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Development of a differentiable virus *via* a spontaneous deletion in the nsp2 region associated with cell adaptation of porcine reproductive and respiratory syndrome virus

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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) is renowned for its genetic, antigenic, and pathogenic heterogeneity. As a consequence, highly pathogenic PRRSV (HP-PRRSV) has emerged and caused tremendous economic losses in the swine industry. In this study, a Chinese HP-PRRSV JX143 isolate was serially passaged in MARC-145 cells up to 100 times. We found that phenotypic changes involved with the cell adaptation process of PRRSV JX143 were characterized by higher titers, faster growth kinetics, and larger plaque sizes as the passage number increased compared with the parental virus. We found that the virulence of the JX143 strain in piglets was decreased greatly at passage 100 (JXM100). The attenuated strain JXM100 could protect piglets from lethal challenge by HP-PRRSV JX143. Genome-wide analysis showed that JXM100 contained a continuous 264 nucleotide (88 amino acids; 88 aa) deletion in the nsp2 region and 75 random nucleotide mutations throughout the genome. The nucleotide changes that arose during MARC-145 passaging of HP-PRRSV JX143 provide a potential molecular basis for the observed cell-adapted phenotype in MARC-145 cells and the attenuated phenotype *in vivo*. We also showed that pigs inoculated with JXM100 with an 88 aa deletion (del88) in nsp2 elicited a strong antibody response against the N protein but they did not develop antibody against the del88, whereas strong reactivity was observed in sera obtained from piglets infected with JX143 using the same del88-based ELISA. This suggests that del88 can be used as a genetic marker for serologically differentiating JXM100 from JX143.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) was first recognized in 1987 in the USA and shortly afterwards in the Netherlands (Wensvoort et al., 1991), and it has spread worldwide to become the most economically significant swine disease (Neumann et al., 2005). More recently, highly pathogenic PRRSV (HP-PRRSV) has emerged in China (An et al., 2007; Lv et al., 2008; Tian et al., 2007; Zhou et al., 2008) and its neighboring countries including Vietnam, Laos (Feng et al., 2008; Ni et al., 2012; Normile, 2007), and the Philippines (Normile, 2009), which has caused disastrous damage to the swine industry. The genetic basis of the hypervirulent PRRSV remains to be determined.

PRRSV is an enveloped virus with a single-stranded, non-segmented and positive-sense RNA genome that measures about 15 kb in size, which encodes at least nine open reading frames (ORFs) (Conzelmann et al., 1993; Nelsen et al., 1999). Approximately two-thirds of the 5′ end of the viral genome contains ORFs 1a and 1b, which are expressed *via* a ribosomal translational frameshift mechanism. The translated polyproteins (pp1a and pp1ab) undergo proteolytic maturation to form 14 predicted non-structural proteins (nsps), including RNA-dependent RNA polymerase (Snijder and Meulenberg, 1998). The 3′ third of the genome contains the ORFs 2a, 2b, and 3–7, which encode the PRRSV structural proteins including glycoprotein 2 (GP2), nonglycosylated protein E, GP3, GP4, GP5, ORF5a protein, matrix protein (M), and the nucleocapsid (N), respectively (Firth et al., 2011; Johnson et al., 2011; Snijder and Meulenberg, 1998; Wu et al., 2001).

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The development of an effective PRRSV vaccine has been problematic, mainly due to its intrinsically high degree of genetic and antigenic diversity (Dee et al., 2001; Meng, 2000; Stadejek et al., 2006). Furthermore, the use of vaccination to control outbreaks has been complicated by the subsequent differentiation between vaccinated pigs and pigs recovering from infections, or those with subclinical, persistent post-vaccination infections. The differentiation between infected and vaccinated pigs is a problem that many researchers have tried to address in recent years. It has been shown that PRRSV nsp2 protein is an excellent candidate site for marker modification. The nsp2 gene is the most variable region in the PRRSV genome and substitutions, deletions, and insertions have been observed in the nsp2 coding region (Han et al., 2006; Shen et al., 2000; Tian et al., 2007; Tong et al., 2007; Yoshii et al., 2008; Yuan et al., 2001a). Several lines of evidence have demonstrated that nsp2 contains a long coding region stretch that is dispensable for virus viability (Han et al., 2007; Kim et al., 2009; Leng et al., 2012b; Ni et al., 2011; Tian et al., 2007). Another important property of nsp2 that is relevant for marker engineering is the presence of several immunodominant epitopes in this region (Oleksiewicz et al., 2001; Yan et al., 2007). As a result, PRRSV strains that can differentiate infected and vaccinated animals (DIVA) might potentially be developed *via* engineered deletions of specific individual immunodominant epitopes or large fragments in nsp2 (de Lima et al., 2008; Fang et al., 2008; Kim et al., 2009; Xu et al., 2012). These rationally designed marker viruses will provide a basis for further development of DIVA vaccines to assist the control of PRRS.

In this study, we developed a cell-adapted strain, JXM100 *via* 100 serially cell passages of a HP PRRSV JX143 strain. Piglets inoculated with JXM100 were virologically and clinically protected from challenge with an HP PRRSV isolate. JXM100 also had an 88 amino acid (aa) deletion in the nsp2. Antibodies against this 88 aa region were found in animals infected with the parental strain (JX143) and another heterologous virus (vAPRRS).

2. Materials and methods

2.1. Cells and viruses

The MARC-145 cell line was propagated in Eagle's minimal essential medium (EMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Gibco-BRL, Gaithersburg, MD). The HP-PRRSV JX143 strain was isolated from the lung tissue homogenate of a dying piglet infected with PHFD in 2006, Jiangxi, China (Lv et al., 2008), and it was used as the parental virus (GenBank accession no. EU708726).

2.2. Serial passaging of the virus

A sub-confluent monolayer of MARC-145 cells was inoculated with 100 μl of 100-fold diluted PRRSV JX143 (P0) virus in a six-well-plate (Cornings) and incubated at 37 °C for 1 h, with redistribution of the inoculum every 15 min. The cells were washed twice with 1 ml of phosphate-buffered saline (PBS) and overlaid with 3 ml of EMEM containing 2% FBS, followed by incubation at 37 °C in 5% CO_2. When 80% cytopathic effects (CPE) were observed, the supernatant was harvested from the cell culture, aliquoted and stored at −70 °C, and designated as passage 1 (JXM1). In the same manner, 100 subsequent passages were produced and designated JXM2 to 100, of which JXM20, JXM40, JXM60, JXM80, and JXM100 were expanded in T75 flask (Corning) as viral stocks.

2.3. Viral plaque assay

The 85% subconfluent MARC-145 cell monolayers in six-well plates were inoculated with 200 μl of 10-fold serial dilutions of virus suspensions and adsorbed for 1 h at 37 °C. The cell culture wells were washed twice with 1 ml PBS and overlaid with 2 ml of premixed EMEM containing 2% FBS and 1% SeaKem agarose (FMC). The solidified plates were then placed in a humidified incubator at 37 °C in 5% CO_2. The plaque morphology was assessed by staining with 2% crystal violet in ethanol at 96 h post inoculation (hpi).

2.4. One-step growth curve

Subconfluent monolayers of MARC-145 cells in six-well plates were infected with each virus at 0.1 MOI. After 1 h incubation at 37 °C, the inoculum was removed and cells were washed twice with PBS, then incubated in 3 ml of EMEM containing 2% FBS in a CO_2 incubator at 37 °C. The supernatant was collected from the infected cells every 2 h while replenishing with the same volume of fresh media, and it was stored at −70 °C. All samples were titrated using a viral plaque assay of the MARC-145 cells and the growth curves were determined after measuring the mean titers of four duplicates at each time point. The virus titers were determined according to the Reed–Muench method.

2.5. Indirect immunofluorescence assay

Indirect immunofluorescence assays (IFA) were used to detect antigens in cells infected with the corresponding virus, as previously described (Wei et al., 2012b). Briefly, confluent monolayers of MARC-145 cells were infected with the transfectant (P0) virus or passaged virus at 0.1 MOI. At 72 hpi, the infected cells were washed twice with PBS, followed by fixation in cold methanol at room temperature. The fixed cell monolayers were washed twice with PBS and incubated in 0.1% BSA at room temperature for 30 min. After two washes with PBS, the cells were incubated at 37 °C for 2 h with anti-del88, a polyclonal rabbit antibody against the del88, which was tailor-made by Shanghai Immunogen Inc., or anti-N protein monoclonal antibody for PRRSV (a kind gift from Dr. Kegong Tian, China Animal Disease Control Center) at 1:600 dilutions. Next, the cells were washed five times with PBS and incubated at 37 °C for 1 h with fluorescein isothiocyanate-conjugated (FITC) secondary goat anti-rabbit antibody or goat anti-mouse antibody. The cell monolayers were washed five times with PBS and visualized under an inverted Olympus fluorescence microscope equipped with a video imaging system.

2.6. RT-PCR and nucleotide sequencing

Viral RNA was extracted using a QIAprep Viral RNA Mini Kit (QIAgen, Hilden, Germany), according to the manufacturer's instructions. RNA was suspended in RNase-free water, quantified using a UV spectrometer, aliquoted, and stored at −70 °C. RT-PCR was performed as previously described (Lv et al., 2008). Briefly, cDNA was synthesized using an anchored primer QNT (Table 1) and used as the template to amplify four overlapping genomic RNA fragments, among which there was a single restriction enzyme site for the downstream assembly of the full-length genomic cDNA clone. The exact 5′ end of the viral genome was obtained using the 5′ RACE kit according to the supplier's instructions (TaKaRa). The PCR products were gel-purified and cloned into the pCR-Blunt II-TOPO vector (Invitrogen). The clones were screened by AGE and restriction enzyme mapping. Three clones for each fragment were subjected to nucleotide sequencing. Additional sequencing primers have been described previously (Lv et al., 2008) and are available upon request. The resultant nucleotide sequences were assembled by SeqMan and aligned using the Lasergene© software package.

Table 1
Primers used in this study.

Name[a]	Sequence (5′–3′)	Application
SR2573	CTGCCCAGGCCATCATGTCCGAAGTC	5′ RACE
5′ outer primer	catggctacatgctgacagccta	5′ RACE
SR1124	CTTGCAGCCTCCGCTGTAGGTA	5′ RACE
5′ inner primer	cgcggatccacagcctactgatgatcagtcgatg	5′ RACE
SR683	GAGCGGCAGGTTGGTTAA	5′ RACE
SMF	gg***TTAATTAA***CG***ACGCGT***ATGACGTATAGGTGTTGGC	RT-PCR
SR2949	CTGGTGCGTCAGCGTTGTTGTC	RT-PCR
SF2230	GCGAATCAGACAACCGAACAACC	RT-PCR
SR6670	ACACCCCTTCCCTCAACTTTCCCTC	RT-PCR
SF6279	CTCCTTTGGGATGTTTGTGC	RT-PCR
SR12186	CAGGGTGAACGGTAGAGCG	RT-PCR
SF10958	CGTGGTGACAACCCAGAACAATG	RT-PCR
QNT	GAGTGACGAGGA***GCGGCCGC***TTTTTTTTTTTTTT	RT-PCR
SFc2325	GA***AGATCTA***CCGCCTTCTCACTGTCCAATTG	Protein expression
SFcc2325	GA***AGATCT***GGTGGTGGTGGTTCCACCGCCTTCTCACTGTCCAATTG	Protein expression
SRc2588	CCG***CTCGAG***CGG***GGATCC***ATCGACCTGCTCAGCTGCCCAGGC	Protein expression
ORF5F	GGCAATGTGTCAGGCATCGTGG	Viremia detection
ORF5R	GCGACTTACCTTTAGAGCATA	Viremia detection

[a] Primer names are organized in groups. Prefixes: F, forward PCR primer; R, reverse PCR primer. Restriction sites introduced by PCR are indicated in italic and black fonts. Small case letters denote non-viral sequence. The nucleotide positions are based on the full-length PRRSV sequence EU708726.

2.7. Animal inoculation and virus challenge

Twenty-four 28-day-old PRRSV-free piglets were obtained and divided randomly into four groups, *i.e.*, six piglets in each group. Each treatment group was housed individually. The six piglets in group 1 (JXM100-JX143) were injected intramuscularly on day 0 with 3 ml of diluted JXM100 (1×10^6 $TCID_{50}$) and challenged on day 28 with 3 ml of diluted HP PRRSV (JX143) (1×10^5 $TCID_{50}$). The six piglets in group 2 (JX143) were injected intramuscularly on day 0 with 3 ml of diluted JX143 (1×10^5 $TCID_{50}$). The six pigs in group 3 (EMEM-JX143) were injected with 3 ml of EMEM on day 0 and inoculated with 3 ml of diluted PRRSV JX143 (1×10^5 $TCID_{50}$) on day 28. The six piglets in group 4 (EMEM) were injected on day 0 and day 28 with 3 ml of EMEM as blank controls. The piglets were monitored daily to assess their general health status and rectal temperature, while they were also weighed at 0, 7, 14, 21, 28, 35, 42, and 49 days post-challenge (dpc). Clinical signs were recorded daily prior to feeding, *i.e.*, coughing, dyspnea, anorexia, diarrhea, lameness, shivering, and fever. Serum samples were collected from all piglets at 0, 3, 7, 14, 18, 21, 28, 35, 42, and 49 dpc.

2.8. Serological examination

PRRSV-specific antibody responses were analyzed in sera collected at 0, 3, 7, 14, 21, 28, 35, 42 and 49 dpi using a commercial ELISA kit (IDEXX Laboratories Inc., Westbrook, ME), according to the manufacturer's instructions. The PRRSV-specific antibody titer was reported as the S/P ratio and the serum samples were considered positive if the S/P ratio was $\geq$0.4. Viral RNA in the serum samples was detected by RT-PCR to assess viremia in the challenged piglets, as previously described (Wei et al., 2012a). Viremia in the challenged piglets was also detected by inoculating MARC-145 cells with serum samples collected during the infection procedure. CPE were observed in each MARC-145 culture well at 5 dpi.

2.9. Construction and expression of recombinant expression plasmids carrying a tandem repeat, nsp2del88

The del88 aa-coding region was amplified using the forward primer SFc2325 and the backward primer SRc2588. The PCR product was digested using *Bgl*II and *Xho*I, and cloned into the expression vector pET-28a (+) (Novagen), which had been digested with *Bam*HI and *Xho*I. This recombinant plasmid was designated as pET-28a-nsp2del88 (+1). The recombinant plasmid pET-28a-nsp2 del88 (+1) coding region was digested using *Bam*HI and *Xho*I, then ligated with the nsp2 del88 coding region, which was amplified using the forward primer SFcc2325 and the backward primer SRc2588, and digested with *Bgl*II and *Xho*I. This recombinant plasmid was designated as pET-28a- nsp2del88 (+2), which had two nsp2del88 coding regions. The recombinant plasmid containing the tandem repeat del88 coding region was designated as pET-28a- nsp2del88 (+3). The recombinant plasmid pET-28a-nsp2del88 (+3) was transformed into *Escherichia coli* BL21 (DE3) and grown at 28 °C until the OD at 600 nm reached 0.6, before protein expression was induced with 1 mM IPTG for 4 h. The expressed protein was analyzed using 12% SDS–PAGE. Recombinant nsp2del88 (+3) protein was purified with an HIS binding kit (Novagen) and used as the antigen for the ELISA.

2.10. ELISA analysis

ELISA analyses were performed using 96-well microtitre plates (Costar). The recombinant protein del88 was diluted with coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.6) and the plates were coated with 5 ml of diluted antigen in columns 1, 3, 5, 7, 9, and 11. Columns 2, 4, 6, 8, 10, and 12 were treated with 5 ml coating buffer as a background control. The plates were incubated at 37 °C for 2 h and excess protein-binding sites were blocked with 10% skimmed milk in PBST buffer (PBS with 0.05% Tween-20) at 4 °C overnight. The test sera were applied at 1:100 dilutions in PBST buffer with 5% skimmed milk. After 2 h incubation at 37 °C, the plates were washed with PBS and HRP-conjugated goat anti-swine IgG (Jackson Laboratories) was added to detect any serum antibodies that were bound to the antigen on the plates. The plates were incubated at 37 °C for 1 h and washed, before the peroxidase substrate TMB was added to achieve color development. Color development was conducted for 10 min and 2 M sulfuric acid was used to stop the reaction. The results were quantified by reading at 450 nm using an EL800 microplate reader (BioTek Instruments).

3. Results

3.1. Serial passaging of HP-PRRSV in MARC-145 cells and the phenotypic characteristics of the cell-adapted viruses

Previously, we demonstrated that PRRSV JX143 strain, which was isolated from a clinical sample from a piglet with "porcine

high fever disease (PHFD)," was indeed hypervirulent and that it was the major etiological agent causing the PHFD outbreak (Lv et al., 2008). To further investigate the genetic basis of HP-PRRSV, we conducted 100 serial passages using a limited dilution method with MARC-145 cells. The virological characteristics and full-length genomic sequenced were determined every 20 passages, *i.e.*, after 20, 40, 60, 80, and 100 passages, and virus stocks were produced that were designated as JXM20, JXM40, JXM60, JXM80, and JXM100, respectively.

One-step growth curves were produced for each virus to evaluate the growth kinetics of the cell-adapted viruses and the parental virus JX143. As shown in Fig. 1A, viruses with higher numbers of passages had faster growth kinetics and higher titers at each indicated time point. Thus, JXM100 had the highest levels, which indicated that it gained viral fitness during its gradual cell adaptation. JXM100 had a peak titer of 7.94×10^6 pfu/ml at 24 hpi, which was about 31-fold higher than that of the parental strain JX143. Next, we investigated the plaque morphology of the parental virus JX143, as well as JXM40, JXM60, and JXM100. Fig. 1B shows that the plaques produced by parental virus JX143 were the smallest compared with the cell-adapted viruses. Interestingly, the size of the plaques increased gradually with the passage number and the plaques produced by JXM100 were larger and more homogenous in size. These results demonstrated that cell-adapted viruses had a higher viral fitness in the cultured cells.

3.2. The cell-adapted virus JXM100 was attenuated in vivo and it protected piglets from lethal challenge with HP-PRRSV

To investigate whether the cell-adapted virus JXM100 was attenuated in piglets, six piglets from group 1 were infected with JXM100 while six piglets in group 2 were infected with the parental virus JX143. The results showed that piglets inoculated with JX143 developed high fever and HP-PRRSV clinical symptoms such as lethargy, lack of appetite, coughing, and frequent paralysis and death, which resulted in 100% morbidity and 66.7% mortality in the infected piglets. Two piglets infected with JX143 died at 9 dpc, while two other piglets died at 14 and 16 dpc, respectively (data not shown). As shown in Fig. 2, all of the piglets inoculated with JX143 had a persistently high fever, which started at 3 days pi and lasted for 6–8 days. By contrast, piglets from the JXM100-infected group and the piglets in the control group did not exhibit high fever or other clinical signs at 0–28 dpc. The average body weight of piglets in the JXM100-infected group was similar to that of those in the control group. The average body weights of piglets infected with JX143 were significantly lower ($p < 0.01$) than those in the JXM100-infected group at 28 dpc (Fig. 3). Serum samples were collected from these piglets and assayed using RT-PCR, followed by virus isolation with MARC-145 cells. As shown in Table 2, all of the JX143-inoculated piglets in group 2 developed viremia, which started at 3 days pi (dpi) and lasted until 42 dpi. However, only 4/6 JXM100-inoculated pigs developed viremia that started at 3 dpi and it decreased to an undetectable level at 21 dpi. Viremia was also confirmed in the piglets by virus isolation using MARC-145 cells (data not shown). The results demonstrated that JXM100 was attenuated in piglets.

To confirm that the 88 aa mutations in the nsp2 were maintained in the replicating virus, we collected serum samples from JXM100-infected piglets at 14 dpi and the nsp2 coding region was amplified by RT-PCR and sequenced. The nucleotide sequencing results confirmed the presence of the 88 aa deletion whereas no other mutations were detected in the nsp2 coding region, which suggested that the 88 aa deletion in nsp2 was stable *in vivo* (data not shown).

To test whether the attenuated JXM100 could protect pigs from lethal infection with HP PRRSV, the pigs in group 1 and group 3

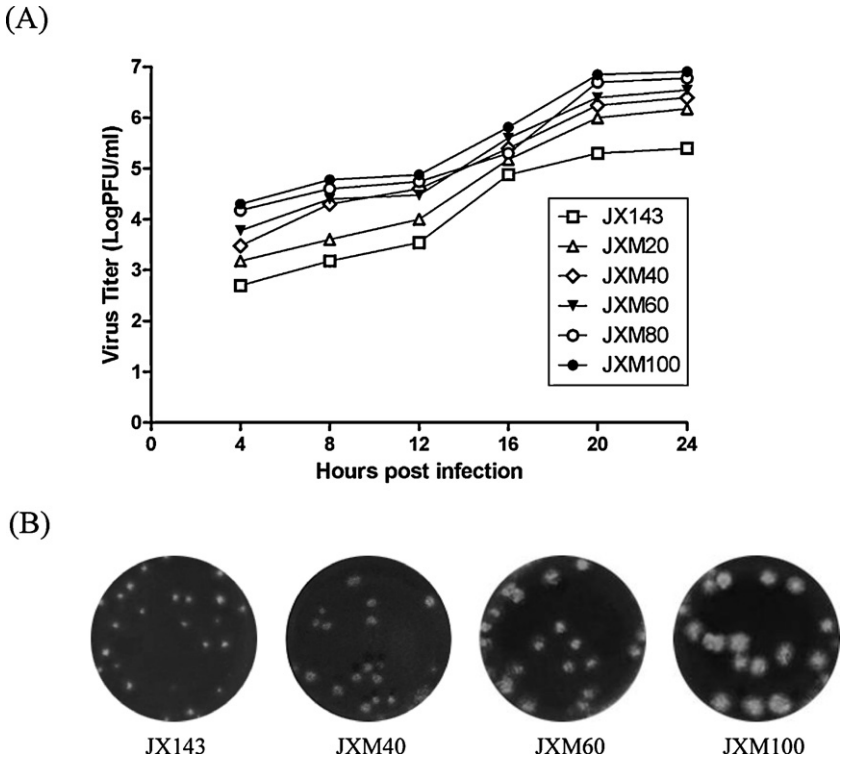

Fig. 1. One-step growth curves and viral plaque morphology of the cell-adapted viruses in MARC-145 cells. The viruses JXM20, JXM40, JXM60, JXM80, JXM100, and the parental strain JX143 were used to infect monolayer cells at 0.1 MOI. Next, 200 μl of the supernatant was collected at the time indicated and stored at −70 °C. (A) The viral titers were determined as plaque forming unit (PFU) and the results were achieved using three separate experiments. (B) Monolayers of MARC-145 cells in six-well plates were infected with JX143, JXM40, JXM60, and JXM100. The cell monolayer was overlaid with 1% agarose and stained with crystal violet at 96 hpi.

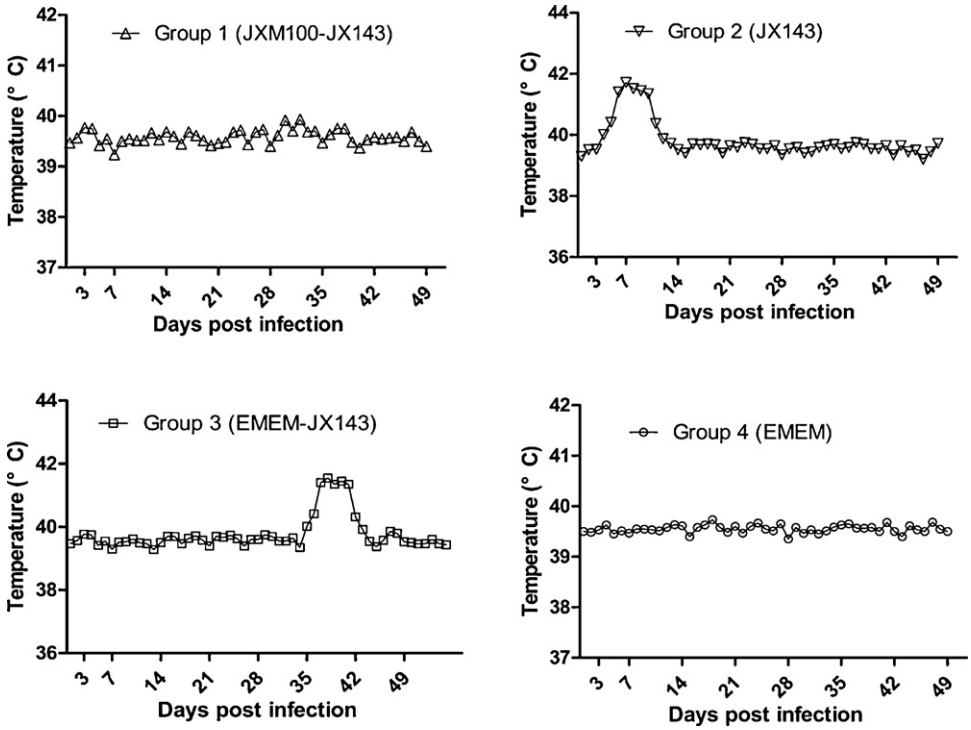

Fig. 2. Rectal temperature of piglets in the different groups. Rectal temperatures of ≥41 °C were defined as fever. Fever that lasted ≥3 days was defined as illness.

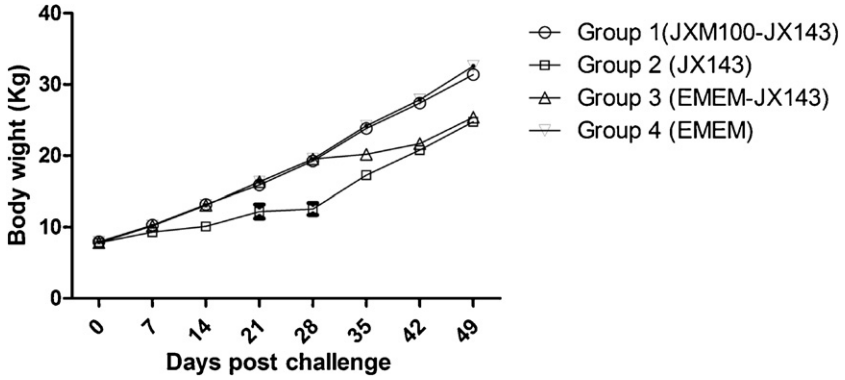

Fig. 3. Body weights of the piglets in different groups. The body weights were measured after inoculation with JXM100 or JX143. The body weights are expressed in kg as the mean ± SD for the pigs that were alive at the time of the measurement.

were inoculated with JX143 at 28 days. As shown in Fig. 2, all of the pigs in group 3 and pigs in group 2 that were inoculated with JX143 at day 0 developed the typical clinical signs of HP-PRRSV and had a persistently high fever that lasted 6 days. Three piglets in the JX143-infected group died at 10, 12, and 16 dpc. By contrast, pigs in the JXM100-infected group 1 that were inoculated with JX143 at 28 dpc, as well as pigs in the control group, exhibited no high fever or other clinical signs throughout the experiment (Fig. 2). The average body weights of the pigs in group 1 were similar to those in the control group (Fig. 3). These results suggested that JXM100 could protect piglets from lethal challenge with JX143 and that it might be a candidate vaccine to protect against HP-PRRSV.

Table 2
Viremia in piglets inoculated with JXM100 or JX143.

Group	Number of RT-PCR positive pigs/total number of pigs[a] (day post challenge)								
	0	3	7	14	21	28	35	42	49
Group 1 (JXM100-JX143)	0/6	4/6	4/6	4/6	0/6	0/6	0/6	0/6	0/6
Group 2 (JX143)	0/6	6/6	6/6	3/3	2/2	2/2	2/2	2/2	0/2
Group 3 (EMEM-JX143)	0/6	0/6	0/6	0/6	0/6	0/6	6/6	4/4	3/3
Group 4 (EMEM)	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6

Viremia in serum samples of the inoculated piglets was analyzed by RT-PCR.
[a] The numbers of dead pigs at the indicated time points were deducted.

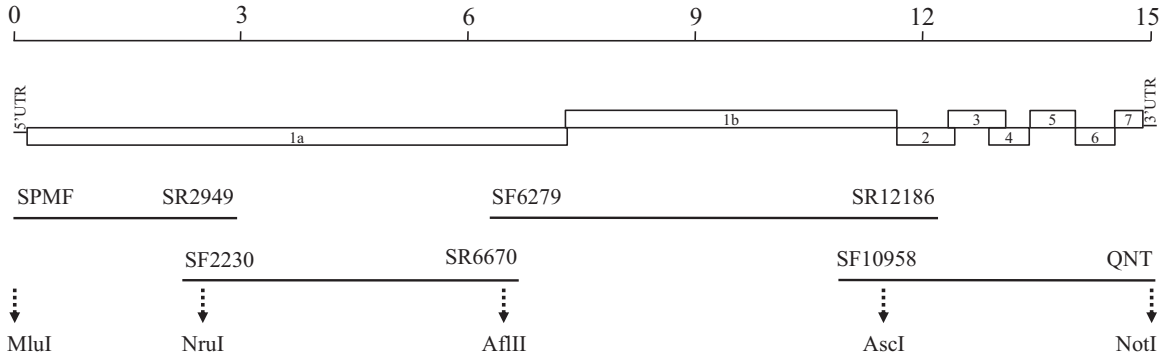

Fig. 4. The genomic organization, nucleotide sequencing approach, and cDNA cloning strategy for the cell-adapted viruses. Viral RNA was purified from the culture supernatants of JXM20, 40, 60, 80, and 100, and used for the synthesis of single-stranded cDNA with the anchored primer QNT. Based on the genomic sequencing and cloning strategy applied to JX143, four overlapping cDNA fragments were amplified using the primer pairs SPMF/SR2949, SF2230/SR6670, SF6279/SR12186, and SF10958/QNT, where single restriction enzyme sites existed to allow the downstream ligation of full-length clones. The RT-PCR products were cloned into the pCR-Blunt II-TOPO vector and three clones of each cDNA fragment were used for nucleotide sequencing.

3.3. Genomic characterization of the cell-adapted viruses showed that mutations were distributed randomly throughout the genome

We then aimed to determine the genetic changes that may have accounted for the viral fitness gained in MARC-145 cells and the *in vivo* attenuation. After RT-PCR amplification of the genomic RNA, the full-length genomic sequences were determined for JXM20, 40, 60, 80, and JXM100. The agarose gel-purified PCR products of four overlapping genomic cDNA fragments (Fig. 4) were subjected to direct nucleotide sequencing with at least three different sequence reads in each position. The PCR products were cloned further with ambiguous sequences and five clones were subjected to nucleotide sequencing. The master genomic sequences of each virus were assembled using the SeqMan program (Lasergene DNAStar package) and deposited in GenBank, *i.e.*, JXM20 (GenBank accession no. GQ499193), 40 (GQ499194), 60 (GQ499195), 80 (GQ499196), and JXM100 (GQ475526). The entire genomic sequences were compared with that of the parental virus JX143 (EU708726) and no less than 99.5% pairwise genetic identity was detected. In particular, JXM100 had a total of 75 nucleotide substitutions compared with the parental JX143, which were distributed randomly throughout the genome (Supplementary Table S1). Specifically, the 5′ and 3′ UTRs were virtually unchanged except for one T to C substitution at position 163 in the 5′ UTR. This mutation did not affect the overall secondary structure of the 5′ UTR (data not shown), which is believed to be crucial for the regulation of viral replication. A total of 33 substitutions were nonsynonymous in the coding regions and the aa substitutions were distributed randomly, as shown in Fig. 5. In the structural protein coding regions (ORF2–7), these mutations were located in ORF2a, ORF3, and ORF4, which contained one, six, and two non-silent mutations, respectively (Table 2). Surprisingly, JXM100 had only two silent mutations in ORF5 (C69T and T345C), which were considered to be highly variable and involved with viral pathogenicity, neutralization, and viral evasion of the host immune system. In addition, a single mutation was identified in ORF6 (Q164R), whereas ORF7 remained unchanged throughout the cell passages.

See Supplementary Table S1 as supplementary file. Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.virusres.2012.11.006.

As expected, most of the aa substitutions (23/33) occurred in ORF1, *i.e.*, the replicase complex coding region, where only the nsp5, nsp6, and nsp8 coding regions contained no changes. The hallmark Chinese HP-PRRSV deletion of 30 aa was stably maintained throughout long-term cell adaptation, which suggested that this deletion may have little involvement with the observed hypervirulence of HP-PRRSV. As summarized in Fig. 5 and Table 3, the aa differences between JXM100 and the JX143 were distributed randomly throughout the genome, especially when the size of the respective coding sequence was taken into consideration.

We also analyzed the timing and stability of the nonsynonymous substitutions during the passaging process. The 33 aa found in JXM100 were compared to those in JX143 and other passaged viruses. As shown in Table 3, 11/33 substitutions occurred in JXM20, *i.e.*, in ORF1a (P108A, D708G, C1681W, I1712V, V1723I, and D1839A), ORF1b (G1R and K450R), ORF2a (R239S), ORF3 (T225A),

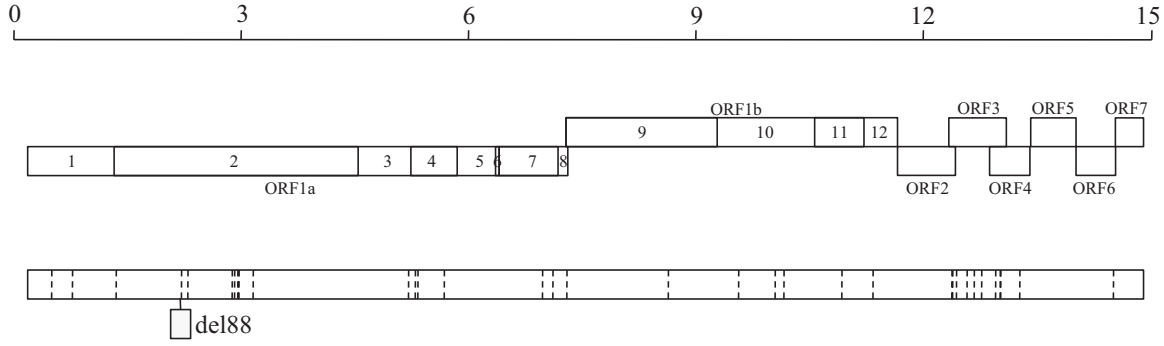

Fig. 5. Amino acid differences between JXM100 and its parental strain. The genomic sequences were determined as described in Fig. 3. The middle panel shows a schematic diagram of the JX143 genomic organization, where the ORFs are indicated and the numbered box represents the coding region for each nsp. The lower panel shows the JXM100 genome, where the vertical dotted line represents 1 aa substitution relative to the predicted aa sequence of JX143. The shaded box labeled as del88 denotes the unique nsp2 deletion found in JXM100.

Table 3
Summary of aa mutation upon cell adaptation.

ORF	aa[a]	JX143	JXM20	JXM40	JXM60	JXM80	JXM100
ORF1a							
Nsp1	108	P	***A***[b]	***A***	***A***	***A***	***A***
	202	A	A	A	A	***V***	***V***
Nsp2	385	G	G	G	G	G	***R***
	680	A	***P***	***S***	A	A	***P***
	708	D	***G***	***G***	***D***	***G***	***G***
	904	A	***T***	***T***	***T***	***T***	***T***
	914	T	T/***I***	***I***	T	***I***	***I***
	927	S	S	S	S	S	***P***
	934	Y	Y	Y	Y	Y	***H***
	996	I	I	I	I	I	***V***
Nsp3	1681	C	***W***	***W***	***W***	***W***	***W***
Nsp4	1712	I	***V***	***V***	***V***	***V***	***V***
	1723	V	***I***	***I***	***I***	***I***	***I***
	1839	D	***A***	***A***	***A***	***A***	***A***
Nsp7	2273	S	S	S	S	S/***G***	***G***
	2319	N	N	N	***S***	N	***S***
ORF1b							
Nsp9	1	G	***R***	***R***	***R***	***R***	***R***
	450	K	***R***	***R***	***R***	***R***	***R***
Nsp10	760	D	D	D	D	D	***N***
	921	T	T	***A***	T/A	***A***	***A***
	959	Y	Y/***H***	***H***	***H***	***H***	***H***
Nsp11	1212	E	***K***	K	E	E	***K***
Nsp12	1353	T	T	T	T	T	***A***
ORF2a	239	R	***S***	***S***	***S***	***S***	***S***
ORF3	31	T	***K***	***K***	***K***	T	***K***
	79	H	H	H/***Y***	***Y***	***Y***	***Y***
	111	L	L	L	L	L	***S***
	143	F	F	F	***L***	F	***L***
	206	N	***S***	***S***	N/S	***S***	***S***
	225	T	***A***	A	A	A	A
ORF4	43	D	***G***	***G***	***G***	***G***	***G***
	129	V	V	V	V	V	***I***
ORF6	164	Q	Q	Q	***R***	***R***	***R***

[a] Number indicates the aa position in the predicted respective ORF of JX143.
[b] aa substitutions compared to initial passage JX143 shown in black and italic font.

and ORF4 (D43G), which suggested that these mutations may have been related to viral cell adaptation or attenuation *in vivo*. However, eight of the remaining 22 substitutions were only present in JXM100, *i.e.*, the coding region of nsp2 (G385R, S927P, Y934H, and I996H), nsp10 (D760N), nsp12 (T1353A), ORF3 (L11S), and ORF4 (V129I). As expected, quasispecies were also detected where substitutions co-existed at some positions (*e.g.*, 914T/I in JXM20), whereas some other substitutions fluctuated between different passages, *e.g.*, nsp1 P680A in JXM60 reverted to the JX143 sequence after an A–P substitution in JXM20 and A–S in JXM40.

3.4. A unique deletion in the nsp2-coding region of highly cell-adapted viruses

Comparative genomic analysis showed that JXM100 contained an 88 aa deletion in nsp2 that was located immediately upstream of the 1 + 29 aa deletion, which is a hallmark of Chinese HP-PRRSV. We aimed to determine the stage when the unique nsp2 del88 emerged during the serial passages. Thus, we determined the nucleotide sequences of the del88 flanking regions for JXM20, 40, 60, 80, and 100. The PCR products were cloned into a plasmid vector and at least five clones were used for nucleotide sequencing. Relative to the parental JX143, the del88 started to emerge in JXM60 when 2/5 clones contained the deletion, whereas 3/5 JXM60 sequences retained del88 (Fig. 6). JXM80 was still a mixed population because 3/8 cloned sequences resembled that of JX143. However, all nine of the cloned sequences of JXM100 contained del88. These results demonstrated that del88 occurred during the middle stage of the serial passages, and that it out-competed other non-deletion viruses.

3.5. del88 could be used to differentiate the attenuated virus JXM100 from the parental virus in vitro and in vivo

A rabbit polyclonal antibody, anti-del88, was prepared and used for IFA to explore the possibility of using del88 as an antigenic marker to differentiate JXM100 from JX143. MARC-145 cells were infected with JX143 and JXM100, and fixed at 36 hpi. As shown in Fig. 7, there was positive intracellular expression of del88 in the JX143-infected cells but not in the JXM100-infected cells, although there was a similar IFA pattern against anti-N monoclonal antibody in cells infected with JX143 or JXM100. These results demonstrated that anti-del88 can be used to differentiate the cell-adapted virus JX100 from its parental virus JX143.

We developed a del88-based ELISA assay for marker detection to assess the ability of the JXM100 marker to differentiate viruses from JX143 in infected pigs. del88 was expressed as a soluble recombinant protein (data not shown). We evaluated the ELISA test for the detection of specific antibodies. As shown in Fig. 8A and B, piglets infected with JX143 virus produced a detectable antibody response against the deleted 88 aa at 14–49 dpi and they also produced a strong antibody response against the N protein antigen, which started at 14 dpi and continued for the duration of the experiment, *i.e.*, up to 49 dpi. By contrast, we observed that pigs inoculated with JXM100 with the 88 aa deletion in nsp2 produced a strong antibody response against the N protein antigen but they did not develop antibodies against del88 until they were challenged with JX143 at day 28. However, a strong reactivity was observed using the same del88-based ELISA in the sera obtained from animals infected with JX143 in groups 1 and 3. These results indicated that the 88 aa deletion in nsp2 did not negatively affect the integral humoral immune

```
PVTQEPLGKDSVPLTAFSLSNCYYPAQGDEVHHRERLNSVLSKLEEVVLEEYGLMSTGLGPRPVLPSGLDELKDQMEEDLLKLANTQATSEMMAWAAEQVDLKAW JX143 NSP2
.........G.....................................................................T..................... JXM20 2#
.........G.....................................................................T..................... JXM20 3#
.........G.....................................................................T..................... JXM20 5#
.........G.....................................................................T..................... JXM20 6#
.........G.....................................................................T...V.................. JXM20 8#
.........G.................K.....................................................T..................... JXM40 1#
.........G.....................................................................T..................... JXM40 2#
.........G................G.....................S.................T..................... JXM40 3#
.........G.....................................................................T..................... JXM40 7#
.........G.....................................................................T..................... JXM40 8#
.........G...-------------------------------------------------------------------------------------.... JXM60 1#
.........G.....................................P.....................T..................... JXM60 2#
.........G...-------------------------------------------------------------------------------------.... JXM60 5#
.........G.....................................................................T..................... JXM60 6#
.........G.....................................................................T..................... JXM60 7#
.........G...-------------------------------------------------------------------------------------.... JXM80 2#
.........G.....................................................................T..................... JXM80 3#
.........G...-------------------------------------------------------------------------------------.... JXM80 4#
.........G...-------------------------------------------------------------------------------------.... JXM80 6#
.........G.....................................................................T..................... JXM80 8#
.........G.....................................................................T..................... JXM80 10#
.........G...-------------------------------------------------------------------------------------.... JXM80 17#
.........G...-------------------------------------------------------------------------------------.... JXM80 19#
.........G...-------------------------------------------------------------------------------------.... JXM100 1#
.........G...-------------------------------------------------------------------------------------.... JXM100 2#
.........G...-------------------------------------------------------------------------------------.... JXM100 6#
.........G...-------------------------------------------------------------------------------------.... JXM100 7#
.........G...-------------------------------------------------------------------------------------.... JXM100 8#
.........G...-------------------------------------------------------------------------------------.... JXM100 13#
.........G...-------------------------------------------------------------------------------------.... JXM100 14#
.........G...-------------------------------------------------------------------------------------.... JXM100 15#
.........G...-------------------------------------------------------------------------------------.... JXM100 17#
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Fig. 6. The unique 88 aa deletion in nsp2 appeared during the later stages of cell adaptation. The cDNA templates of JX20, 40, 60, 80, and JXM100 were used for RT-PCR amplification with the primer pair STF/SR2949, and the amplified products containing the del88 region were cloned into the pCR-Blunt II-TOPO vector. Multiple clones were subjected to nucleotide sequencing and the cloned sequences were aligned with the JX143 sequence. The predicted aa sequence alignment is shown here. The top line represents the JX143 aa sequences, which is followed by those of different clones (#) of the cell-adapted viruses. The nsp2 del88 deletion is denoted by the dashed line (—) in the black-lined frame.

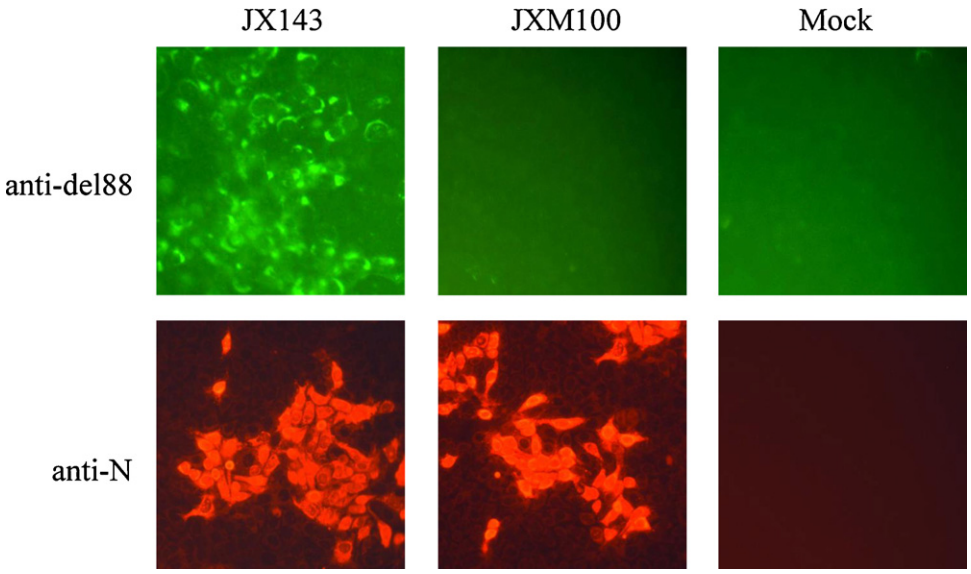

Fig. 7. del88 can be used to differentiate the cell-adapted virus JXM100 from its parental virus *in vitro*. JXM100 and JX143 were inoculated in duplicate MARC-145 cell monolayers at 0.1 MOI. At 72 hpi, the infected cells were fixed and tested to determine the expression of PRRSV N protein and the nsp2del88. The fixed cell monolayer was stained using the N-specific MAb or the polyclonal rabbit anti-nsp2del88 primary antibody, followed by labeled secondary antibody staining as described in the text, and observation using an immunofluorescence microscopy.

response elicited by the JXM100, which was assayed using a commercial ELISA that detected antibody against the N antigen. Thus, del88 can be used as a genetic marker to serologically differentiate JXM100 from JX143.

4. Discussion

PRRSV exhibits high levels of genetic, antigenic, and pathogenic variability (Meng, 2000), which presents a major problem for the effective control of the most economically significant disease that affects the swine industry. Newly emerged HP-PRRSV isolates, such as JXA1, HuN4, and TJ, are highly pathogenic to piglets and can cause 100% morbidity with >60% mortality in infected piglets (Leng et al., 2012b; Tian et al., 2007; Tong et al., 2007). JX143 was isolated from a dying piglet infected with PHFD in 2006 and it shared the same genetic makeup with JXA1, *i.e.*, approximately 99.6% identity at the nucleotide level. JX143 has also been shown to be highly pathogenic in piglets (Lv et al., 2008). In this study, we conducted 100 serial cell passages using a HP PRRSV JX143 isolate to determine the phenotypic and genetic changes associated with the cell

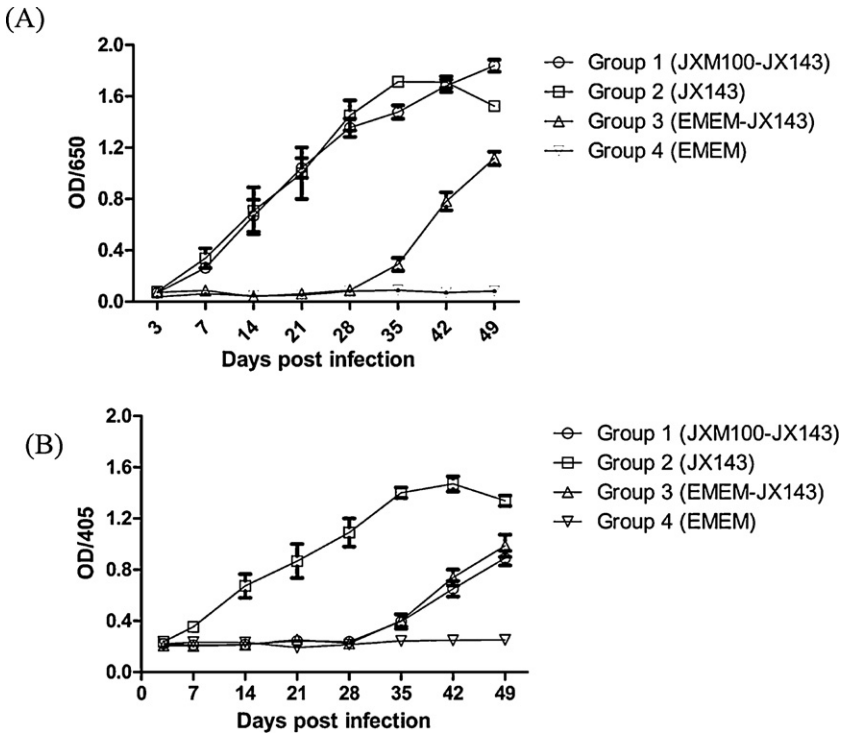

Fig. 8. The serological responses of pigs after experimental infection with JXM100 or JX143. (A) Mean anti-N antibody levels. PRRSV-specific N antibody development was monitored throughout the challenge periods and reported as S/P ratios (S/P ratios >0.4 were considered positive). (B) The del88-based ELISA was performed to differentiate animals infected with JXM100 from those infected with JX143, by detecting the specific antibodies raised against del88.

adaptation process. Indeed, prolonged *in vitro* viral passaging is the usual method of choice for attenuating HP-PRRSV and the development of live-attenuated vaccines (JXA1-R, HuN4-F112, and TJM) (Han et al., 2009; Leng et al., 2012a, b; Tian et al., 2009). We found that the virulence of the JX143 strain in piglets decreased greatly up to passage 100 (JXM100). The piglets in the JXM100 group exhibited no high fever or other clinical signs throughout the experiment. The average body weights of pigs in the JXM100-infected group were similar to those in the control groups. We found that 2/6 pigs inoculated with JXM100 exhibited no viremia, but they developed immune response and protected pigs from lethal challenge, virologically and clinically. These results agreed with a previous study were 4/9 adult pigs infected with a vaccine strain of PRRSV never exhibited viremia, although all seroconverted at the same rate as the viremic pigs (Klinge et al., 2009). The JXM100-infected group could resist a lethal challenge with JX143 and they exhibited no changes in body temperature or clinical signs, while they gained weight in a similar manner as the controls. These results suggest that JXM100 can provide piglets with virological and clinical protection from a lethal challenge and it might be a vaccine candidate to protect against HP-PRRSV. A subsequent study will be conducted to demonstrate that JXM100 is sufficiently safe for use, *i.e.*, the absence of transplacental spread and negative effects in fetuses, and no induction of histopathological lesions in the lungs.

It has been difficult to correlate the genetic changes with the phenotypic properties of PRRSV. Several previous studies were conducted that compared virulent virus with its derived avirulent virus, but there were few consensus genetic changes among different pairs of viruses (Allende et al., 2000; An et al., 2011; Grebennikova et al., 2004; Han et al., 2009; Leng et al., 2012b; Nelsen et al., 1999; Nielsen et al., 2001; Tian et al., 2009; Yuan et al., 2001b). It is possible that any virulence determinant(s) could be virus- and/or strain-specific, or the virulent phenotype could be caused by a combination of protein-protein tertiary interactions between different viruses and/or host proteins. In this study, we identified a total of 75 random nucleotide substitutions throughout the genome, including 33 nonsilent mutations located in the ORF1, ORF2, ORF3, ORF4, and ORF6 regions, which suggested that some of these mutations may have had a role in virus adaptation in the cultured cell system. The ORF3 H79Y mutation was detected during later passage (JXM40) and it could be more strongly related to viral virulence. Several reports have suggested that mutations in structural proteins may be responsible for viral virulence (Allende et al., 2000; Chang et al., 2002; Kwon et al., 2008; Yuan et al., 2001b). In particular, E83G in ORF3 and R151G in ORF5 were considered to be associated with virulence (Allende et al., 2000; Chang et al., 2002). In this study, we failed to observe such mutations because there were no aa changes in ORF5, whereas 5 aa substitutions detected in JXM100 were all different from the E83G described in previous studies. UTRs in the genome are critical for the regulation of virus replication and the virulence determinant of poliovirus and alphavirus (Chumakov et al., 1992; Kinney et al., 1993). Tan et al. (2001) reported that a bulge in the 5′ UTR RNA secondary structure could be used as a differential marker to distinguish field isolates from cell-adapted strains. In this study, we observed a single T163C substitution downstream of the proposed bulge in the 5′ UTR of JXM100, and *in silico* analysis revealed no significant change in the overall secondary structure of the 5′ UTR. In addition, there were no mutations in the UTRs of PRRSV JXA1, TJ, and HuN4 isolates throughout the attenuation process (Han et al., 2009; Leng et al., 2012a, b; Tian et al., 2009). Thus, we assumed that the UTR may have little effect on PRRSV virulence. Therefore, the genetic changes associated with virulence traits in PRRSV are possibly virus strain-specific, or more likely polygenic.

Nsp2 is the largest processed protein that is produced proteolytically by virally encoded proteinases during infection (den Boon et al., 1995). Nsp2 is significantly different in length due to the presence of insertions and deletions in the genotypes of PRRSV isolates, which are found in the middle or near the N-terminus of nsp2 in field isolates (den Boon et al., 1995; Shen et al., 2000; Tian

et al., 2007). Intriguingly, most of the so-called hypervirulent PRRSV strains were found to have a variety of deletions in the nsp2 coding region, *e.g.*, MN184 and Chinese PRRSV isolates (An et al., 2007; Han et al., 2006; Lv et al., 2008; Tian et al., 2007; Tong et al., 2007). The Chinese HP-PRRSV isolates all contained a discontinuous deletion of 30 aa in the variable region of nsp2 and this hallmark deletion was proposed to be a factor that determines high pathogenicity (Tian et al., 2007). However, a recent study showed that the same engineered 30 aa deletion in the backbone of a low virulence PRRSV HB-1 strain did not result in increased virulence (Zhou et al., 2009). In our study, the 30 aa deletion was retained throughout the cell adaptation process, which suggests that the deletion *per se* may not have insignificant effects on virulence. Interestingly, an extra 88 aa deletion located immediately upstream of the 30 aa deletion in nsp2, was detected in the highly cell-adapted virus (P60), which suggests that continuous deletion may be involved in the attenuation of HP PRRSV *in vivo*. Interesting, a spontaneous nsp2 deletion (120 aa) located downstream of the 30 aa deletion in nsp2 region was found in another attenuated virus, TJM, at after 19 passages (P19) of a cell-adapted virus of HP-PRRSV TJ stain (Leng et al., 2012b). These results are consistent with a previous study, which showed that a mutant carrying a 131 aa deletion that partially overlapped with the 120 aa deletion of TJM was less virulent than the parental virus (Kim et al., 2009). However, a large 145 aa deletion was identified in the C-terminal of nsp2 after continuous passaging of the VR2385 virus in cell culture, which showed that this spontaneous nsp2 deletion had a role in enhanced PRRSV replication in cultured MARC-145 cells whereas it had no effect on the pathogenicity of the virus *in vivo* (Ni et al., 2011). Overall, the mechanism underlying spontaneous deletions during viral passages in cells or *in vivo* and their effect on viral replication and pathogenicity remain unclear.

The nsp2 protein contains a large number of linear B-cell epitopes (de Lima et al., 2008; Yan et al., 2007). It has been suggested that the nsp2 protein of PRRSV is immunogenic and capable of eliciting specific antibody production during viral infection. It has been shown that the PRRSV nsp2 protein is an important target for the development of marker vaccines. DIVA PRRSV strains may potentially be developed *via* the engineered deletion of specific individual immunodominant epitopes or large fragments in nsp2 (de Lima et al., 2008; Fang et al., 2008; Kim et al., 2009; Xu et al., 2012). In agreement with previous studies, our study showed that the natural deletion of 88 aa in nsp2 is immunogenic and capable of eliciting a specific antibody *in vivo*. The piglets inoculated with JX143 elicited a robust antibody response against N protein and del88. Sera collected from pigs infected with the heterologous strain vAPRRS could be detected using the anti-del88 antibody (data not shown). However, piglets injected with the attenuated virus JXM100 with 88 aa deletion in nsp2, only elicited antibody against N protein and not against del88 in our experiments. It is suggested that del88 can be used as a genetic marker for serologically differentiating JXM100 from JX143 and other strains.

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