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Nonstructural protein 9 residues 586 and 592 are critical sites in determining the replication efficiency and fatal virulence of the Chinese highly pathogenic porcine reproductive and respiratory syndrome virus

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ABSTRACT

The highly pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV) has caused huge economic losses to the swine industry in China. Understanding the molecular basis in relation to the virulence of HP-PRRSV is essential for effectively controlling clinical infection and disease. In the current study, we constructed and rescued a serial of mutant viruses in nsp9 and nsp10 based on the differential amino acid sites between HP-PRRSV JXwn06 and LP-PRRSV HB-1/3.9. The replication efficiency in pulmonary alveolar macrophages (PAMs) and the pathogenicity of the mutant viruses for piglets were analyzed. Our results showed that the mutation of Thr to Ala in 586 and Ser to Thr in 592 of nsp9 decreased the replication efficiency of HP-PRRSV in PAMs, and could attenuate its virulence for piglets, suggesting that the residues 586 and 592 of nsp9 are critical sites natively in determining the fatal virulence of the Chinese HP-PRRSV for piglets.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV), a devastating pathogen for global swine industry, causes severe reproductive failures in sows and respiratory distress in growing pigs (Albina, 1997; Rossow, 1998). The virus remains a threat to pig production since it was first recognized in the early 1990s (Benfield et al., 1992; Wensvoort et al., 1991). PRRSV is an enveloped virus with a single positive-stranded genomic RNA, which is newly proposed to be classified into the genus *Porartevirus* of the family *Arteriviridae* in the order *Nidovirales*, together with lactate dehydrogenase-elevating virus (LDV) and rat arterivirus 1 (Kuhn et al., 2016; https://talk.ictvonline.org/ictv-reports/ictv_online_report/). PRRSV has two species (PRRSV1 and PRRSV2) (Kuhn et al., 2016), namely the European type (type 1) and North American type (type 2), based on genetic and antigenic differences (Allende et al., 1999; Mardassi et al., 1994; Meng et al., 1995; Murtaugh et al., 1995; Nelsen et al., 1999). The PRRSV genome is approximately 15 kb in size, and contains 12 known open reading frames (ORFs). The ORF1a and ORF1b situated in the 5'-proximal three quarters of the genome encode two large polyproteins, pp1a and pp1ab, which can be processed into 16 nonstructural proteins (nsp), including nsp1α/β, nsp2-6, nsp7α/β, nsp8-12, as well as nsp2TF and nsp2N (Fang et al., 2012; Fang and Snijder, 2010; Li et al., 2015; Mardassi et al., 1995; Meulenberg et al., 1995). The ORF2a, ORF2b, ORFs3-7, and ORF5a encode viral structural proteins (Johnson et al., 2011; Mardassi et al., 1995; Meulenberg et al., 1995; van Nieuwstadt et al., 1996; Wu et al., 2001). The majority of PRRSV nsps have been shown to play important roles in viral replication, genomic transcription and the modulation of innate immune responses (Fang and Snijder, 2010; Yoo et al., 2010).

Tremendous molecular epidemiological data have shown that either type 1 or type 2 PRRSV exhibits broad genetic variation and diversified strains (Shi et al., 2010). The persistent evolution nature of PRRSV has led to the emergence of novel and variant strains with higher pathogenicity or virulence (Han et al., 2006; Karniychuk et al., 2010; Tian et al., 2007; Wang et al., 2015; Zhou et al., 2015). The Chinese highly pathogenic PRRSV (HP-PRRSV) with a unique hallmark of 30-amino acid deletion in the viral nsp2 emerged and prevailed in 2006 (Tian et al., 2007), causing colossal economic losses to the swine production (Zhou and Yang, 2010). A number of studies have provided essential evidence for understanding the pathogenesis of the Chinese HP-PRRSV (Han et al., 2017). HP-PRRSV infection is shown to display expanded tissue tropism and increased virus loads in a variety of organs (Hu et al., 2013; Li et al., 2012), and cause more severe lung injuries and histopathological changes (Han et al., 2014; Hu et al., 2013), and thymus atrophy, depletion of thymocytes, and apoptotic cell death of thymic

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epithelial cells (Guo et al., 2013; He et al., 2012; Li et al., 2014b). Importantly, much attention has been paid to the molecular basis in relation to its virulence. Our previous study indicated that the 30-amino acid deletion in its nsp2 is not related to the fatal virulence for piglets (Zhou et al., 2009). Subsequent works clearly revealed that the nsp9 and nsp10 together are closely related to the replication efficiency both *in vitro* and *in vivo*, and contribute to the fatal virulence of the Chinese HP-PRRSV (Li et al., 2014c). However, it remains unclear whether the Chinese HP-PRRSV shares the amino acid sites in nsp9 or/and nsp10 determining its fatal virulence.

In arterivirus, the nsp9 possesses RNA-dependent RNA polymerase (RdRp) activity, and the nsp10 contains metal binding region with RNA helicase activity, which are considered to be assembled into the replication and transcription complex (RTC), and to play a crucial role in virus replication (Beerens et al., 2007; van Dinten et al., 2000). In terms of the structural model of coronavirus nsp12 (Gorbalenya et al., 1989; Xu et al., 2003), the nsp9 of PRRSV is predicated to consist of at least two domains, an N-terminal domain with RdRp-associated nucleotidyltransferase (NiRAN) activity and a canonical RdRp occupying its C-terminal domain (Lehmann et al., 2015a). A conserved SDD motif and Asp residue within the RdRp domain of nsp9 are recognized to be critical for RNA polymerase activity and RNA synthesis (Lehmann et al., 2016; Snijder et al., 1990; Subissi et al., 2014). The nsp10 of arterivirus belongs to superfamily 1 helicase (HEL1), and its helicase activity domain has similar structure to a cellular helicase Upf1 that participates in nonsense-mediated mRNA decay pathway, implying that the nsp10 might also play a role in post-transcriptional quality control of viral RNA (Deng et al., 2014; Lehmann et al., 2015b). In the present study, we constructed a serial of mutant viruses with the mutated amino acid residues in the nsp9 and nsp10 of PRRSV by using reverse genetic approach, based on the differential amino acid sites in nsp9 and nsp10 between HP-PRRSV and low pathogenic PRRSV (LP-PRRSV), and analyzed the replication efficiency and pathogenicity of mutant viruses for piglets, in an attempt to identify the critical amino acids in relation to the fatal virulence in nsp9 or/and nsp10 of HP-PRRSV.

2. Materials and methods

2.1. Ethics statements

The animal experiments in this study were approved by the Laboratory Animal Ethical Committee of China Agricultural University with the license number (CAU20160828-2). All animal experiments were performed according to the Chinese Regulations of Laboratory Animals—The Guidelines for the Care of Laboratory Animals (Ministry of Science and Technology of People's Republic of China) and Laboratory Animal-Requirements of Environment and Housing Facilities (National Laboratory Animal Standardization Technical Committee).

2.2. Cells, infectious cDNA clones of PRRSV and viruses

MARC-145 cells (ATCC CRL-12231), a subclone of the African green monkey kidney epithelial cell line, were used for PRRSV propagation. The cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Fisher Scientific, Waltham, MA), supplemented with 10% fetal bovine sera (FBS) (HyClone Laboratories Inc., South Logan, UT) at 37 °C under a humid 5% CO_2 atmosphere. Porcine pulmonary alveolar macrophages (PAMs) were prepared as previously described (Zhang et al., 2009), and used for growth efficiency analysis of the viruses. PAMs were maintained in RPMI 1640 medium (Fisher Scientific) supplemented with 10% FBS, 50 U/ml penicillin, 50 mg/ml streptomycin, at 37 °C under a humid 5% CO_2 atmosphere. The full-length infectious cDNA clones of HP-PRRSV JXwn06 and the chimeric infectious cDNA clones between JXwn06 and LP-PRRSV HB-1/3.9 were used in this study, including pWSK-RvJXwn, pWSK-RvJHn10, pWSK-RvJHn9n10, pWSK-RvHJn9n10 and pWSK-RvHJn10, which were constructed previously in our laboratory (Li et al., 2014c; Zhou et al., 2009). For virus growth efficiency analysis and animal experiments, the rescued viruses (RvJXwn, RvJHn9n10, RvJHn10, RvHB-1/3.9, RvHJn9n10 and RvHJn10) conserved in our laboratory were utilized in this study (Li et al., 2014c; Zhou et al., 2009).

Table 1
Differential amino acid sites in nsp9 and nsp10 between PRRSV JXwn06 and HB-1/3.9.

Amino acid positions[a]	JXwn06 (nt/aa)	HB-1/3.9 (nt/aa)
nsp9		
427	GCC/Ala	ACC/Thr
586	ACC/Thr	GCC/Ala
592	TCA/Ser	ACA/Thr
609	GAC/Asp	GGC/Gly
nsp10		
11	GGG/Gly	ATG/Met
51	AGT/Ser	GGT/Gly
69	GAA/Glu	GGA/Gly
296	ATG/Met	GTG/Val
408	AGA/Arg	AAA/Lys

[a] The positions are determined based on amino acid sequence of nsp9 and nsp10 of these two strains of PRRSV, respectively.

2.3. Construction of infectious cDNA clones with mutated sites in nsp9 and nsp10 of PRRSV and virus rescue

By aligning the amino acid sequences, totally nine different amino acid residues existed in nsp9, including nsp8 that is considered an N-terminal domain of nsp9 (Lunney et al., 2016), and nsp10 between the HP-PRRSV JXwn06 and LP-PRRSV HB-1/3.9 with an amino acid deletion in nsp9 that is not counted (Zhou et al., 2009). Of them, four amino acids located in the position 427, 586, 592 and 609 of nsp9, and five located in the position 11, 51, 69, 296 and 408 of nsp10 (Table 1). According to the strategies as previously described (Zhou et al., 2009), respective nucleotide mutation targeting the differential residues in the fragment C (containing nsp9- and nsp10-coding region) of infectious cDNA clones was conducted with the unique restriction enzymes *NheI and AscI* (New England Biolabs, Ipswich, MA). The plasmid Pjet1.2/blunt (Fisher Scientific) was used to construct the plasmid Pjet1.2-C, which was then inserted the fragment C. Then the target nucleotides were mutated in the plasmid by fast mutagenesis system (TransGen, Beijing, China). The mutant plasmids and the backbones of infectious cDNA clones were digested by the restriction enzymes *NheI and AscI*, and finally the mutated fragment C was inserted into the backbone to generate the full-length infectious cDNA clone with respective mutated site.

MARC-145 cells were transfected with the mutant full-length cDNA clone by Lipofectamine LTX (Fisher Scientific) and serially passaged for three times in MARC-145 cells. Then the rescued viruses were examined by indirect immunofluorescence assay (IFA) using the PRRSV N-specific monoclonal antibody (mAb) SDOW17 (Rural Technologies, Brookings, SD). The RNAs of third-passage rescued viruses were extracted, and amplified by RT-PCR, and the amplified fragment was sequenced to further confirm the mutated sites.

2.4. Animals and animal trials

Healthy, six-week old, landrace piglets were obtained from Beijing Center for SPF Swine Breeding & Management that is known to be free of PRRSV. All piglets were confirmed to be negative for PRRSV infection by commercial IDEXX ELISA kits and RT-PCR detection in our laboratory. The piglets were raised in the animal facilities at China Agricultural University (CAU).

The piglets were randomly divided into different groups based on the animal experiments design. The animals in each group (n = 5) were