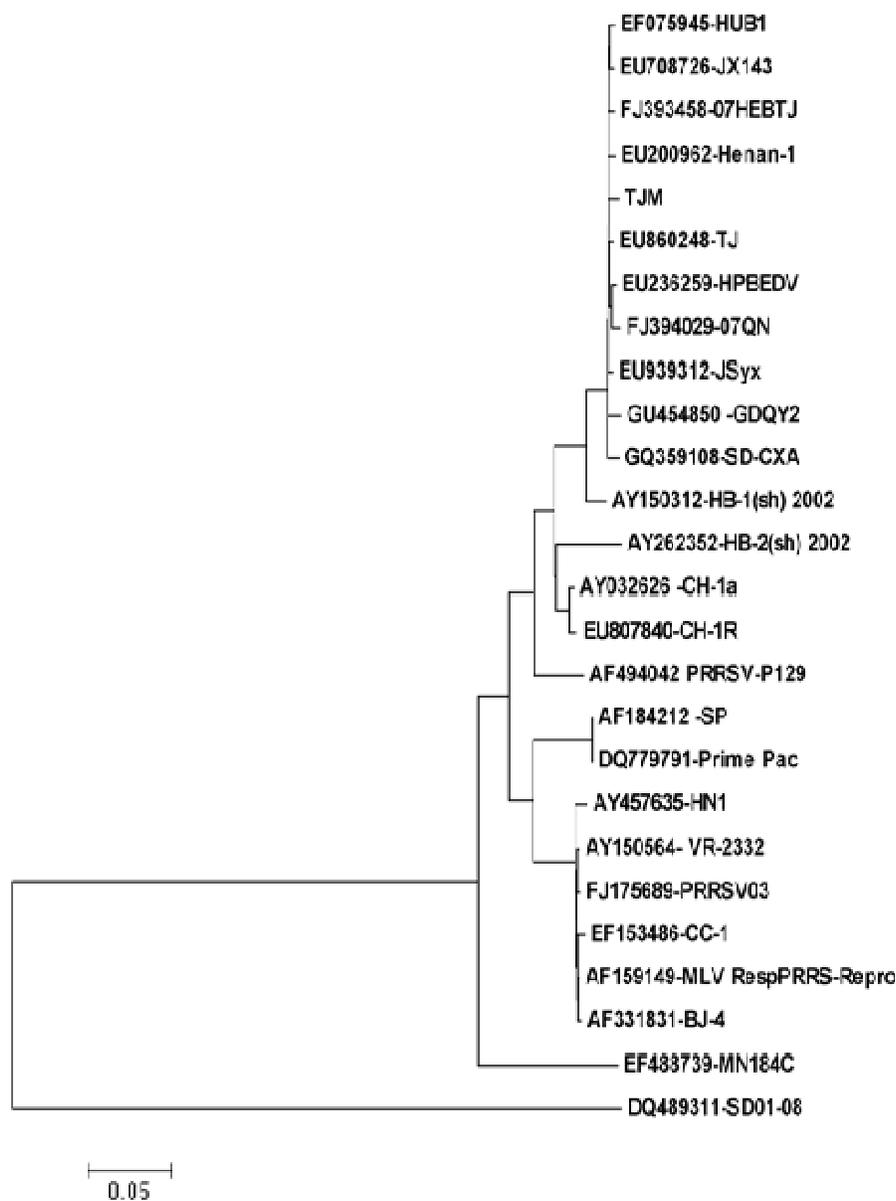
EF075945-HUB1
EU708726-JX143
FJ393458-07HEBTJ
EU200962-Henan-1
TJM
EU860248-TJ
EU236259-HPBEDV
FJ394029-07QN
EU939312-JSyx
GU454850 -GDQY2
GQ359108-SD-CXA
AY150312-HB-1(sh) 2002
AY262352-HB-2(sh) 2002
AY032626 -CH-1a
EU807840-CH-1R
AF494042 PRRSV-P129
AF184212 -SP
DQ779791-Prime Pac
AY457635-HN1
AY150564- VR-2332
FJ175689-PRRSV03
EF153486-CC-1
AF159149-MLV RespPRRS-Repro
AF331831-BJ-4
EF484739-MN184C
DQ489311-SD01-08

0.05

Figure 4. Phylogenetic analysis based on nucleotide sequences of the full-length genome of 27 fully sequenced PRRSV isolates. Multiple-sequencing alignments were performed using the MEGA 5 software. Phylogenic trees were constructed by using the neighbor-joining method.

parent viral strain with that of the cell-passaged attenuated variant. Genomic sequences of the strain TJM was determined and analyzed in this report. It's full-length sequence was compared with the genomic sequences of TJ and the American isolates VR-2332, MN184C and P129, Chinese isolates CH-1a, BJ-4, HN1, HB-2(sh) 2002, JX143 and GDQY2. Excluding the poly A tail, the TJM genome was found to be 14 960 nts in length, which was 451 bases shorter than that of the prototypic VR-

2332 sequences, and 360 bases shorter than its parental virus TJ. As we know the genome of TJM is most shorten in PRRSV. TJM still has high identity to the highly pathogenic strains. The phylogenetic relationship among North American type PRRSV strains isolated in China and other countries were analyzed. The results showed a high degree of genetic homology was observed among most of the Chinese isolates after 2006. Phylogenic tree manifested the highly pathogenic isolates after 2006 in

China and attenuated virus TJM located in one cluster, although the virulence is different. That is similar to strain CH-1R and CH-1a. However, BJ-4, HN1 and CC-1 isolated before 2006 were exception, and they belong to one group with VR-2332 strain.

Complete genome analysis of the parental strain TJ and its cell-culture adapted descendant, strain TJM, revealed differences in their nucleotide and deduced amino acid sequences. Like other strains of PRRSV, ORFs 2a to 7 of TJM, identified immediately downstream of ORF1b, cover the 3' one-third of the genome of PRRSV. These ORFs encode seven envelope-associated proteins GP2a, GP2b, GP3, GP4, GP5, M and a nucleocapsid (N) protein. Comparing to the TJ strain, the nucleotide mutations occurred at 115 positions in complete genome of PRRSV TJM strain. Comparison of Nsp1–Nsp3, Nsp9, Nsp10 was done to find more nucleotide mutations, which lead to more amino acid mutations. The 11 nucleotide mutations described in nsp1 regions of PRRSV TJM and five of the 11 nucleotide changes in strain TJM were missense. EAV Nsp1 was shown to be dispensable for genome amplification but essential for sgmRNA production (Nedialkova et al., 2010; Tijms et al., 2007; Tijms et al., 2001). Confirming the PRRSV Nsp1α structure (Sun et al., 2009) implicated its regulation in sgmRNA synthesis (Kroese et al., 2008). Prominent hydrophobic domains of replicase pp1ab of PRRSV (strain VR-2332) are present in Nsp2, Nsp3, and Nsp5, and are thought to mediate the membrane association of the replication and transcription complex. It is possible that amino acid changes of non-structural proteins might affect viral RNA synthesis. The most interesting finding from this study is that Nsp2 of TJM had a 120-amino acid deletion, which leads to changes of the secondary structures including the absence of three alpha helixes, five extended strands and two beta turns of the Nsp2. The 24 amino acid mutations of Nsp2 and 120 amino acid deletion could make the prominent structural changes. Secondary structure prediction of Nsp2 could be evidence.

ORF1b encodes the key enzymes for RNA-templated RNA synthesis—the viral RNA-dependent RNA polymerase (RdRp; nsp9) and RNA helicase (Hel; nsp10), which originally identified by comparative sequence analysis (Fang et al., 2004; Ropp et al., 2004). They are related to replication and transcription of the viral genome (Bautista et al., 2002; Fang and Snijder, 2010; Snijder and Meulenberg, 1998). TJM manifested higher viral titre and was more adaptive on Marc-145 cell than TJ. The characteristic of TJM could be affected by 15 nucleotide changes of Nsp9 and 17 of Nsp10. It was speculated the changes of these non-structure protein of TJM may play a role in view of its higher titer. Five pairs of attenuated viruses with wild-type parental viruses were compared in previous study (An et al., 2010). Nsp2, Nsp3, Nsp10,

GP2, GP3, and GP5 were found to be susceptible to mutation. However, in this study, results were not same as the reference. Genome sequencing results ultimately revealed that ORF2, ORF3, ORF4 of TJM were most closely related to the corresponding genes from its parental virus TJ strain. No mutations were detected in Nsp 6 and Nsp8 of the five pairs of parental/attenuated vaccine strain pairs(An et al., 2010), and in the current paper, only 1 mutation was found in Nsp 6 and none in Nsp 8. Perhaps such conservation may be a result of the small predicted size of the Nsp 6 and 8 ORFs. That is suggested the ORFs are smaller and thus less likely to acquire random mutations than larger ORFs.

Nsp2 protein size may be smaller or larger, depending on which cleavage sites are used, the presence of insertions and deletions and the genotype of the PRRSV isolate. VR-2332 Nsp2 protein is 980 residues long, while the TJ and TJM Nsp2 proteins are 950 and 830 in this study, respectively. Genetic sequence comparisons of PRRSV isolates identified a hypervariable regions in the Nsp2 that naturally incorporates nucleotide insertions and deletions (Gao et al., 2004; Han et al., 2006; Shen et al., 2000; Zhu et al., 2011). Now, we are reporting a 120-amino acid deletion within Nsp2 of TJM strain when compared to the Nsp2 protein of strain VR-2332. The 120-amino acid deletion of TJM was a most longest contiguous deletion in Nsp2 among these PRRSV strains (Figure 2). And the 120-amino acid deletion was always exist until passages 122. From all deletions in Nsp2 gene of these viruses, we found the deletion mainly occurred at amino acid 320-750. Thus, the region is highly variable. Their deletions were all overlap partly with 30-amino acid discontiguous deletion in Nsp2 of highly pathogenic PRRSV. Interestingly, the novel 120-amino acid deletion is located at following the 30-amino acid deletion, a hallmark of HP-PRRSV. It's important the additional 120-amino acid deletion in this coding region were unique deletion comparing with privious report strains. On the side, Nsp2 genes from wild-type and attenuated virus pairs were compared, and the obvious evolusion relationship from six pairs of parental/ attenuated virus. Our study updated the information of comparision of parental/attenuated vaccine strain pairs.

In the immunology, PRRSV nsp1α/β, nsp2, nsp4, nsp7, and nsp11 have been implicated in the modulation of host immune responses to PRRSV infection. They were determined to have roles in IFN antagonism and inhibition of the IFN-β promoter activities. Within pp1ab, Nsp2 contains a highly immunogenic region that possesses at least eight B cell epitopes (de Lima et al., 2006). The deleted regions and variable parts of Nsp2 were thought to be important for immunity (Fang et al., 2004; Ropp et al., 2004). But this deletion region didn't contain previously identified B-cell linear epitopes (de Lima et al., 2006) and potential T-cell epitopes.

Initial results from our laboratory showed that PRRSV TJM is a live-attenuated virus based on animal experiments (data is going to be published). Previous study concluded that a complex summation of several major effectors contribute to the overall pathogenesis of PRRSV(Beura et al., 2010).Tong et al thought determinants of PRRSV attenuation are multigenic products of both non-structural and structural genes(An et al., 2010). The results in this study revealed that PRRSV TJM strain is a novel strain with unique deletions and Nsp1, Nsp2, Nsp3, Nsp9 and Nsp10 had many mutation sites. One or more factors might affect the virus virulence. Among these non-structure proteins, Nsp2 is especially remarkable. One mutant recovered from an infectious PRRSV cDNA clone containing a 131-amino acid deletion of Nsp2 previously was proved to be less virulent in pigs(Kim et al., 2009). Recombinant viruser△727–813, deleted the amino acid position 727–813 of VR-2332 Nsp2 shad a significant decrease in lymph node enlargement compared to rVR-2332 (Han et al., 2007). The attenuated TJM also has a 120 amino acid deletion (628-747) in Nsp2. These results suggested Nsp2 are likely relavent to the viral virulence. Whether this 120 amino acid deletion has resulted in the virulence from unusually high to lower remains to be determined by reverse genetics.

The remarkable genetic variability of PRRSV possesses difficulties in developing diagnostic assays (serological or RT-PCR) and effectively vaccination. It should be noted that Nsp2 containing deletion region can be used as diagnostic marker for controlling newly emerged PRRSV. The 120 amino acid deletion of TJM was successfully used for differentiating the animals' vaccinated TJM strain from infected field virus, especially HP-PRRSV. With respect to classical PRRSV, we didn't find a mating primers stretching over the 120 amino acid deletion for discriminating TJM from representative strains VR-2332 and CH1a isolated before 2006 causing the variable sequences.

The tolerate deletions in Nsp2 means it can be as a potential target for the construction of a marker virus. Based on this property, we reasoned that Nsp2 could support the insertion of foreign marker proteins. The choice of the Nsp2 as a site for foreign protein insertion was based on its large size and natural capacity to support the insertion of polypeptides. The insertion of foreign oligo- and polypeptide sequences into the Nsp2 of PRRSV provides the opportunity to develop marker viruses for use in studies of virus replication and the Nsp2 function. Developing a vaccine that can differentiate infected and vaccinated animals (DIVA) is a new challenge in the design of a vaccine for porcine reproductive and respiratory syndrome virus. The 120-amino acid deletion of TJM strain means that the location can insert foreign genes without effect on replication.

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