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Construction of infectious cDNA clones of PRRSV: Separation of coding regions for nonstructural and structural proteins

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Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), the causative agent of the ongoing "porcine high fever syndrome" in China, is capable of genetic and antigenic mutations at high frequency. How to design vaccine rationally to keep up with the ever-changing prevalent PRRSV variant is of great interest. We developed an infectious cDNA clone of an attenuated strain of Type II PRRSV, and further manipulated the infectious cDNA clone by inserting polylinker between ORF1 and ORF2, encoding for nonstructural- or structural-protein, respectively. The cDNA was generated from the cell-attenuated virus strain, APRRS, via RT-PCR, and followed by nucleotide sequencing and molecular cloning. The full-length of the APRRS genomic RNA was determined as 15521 nucleotides in length excluding poly(A) tail, which has a 99.7% nucleotide identity with that of PRRSV Nsp strain, also a vaccine strain. Based on the nucleotide sequencing results, the full-length cDNA clone was assembled in pBlueScript vector, under the control of T7 promoter at the immediate 5′ terminus of genome. To discern the rescued viruses from that of parental virus, a *Mlu* I restriction site was engineered into ORF5 coding region. Upon transfection of the in vitro transcripts of both the original and *Mlu* I-tagged cDNAs into MA-104 cells, typical PRRSV cytopathic effects were observed. The rescued viruses from the full-length cDNA clones displayed the same virological and molecular properties. Subsequently, PCR-based mutagenesis was conducted to separate the coding regions between PRRSV nonstructural genes, ORF1, and structural proteins, ORF2—ORF7. The synthetic RNA of such mutant clone, pCSA, was infectious and the rescued virus shared similar properties with that of the parental virus. This study provided a valuable tool for development of chimeric PRRSV as vaccine candidate offering cross-protection to various genetically diversified PRRSV strains, and a platform for further development of PRRSV as a gene expression vector for recombinant vaccines against other significant swine diseases.

PRRSV, reverse genetic system, coding region, nonstructural protein

Porcine reproductive and respiratory syndrome (PRRS) was first recognized in the late 1980's in the United States[1], and now is the most economically significant swine disease worldwide. Since Guo et al. (1996) first reported PRRS-like outbreaks in the mainland China[2], the infection rate of Chinese herds is up to 90%. The causative agent, PRRS virus (PRRSV), was first identified in the Netherlands (designated Lelystad virus, Wensvoort et al., 1991)[3] and followed immediately with the isolation of the North American isolate (VR2332)[4]. These two isolates now represent the two recognized

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genotypes of PRRSV: European (EU genotype, type I) and North American (US genotype, type II)[5]. The principal clinical manifestation of the disease is characterized by a sudden onset of abortion and infertility in infected sows, and increased mortality of piglets that may be the result of secondary respiratory infections. In the past twenty years, PRRS damage becomes more devastating worldwide. A neurotropic PRRSV has been isolated from herds where increased rates of abortion occurred, suggesting that PRRSV may be expanding its tissue tropism[6]. More recently, the swine industry has suffered from a severe form of PRRSV, characterized by increased abortion and mortality in pregnant sows[7]. These so-called "atypical PRRSV" outbreaks have surprisingly been reported in vaccinated herds with the currently commercial live vaccines[7]. Beginning from the summer of 2006, a so-called "Porcine High Fever Syndrome (PHFS)" has spread all over the Chinese swine industry and resulting in culling of an estimated 30 millions of pigs. A highly pathogenic variant of PRRSV has been confirmed to be directly involved in the ongoing PHFS (refs. [8,9]; Yuan et al. unpublished data), against which a panel of vaccines of traditional PRRSV strains were surprisingly ineffective (Tian et al. personal communication). In addition, PRRSV targets porcine Alveolar macrophage, an important player in immune response, thus results in host immunosuppression and leads to secondary infection. It is confirmed that PRRSV infection causes mixed infections such as porcine post-weaning multi-systemic wasting syndrome (PMWS), porcine respiratory disease complex (PRDC), etc. Obviously, PRRS is the top threat to the swine industry worldwide, and prevention and control strategy is in dire need.

PRRSV is a member of the *Arteriviridae*, which also includes *Equine Arterivirus* (EAV), *Mouse Lactate dehydrogenase-elavating Disease Virus* (LDV), and *Simian Hemorrhagic Fever Virus* (SHFV). There are two PRRSV serotypes / genotypes, (European) Type I and (North American) Type II, with the Lelystad virus[4] and VR-2332 strain[5] as the prototype, respectively. The PRRSV genome is a single-stranded, nonsegmented, positive-sense RNA. The genomic RNA contains a 5′-cap and 3′-poly(A), about 15.5 kb in length, encoding at least eight open reading frames (ORFs) (Figure 1). The coding sequences are flanked by untranslated regions (UTR) at both 5′ and 3′ ends of the genome. The viral replicase is encoded by ORFs 1a/1b, of which 1b is expressed via a −1 frameshift mechanism from the genomic RNA. Viral structural proteins are expressed from six subgenomic mRNAs formed by a unique yet unknown discontinous transcription mechanism by which the 5′ leader noncontiguously joins the subgenomic body RNA located at the 3′ genomic end. Open reading frames 2 to 6 encode for the major envelope associated structural proteins, and the multifunctional nucleocapsid (N) protein of 15 kDa is encoded by ORF 7.

As a RNA virus, PRRSV is prone to be highly genetically and immunologically diversified[10]. The two PRRSV genotypes are dramatically different both genetically and immunologically, with only about 60 percent nucleotide identity and little serological cross reaction have been reported[11]. Among the same genotype, up to 20% genetic diversity has been reported[12,13]. The high heterogeneity of PRRSV poses challenge for developing vaccine with a similar genetic trait to the ever-changing of prevalent PRRSV strains, as little cross-protective immunity has been observed for current vaccine against challenge by heterologous virulent strains[10]. The genetic diversity among field isolates of PRRSV is the major obstacle for PRRS control.

Full-length infectious cDNA clones of positive-strand RNA viruses are important tools for the study of the biology of viruses. The so-called "Reverse Genetics System" has been instrumental for the dissection of the virus replication processes for several RNA viruses. Moreover, genetically engineered molecular vaccine with desired genetic and immunological traits can be developed based on RGS manipulation. Although infectious cDNA clones have been developed for the PRRSV European type[14,15] and several North American PRRSV isolates[16–19], there is a need to develop an RGS based on cell culture-adapted avirulent PRRSV, which would be critical for investigating viral virulence factors as well as *cis*- and *trans*-acting elements that controls the discrete steps of viral replication. Furthermore, for development of modified live vaccine (MLV), RGS application has a huge advantage over the traditional cell-attenuation approach, as RGS can be easily employed to change the protective antigen coding sequences to that of the prevalent strain. We reported here that full-length infectious cDNA clone of an attenuated PRRSV strain was developed, based on which further reverse genetics manipulations were conducted for further dissecting the genomic structure and functions. In particular, an infectious mutant clone containing multiple cloning sites between the coding re-

gions for the replicase complex and structural proteins was found to be similar to the parental virus, which provide a platform for engineering attenuated vaccine with protective antigens from prevalent PRRSV.

1 Materials and methods

1.1 Viruses and cells

The original PRRSV strainwas isolated from clinically typical PRRSV infected pigs, followed by serial passage on MA-104 cells for 90 times, designated as APRRS strain. The cells were maintained in essential medium with Eagle's salts (EMEM, Gibco BRL) containing 2% fetal bovine serum (FBS, Gibco BRL). The culture supernatant of the infected cells was harvested 4—5 d post inoculation (dpi), when about 80% of the cells developed cytopathic effect (CPE). The harvested viral suspension was aliquoted and stored at −70℃ as virus stocks.

1.2 RT-PCR amplification of viral cDNA

Viral RNAs were isolated from the supernatant of the APRRS virus infected cells, using the QIAamp viral RNA isolation kit (QIAgen Inc., Valencia, CA). Briefly, 140 μL of viral suspension was used to isolate viral RNA, followed by Reverse transcription (RT) conducted by using Superscript II Reverse transcriptase (Gibco BRL). The synthetic complementary DNA (cDNA) of the viral RNA was initiated with poly(T)-anchored primer, Qvt (Table 1), which contains two restriction endonuclease sites, *Xho* I and *Vsp* I, respectively, immediately following poly(A) tail. The PCR primers (Table 1) were designed mainly based on the consensus sequences of the available GeneBank sequences such as accession number AF184212[20] and U87932[11]. The synthetic T7 promoter (underlined sequence), along with two nonviral Gs, was engineered into primer STL (Table 1) and directly fusing with the first nucleotide of the PRRSV genomic RNA. The anchor primer Qvt contains *Vsp* I and *Xho* I recognition sequences which may be used for linearization of the plasmid with only 2 or 12 nonviral nucleotides beyond the authentic ploy(A) stretch. "SF" indicates the oligonucleotide is a forward primer, with "SR" as a reverse primer. Platinum High Fidelity DNA polymerase (Gibco/BRL) was used for PCR amplification of the cDNA fragments from 2 μL of the RT reaction mixture, using the primers shown in Table 1. A normal PCR reaction included one cycle of 95℃ for 2 min, followed by 30 cycles of 95℃ for 15 s, 64℃ for 30 s, 68℃ for 1 min per kilobasepair (kb), and polished by incubation at 68℃ for 10 min. The PCR products were identified by electrophoresis on a 1% agarose gel. The correct DNA bands were purified using the QIAEX II gel-purification kit (QIAgen Inc., Valencia, CA) according to the supplier's instructions.

1.3 Cloning and nucleotide sequencing of viral cDNA

The purified RT-PCR products were cloned into the pCR-ZeroBlunt-TOPO vector (Invitrogen, USA). The positive clones were first screened by agarose gel electrophoresis of the plasmids isolated with QIAprep Miniprep Spin Kit (QIAgen Inc., USA), followed by restriction endonuclase digestion and/or nucleic acid sequencing. Restriction endonuclases and other DNA modifying enzymes are purchased from commercial sources (Pro-mega, Madison, WI; New England Biolabs, MA), and used according to the suppliers' instructions. Nucleotide sequencing of the clones and/or PCR fragments was performed by Shanghai Auke Inc.(Shanghai, China) using an ABI 377 automatic sequencer. Besides the prim-

Table 1 Primers used for RT-PCR and PCR mutagenesis

Name	Sequence	Position	Application
Qvt	GAGTGACGAGGACTCGAGCGCATTAATTTTTTTTTTTT	5412	T-PCR
STL	CATGCATGCTAATACGACTCACTATAGGTATGACGTATAGGTGTTGGC	1	PCR
R2573 *	CTGCCCAGGCCATCATGTCCGAAGTC	2573	PCR
F1973 *	CGCCACAACGGAGGGAATCAC	1973	PCR
R5609	CGCGGGGGCCACTGGTGTAATGAT	5501	PCR
F5124	GCGGCACCGGCACTAACGAT	5016	PCR
R9753	GTACCCGCACACTCTCGACTTCTTCCCCTCAT	9645	PCR
F7682	CTTTCCGTTGAGCAGGCCCTTGGTATGA	7574	PCR
PSA2F	TTAATTAATTTAAATGGCGCGCCAATGAAATGGGGTCCATGC	12072	Mutagenesis
PSA1R	GGCGCGCCATTTAAATTAATTAATCAATTCAGGCCTAAAGTTGG	12072	Mutagenesis
MLU5F	TGTCCTGGCGCTACGCGTGCACCAGATACA	14194	Mutagenesis
MLU5R	TGTATCTGGTGCACGCGTAGCGCCAGGACA	14194	Mutagenesis

*: F, Forward primer; R, reverse primer.

ers listed in Table 1, a number of sense or anti-sense sequencing primers specific for North American PRRSV genomic RNA, were synthesized and detailed information are available upon request. The resulting sequences were analyzed using the Lasergene Software Package (DNAstar Inc., USA).

1.4 Construction of full-length cDNA clones

As outlined in Figure 1, a total of five RT-PCR amplifications were conducted to obtain DNA fragments TB1 (with primer pair STL/SR2573), TB2 (SF797/SR6589), TB3 (SF4344/SR9573), TB4 (SF7682/SR13334), and TB5 (SF11210/Qvt), followed by cloning into plasmid vector pZero-Blunt and/or pCR-XL using the TOPO-cloning kit (Invitrogen). All five PCR products or their clones were subjected to nucleotide sequencing, based on which the full-length PRRSV genomic sequence was generated and then the full-length cDNA clone was assembled. A series of subcloning steps were conducted by utilizing unique restriction endonuclease cleavage site(s) for assembly of the full-length cDNA clone. In doing so, TB2 and TB3 were connected via a restriction endonuclease Kpn I site at nucleotide (nt) position 5388 of the full-length genome. Accordingly, the *Mlu* I at 2177 was utilized to obtain a clone, designated as pBT123, covering the 5′ half of the genome. The 3′-half clone, pBT45, was constructed by utilizing the *Spe* I site at position nt 13117. Finally, the two intermediate clones were connected via a unique site Pme I at 7800, cloned into pBluescript SK(+) vector (Stratagene). The full-length cDNA clone, pAPRRS, is controlled by T7 promoter followed by two Gs, which is believed to enhance *in vitro* RNA transcription level. The 3′ end of the full-length clone can be linearized by utilizing *Vsp* I or *Xho* I sites, which encoded in the anchor primer Qvt. The full-length nucleotide sequence was verified by restriction nuclease mapping and nucleotide sequencing.

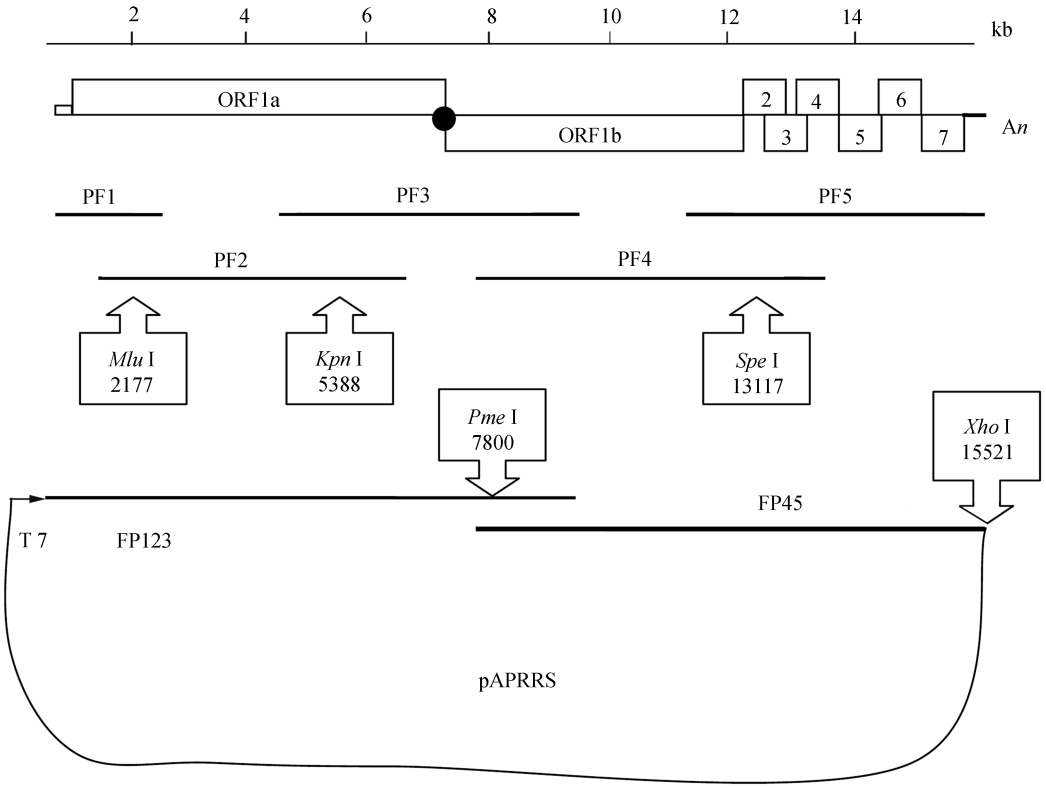

Figure 1 PRRSV's genomic organization and cloning strategy. The viral genomic RNA of PRRSV was isolated, followed by synthesis of the first-strand cDNA, PCR amplification with synthetic primers (Table 1). The primary clones of the RT-PCR products designated as PF1, PF2, PF3, PF4, and PF5, were serially assembled into pBluescript SK(+) vector generating the full-length cDNA clone, pAPRRS, under control by T7 promoter located immediately upstream of the first nucleotide (T) of the 5′ UTR. The 3′ end of the full-length clone can be linearized by utilizing *Vsp* I or *Xho* I sites, which were encoded in the anchor primer Qvt.

1.5 Mutagenesis of full-length cDNA clones

Mutagenesis was conducted according to the instructions of the Quikchange Site-directed Mutagenesis kit (Stratagene, CA) with the 3′-half pBT45 as the template plasmid, and a pair of synthetic forward and reverse primers (PSA1F and PSA1R, Table 1) containing engineered *Pac* I, *Swa* I and *Asc* I recognition sites, which were inserted between ORF1 and ORF2. Briefly, plasmid-based PCR amplification was carried out with the corresponding primer pairs containing the targeted mutations (Table 1). A typical thermal cycling consisted of, 95℃ for 5 min, followed by 18 cycles of 95℃ for 30 s, 55℃ for 30 s, and 68℃ for 20 min extension, and the amplified products were further polished at 68℃ for 10 min, followed by salking at 4℃ until further treatment. To eliminate the plasmid template, 50 units of restriction endonuclease *Dpn* I (Invitrogen, MA) was added to a 50 μL PCR reaction and incubated at 37℃ for at least 4 h. Subsequently, two μL of *Dpn* I-digested mixture was directly used to transform *E. coli* TOP 10 cells. Upon verified by restriction endonuclase mapping and nucleotide sequencing, the mutated clone was selected and swapped back into the corresponding position in the parental pAPRRS utilizing the appropriate restriction enzyme cutting sites in the flanking region, and the desired mutations were verified through RFLP mapping and nucleotide sequencing.

1.6 *In vitro* transcription and transfection

RNA transcripts were generated *in vitro* from 1 μg of *Vsp*I-linearized DNA template by T7 RNA polymerase provided in the mMessage mMachine kit (Ambion Inc., USA). After transcription, RNase-free DNase I was added to eliminate the DNA template. Native RNA agarose gel electrophoresis was carried out to check the quality and quantity of the synthesized RNAs, which were either used immediately or stored at −80℃. One μg of *in vitro* RNA transcripts was transfected into MA-104 cell monolayers in 6-well-plates, using DMRIE-C tranfection reagent (Invitrogen, MA) according to the instructions. The transfection mixture was replaced with EMEM containing 2% FBS at 4 h post transfection and further incubated at 37℃, in the presence of 5% CO_2 for 24 h. One hundred μL of the culture supernatant was used to inoculate fresh MA-104 cells for 1 h adsorption at room temperature, followed by the addition of 3 mL of EMEM containing 2% FBS and incubated as above. The culture supernatants, harvested at 4 or 5 d post inoculation when 80% of CPE developed, were saved at −80℃ until further use. To confirm the transfectant virus was indeed generated from reverse genetics manipulations, the rescued viral genomic RNA was isolated, and subjected to RT-PCR amplification of the targeted genomic region as described above. PCR was conducted with primer pairs SF11210 / SR 13334 for mutations in the range of the end of the ORF1 thorough ORF3, followed by nucleotide sequencing for verification of the desired mutation.

1.7 Immune fluorescence assay

To investigate if the transfected RNA can express authentic viral protein, direct immune fluroscence assay (FA) was employed to detect the ORF 7-encoded Nucelocapsid (N) protein with a monoclonal antibody conjugate, SR30-F (Rural Technologies Inc., IA), according to the supplier's instruction. Briefly, monolayer cells of MA-104 in four-well slide chamber (Nalgene) was infected at 0.1 MOI and fixed with 1∶1 of methanol: acetone for 15 min. The fixed slide was incubated with the anti-PCV II genotic serum for 30 min at 37℃, followed by staining the slide with secondary goate anti- porcine antibody conjugate. The unbinding antibodies were washed off using 3 rounds of PBS flushes.

1.8 Northern blot analysis

The RNA profiles of the infected cells by transfectant viruses were further analyzed by RNA blot analysis. QIAgen RNeasy Mini kit (QIAgen) was employed for isolation of total cellular RNAs from virus-, or mock-infected cells at 36 h post inoculation at 0.5 multiplicity of infection (MOI). Ten μg of total RNAs were separated on formaldehyde-denatured RNA gel, and transferred to Nylon membrane, in according with the instruction manual of NorthernMax system (Ambion). Anti-sense oligonucleotides PSA1R (Table 1), was 3′ end-labeled with digoxigenin-11-2′, 3′-dideoxy-uridine-5′-triphosphate (DIG-11-ddUTP, Roche, Germany) by using terminal dideoxylnucleotide transferase (TdT, New England Biolabs) according to the suppliers' instructions. The hybridization was carried out at 42℃ in the ULTRAhyb-Oligo solution with the NorthernMax blotting kit (Ambion). After low and high stringency hybridization washes at 42℃ twice for 30 min, the color development of hybridization signal was conduct-

ed according to the procedure of DIG Nucelic Acid Detection Kit (Roche).

2 Results

2.1 Complete genomic sequence of the attenuated PRRSV

The virulent strain of PRRSV was isolated from clinical infected pigs, and identified with virological and molecular assays, the virus was attenuated upon serial passage for 90 times on MA-104 cell-culture, designated as APRRS strain, and was proved to be efficacious as a vaccine candidate against challenge with virulent PRRSV (Yuan et al. unpublished data). For complete genomic sequencing, a panel of primers (Table 1) was synthesized based on the consensus sequence among available GeneBank data, mainly accession numbers AF184212 and U87392. The RT-PCR products were cloned and nucleotide sequence was determined, based on which the full-length genomic sequenced was assembled using SeqMan program. The result showed that the attenuated PRRSV has a genome size of 15521 nucleotides in-length excluding poly(A), and shares 99.6% nucleotide identity with that of Nsp strain, also a vaccine virus commercially named as PrimePac (AF184212). Compared to the prototype Type II strain, VR-2332 and its vaccine strain RespPRRS, APRRS shares 96% ORF5 homology, has a unique insertion of 108 nucleotides in nsp2 coding region, as does the Nsp strain.

2.2 Construction of full-length PRRSV cDNA clones

Based on the nucleotide sequence and restriction site information of the individual clones, as shown in Figure 1 and described in section 1, the full-length cDNA clone pAPRRS driven by T7 promoter was generated by a series subcloning steps. To discern pAPRRS with its parental sequence, a *Mlu* I restriction enzyme site was engineered into ORF5 coding region using PCR-based mutagenesis and subclonings; As a result, substitution mutation was used to create a *Mlu* I site by mutations at position 14035 (T to G) and 14037 (A to G) thus generate pORF5M. To separate the coding regions between nonstructural protein and structural proteins, pCSA was constructed by inserting restriction sites of *Pac* I, *Swa* I and *Asc* I at the end of ORF1 and the immediately upstream of ORF2. All plasmids were verified by restriction endonuclease mapping and nucleotide sequencing.

2.3 PRRSV was rescued from *in vitro*-generated RNAs

To test the infectivity of the full-length cDNA, *in vitro* transcripts with 5′ cap were generated from *Xho* I linearized plasmid DNA templates of pAPRRS, pORF5M and pCSA. As a negative noninfectious control, BTSX synthetic RNA from pAPRRS with the *Spe* I / *Xho* I fragment encoding the main structural proteins, ORF4 - 7, and 3′ UTR deleted, were also generated. The *in vitro* synthesized RNAs were transfected into the MA-104 monolayer cell-culture reaching 80% confluency, with the use of the DMRIE-C reagent (Invitrogen). Upon incubation, the synthetic RNAs from pAPRRS, pORF5M and pCSA, but not that of BTSX, induced typical PRRSV CPE beginning at 2 or 3 d post transfection (dpt). The supernatant of cell culture were harvested on 4 dpt or when 80% CPE of the infected MA-104 cells were developed. Furthermore, the BTSX mutant RNAs with larger deletions in structural protein coding region did not generate CPE in both assays even for prolonged incubation for 14 d post treatment. To further passage the rescued viruses, 200 μL of the transfection supernatants were inoculated into fresh MA-104 cells, on which CPE began at 2－3 d post inoculation (dpi). Meanwhile, to test the specificity of the rescued viruses, immune fluorescence assay was conducted with FITC-labeled monoclonal antibody against N, SR-30F. As shown in Figure 2, all passage 1 of the rescued viruses including vAPRRS, vORF5ME, and vCSA displayed the same anti-PRRSV N specificity. These results demonstrated that the full-length cDNA clone pAPRRS and their derivatives harboring minor mutations can mimic the virus infection cycle of their parental PRRSV.

2.4 The rescued viruses contained the engineered mutations and separation of coding regions between nonstructural and structural proteins

To confirm that the transfectant virus contained the expected mutation, RT-PCR was conducted as described above by using the transfectant viral RNA. After purification of the amplified products, nucleotide sequencing was conducted and compared with the plasmid mutant and the parental viral sequences. As shown in Figure 3, the nucleotide sequences from RT-PCR products of pORF5ME (Figure 3(a)) and pCSA (Figure 3(d)) RNA transfectants were all conformed with their parental

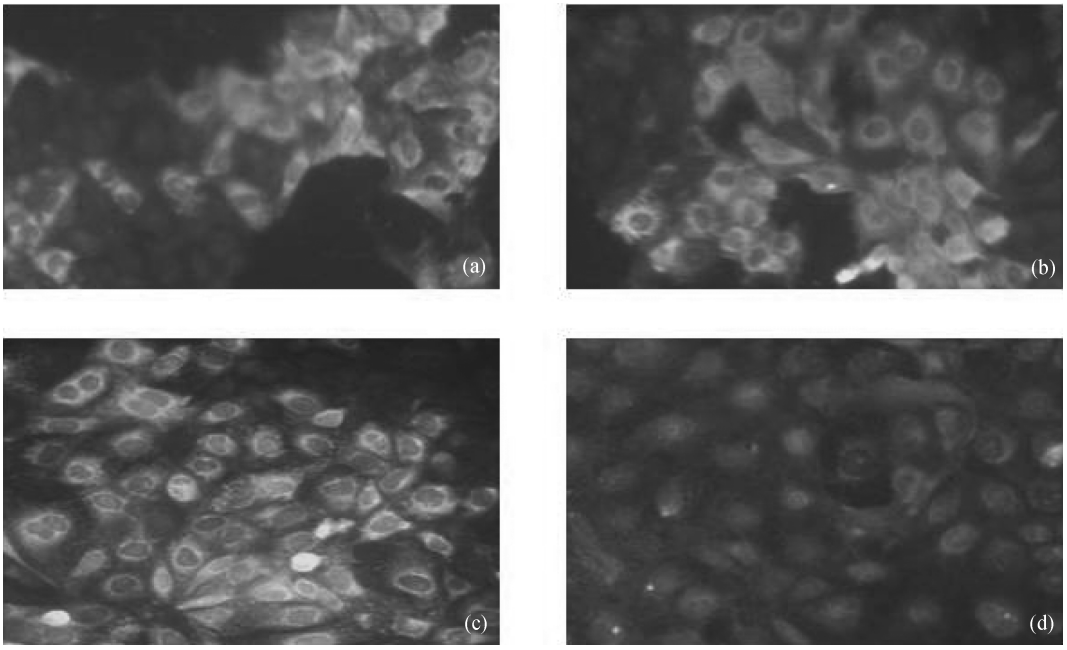

Figure 2 Immmunofluroscence analyses of the transfected viruses. At 24—48 h post transfection (hpt), 200 microlilter of the resultant supernatants were used to inoculate fresh MA-104 cells. At 48 h post infection (hpi), the virus- or negative control infected cell monolayers were subjected to immunofluroscence assay using anti-PRRSV monoclonal antibody SR-30F, labeled with FITC, and images were observed under magnification of 400 (20×20) fold. (a) vAPRRSV; (b) vORF5ME; (c) vCSA; (d) BTSX-transfection control.

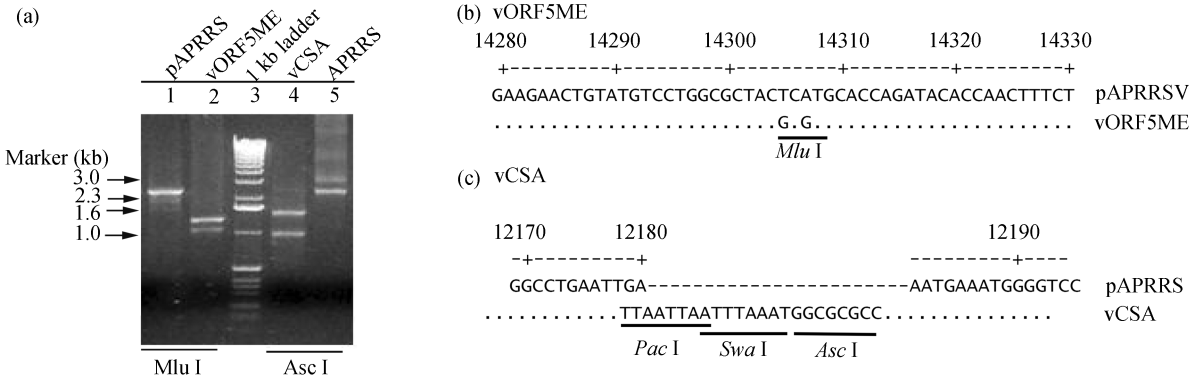

Figure 3 The rescued viruses were stable and contain the desired mutations. The rescued viruses were passaged on fresh MA-104 cells for 5 times. First-strand cDNA was synthesized using superscript II reverse transcriptase and an anti-sense anchor primer Qvt from the viral RNA. RT-PCR with primer pair SF11210 / SR13334 (Table 1) was conducted from the purified viral RNAs from the supernatants of the transfectant virus cultures at passage 2. (a). The purified amplification products were subjected to restriction endonuclease digestion with *Mlu* I for those of vARRS (lane 1) and vORF5ME (lane 2), or Asc I for vCSA (lane 4) and parental virus APRRS (lane 5), analyzed on 1% agarose gel electrophoresis. The amplified PCR products were purified using QIAgen PCR cleanup kit and subjected to nucleotide sequence analysis by ABI 377 sequencer. The lineups of the nucleotide sequences were done by using Lasergene DNA package (DNASTAR, Madison, WI). (b) vORF5ME; (c) vCSA. The engineered restriction endonuclease recognition sequences were underlined.

plasmids, indicating that these mutant plasmid clones generated transfectant viruses. As shown in the Figure 3(b), pCSA contains a polylinker separating coding regions of ORF1 and ORF2, the nonstructural and struc--tural protein coding regions of PRRSV.

2.5 Subgenomic RNA profile of the rescued viruses

To investigate if the inserted sequence can be expressed in the form of subgenomic mRNAs, Northern blotting analyses were conducted. Of most interest, we wanted to know that if the PSA linker inserted at the junction of the ORF 1 and 2 can be transcribed as a subgenomic mRNA. The total cellular RNAs from mock-(lane 3), pAPRRS (lane 2), and vCSA (lane 1) infected cells, were blotted against an oligonucleotide probe PSA1R

(Table 1) labeled with DIG-11-ddUTP. As shown in Figure 4, vCSA did express PSA linker at approximately size of mRNA 2 in the parental virus, which showed weak hybridization signal because that the PSA1R probe contain 14 nucleotides of the parental sequence, partially due to the need of increasing the melting temperature of the probe.

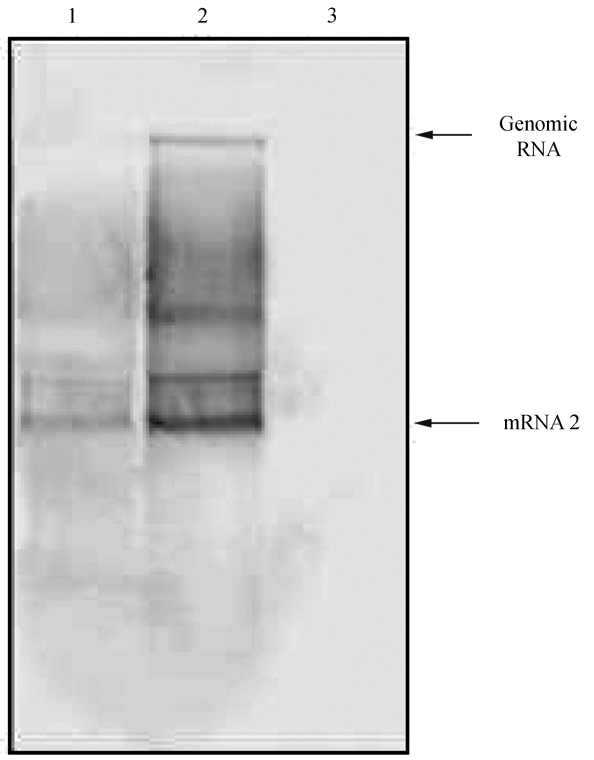

Figure 4 Northern blot analysis of the rescued viruses infected cells. Total cellular RNAs were isolated from the infected cells at 36 hpi, conducted with QIAgen RNAeasy Kit (QIAgen). Denatured RNA gel electrophoresis and Northern blotting were done by using NorthenMax system (Ambion), using DIG-labeled oligonucleotide probes (Roche). DIG Nucleic Acids Detection kit (Roche) was used for detection of the blotted RNAs. PSA linker insertion in pCSA, with PSA1R (Table 1) as probe aginst RNAs from cells infected by vCSA (lane 2), pAPRRS (lane 1), and mock-infected (lane 3).

3 Discussion

The development of reverse genetic system is critical for better understanding the processes of PRRSV replication and infection, and rational design of new-generation vaccine against the ever-changing PRRS. In this report, we obtained an infectious cDNA clone of a PRRSV vaccine strain (unpublished data), APPRS. To our knowledge, this is the first reverse genetics system of PRRSV vaccine strain, which offers a valuable tool for further improvement of vaccine efficacy against highly-pathogenic PRRSV, against which a variety of vaccines were proved to be ineffective (Tian et al, personal communication). To do so, we constructed mutant infectious cDNA clone containing polycloning sites between coding regions of nonstructural proteins (ORF1) and that of the structural protein (ORF2－7). One can simply replace the coding sequence for PRRSV structural protein(s) to develop chimeric vaccine with higher cross-protective immunity against HP PRRSV, provided the virulence determinant of PRRSV can be defined. Furthermore, the infectious cDNA clone provides a powerful tool for molecular dissection of the mechanism of pathogenesis of the increased virulence of the on-going prevalent PRRSV in China.

3.1 The relationship of nsp2 genetic diversity with viral virulence

APRRSV attenuated strain and its parental virus all contains an insertion of 108 nucleotides in the middle of the nsp2, as does the PrimePac Nsp strain[20]. Surprisingly, a discontinuous deletion of a 90 nucleotides of nsp2 was identified (ref. [8]; Liu et al. unpublished data) was identified as the unique hallmark of the HP PRRSV strains isolated during the Chinese Porcine High Fever Syndrome outbreaks. Tian et al.[8] speculated on the possibility of such an nsp2 deletion could correlate to the increased virulence of HP PRRSV. Interestingly, larger nsp2 deletion was also found in previously isolated highly virulent PRRSV MN184[21]. However, serial deletion has also been found in normal virulent PRRSV isolates such as Type II[22] (Yang et al. 2006), and a type I strain isolated in North America[15]. Therefore, it remains further experimental evidence that if nsp2 or other component relates to the increased pathogenecity of the HP PRRSV.

3.2 PRRSV heterogeneity and cross-protection of live PRRSV vaccine

As a RNA virus, PRRSV replication is error prone and cause genetic variation and antigenic drift at high frequency, which poses problem for immune prevention against prevalent PRRS[10]. In fact, the current commercial vaccines made of traditional PRRSV strains were proved to be ineffective against the on-going endemic HP PRRSV in China (Tian et al., personal communication). A killed vaccine was developed and put on clinical trial against HP PRRS under the conditional provisional

license by the Chinese Ministry of Agriculture. It remains to be known if the provisional vaccine is effective against the on-going endemic in China, yet several lines of evidences have proved that PRRSV inactivated vaccine is much more inferior to modified-live vaccine (MLV) in terms of immune efficacy[23]. However, the current MLV was made of a traditional PRRSV strain and displayed a mere 89% of genetic identity of to the HP PRRSV, which likely is the reasonthat the commercial MLV offers little protection against HP PRRS. It is believed that specific protective immunity against PRRSV consists of cellular and humoral responses, mainly stimulated by nonstructural and structural proteins, respectively. The nonstructural proteins, coding the viral replicase complex, are relatively conserved and believed to be the main viral components stimulating cellular and innate immunity of the host; while protective humoral immunity are provoked by the structural proteins, coded by ORF2—ORF7. Therefore, a rational design of PRRSV vaccine would require cellular immunity components as well as the humoral protective antigens. To this end, we developed a reverse genetic system of an attenuated PRRSV, based on which new generation of MLV vaccine against HP PRRS can be developed.

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