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Research paper

Genetic characteristics of porcine epidemic diarrhea virus in Chinese mainland, revealing genetic markers of classical and variant virulent parental/attenuated strains

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ABSTRACT

Since October 2010, porcine epidemic diarrhea (PED) caused by variant porcine epidemic diarrhea virus (PEDV) has led great economic losses to the global pig industry, especially in China. To study the genetic characteristics of PEDV strains in Chinese mainland, a total of 603 clinical samples from nine provinces/districts of Chinese mainland from January 2014 to December 2015 were collected for RT-PCR detection and 1-1323 bp of *S* gene of 91 isolates and *ORF3* gene of 46 isolates were sequenced. The results showed that the variant PEDV were the dominant pathogens of viral diarrhea diseases in these areas. Six novel variant PEDV strains (FJAX1, FJAX2, HeNPDS1, HeNPDS2, HeNPY3, and HeNPY4) with two amino acids (aa) deletion at the 56–57 aa of S protein were identified. A total of 405 Chinese PEDV strains were subjected to phylogenetic and phylogeographic analysis. The results revealed that the subgroup Va in variant PEDV group were the dominant subgroup and the spread trend of variant PEDV strains seemed to be from the southeast coastal districts to other coastal districts and interior districts. The N-terminal of *S* gene (1–750 bp), to some extent, could represent *S1* or full length *S* gene for phylogenetic, similarity, antigen index, hydrophilicity plot, and differentiation analyses. The 404–472 bp of *S* gene contained the three genetic markers, i.e., "TAA" insertion at 404–405 bp, "ACAGGT" deletion at 430–435 bp, and "ATA" deletion at 455–457 bp can be used to differentiate the classical and variant virulent parental/attenuated PEDV strains and help us to learn the infectious and genetic characteristics of PEDV strains more convenient and cheaper. This study has important implication for understanding the infectious, genetic, and evolutionary aspects of PEDV strains in Chinese mainland.

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1. Introduction

Porcine epidemic diarrhea virus (PEDV) belongs to the group I, genus *Alphacoronavirus*, family *Coronaviridae*. It is an enveloped, single-stranded, positive-sense RNA virus with a genome about 28 kb (Song and Park, 2012). It is the pathogen of porcine epidemic diarrhea (PED) that is a highly contagious, acute enteric tract infectious disease. PED is characterized by watery diarrhea, dehydration and weight loss in adult pigs and lethal in piglets (about 100% morbidity and about 80%–100% mortality) (Chen et al., 2010; Li et al., 2012). Since first reported in England in 1971 (Oldham, 1972), PED has been reported in the worldwide pig industry (Huang et al., 2013; Lee and Lee, 2014; Li et al., 2012; Song and Park, 2012; Song et al., 2015; Sun et al., 2012; Van Reeth and Pensaert, 1994; Wang et al., 2013). The heterogeneity in spike (*S*) gene of PEDV strains was first reported in Korea in 2010 (Lee et al., 2010). PED was re-emerged in China since October 2010, and caused devastating economic losses to the piggeries in the southeast provinces of China (Sun et al., 2012). Since October 2010, PED caused by variant PEDV has led great economic losses to world pig industry, especially in China (Song et al., 2015). The PED was reported in some pig farms that were vaccinated with inactivated CV777-based vaccines (Chen et al., 2010; Ge et al., 2013). The variant PEDV strains with five amino acids (aa) insertions at about 56–60 aa and one aa insertion at 141 aa of S protein comparing with CV777 were identified as causative organisms of this re-emerging of PED (Li et al., 2012). During the next two and half years after October 2010, the diseases caused by the variant PEDV strains were mainly prevailing and

Abbreviations: PED, porcine epidemic diarrhea; PEDV, porcine epidemic diarrhea virus; TGEV, porcine transmissible gastroenteritis virus; RVA, porcine group A rotavirus; US, United States; aa, amino acid; *S* gene, spike gene.

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led millions of piglet deaths in China (Song et al., 2015). After the first case reported in the United States (US) in the April 2013, the Chinese AH2012 strain like variant PEDV strains were quickly identified in the piggeries of the whole US and then emerged or re-emerged in world-wide pig industries (Cima, 2013; Hao et al., 2014; Huang et al., 2013; Lee and Lee, 2014; Li et al., 2012; Lin et al., 2014; Marthaler et al., 2013).

The *S* gene sequence is a determining feature of PEDV's virulence and evolution (Park et al., 2007b). The S glycoprotein (surface antigen) is the major surface protein in PEDV, which is type-I transmembrane glycoprotein that responds for viral receptor binding, induction of the neutralizing antibody and host-virus fusion (Park et al., 2007a; Puranaveja et al., 2009; Sato et al., 2011). The S protein of PEDV could be artificially defined by the conserved nonamer and the GxCx motifs into S1 (residues 1–789 aa) and S2 (residues 790–1383 aa) subunits. Although lack of proteolytic cleavage sites (Park et al., 2007a; Sun et al., 2008), S1 subunit contains the receptor-binding site and main neutralizing epitopes and thus defines the binding between host and virus and neutralization activity. The phylogenetic analysis based on *S1* N-terminal of PEDV was similar to that based on full *S* gene (Lee et al., 2010; Sun et al., 2015). The *S1* N-terminal has been used in conforming variant PEDV strains, phylogenetic analysis and differentiating PEDV strains (Gao et al., 2013; Li et al., 2012; Wang et al., 2014). The ORF3 protein was reported to function as an ion channel and can prolongs S-phase, facilitates formation of vesicles and thus to regulate virus production (Wang et al., 2012; Ye et al., 2015). The PEDV strains with about 49 nt deletion at 245–293 bp of *ORF3* gene were identified as vaccine PEDV strains or PEDV strains derivative from vaccine strains (Chen et al., 2010).

In this study, a total of 603 samples (feces and intestines) were collected from 39 farms in the Hebei, Henan, Beijing, Hubei, Fujian, Zhejiang, Guangdong, Anhui, and Heilongjiang areas of China during the January 2014 to December 2015. In which, the *S1* N-terminal of 91 isolates and *ORF3* gene of 46 isolates (see Table S1 in the Supplemental material) from Hebei, Henan, Beijing, Hubei, Fujian, Zhejiang, Guangdong, Anhui, and Heilongjiang areas of China were sequenced. The *S1* N-terminal region sequences and *ORF3* gene sequences of different isolates were used to determine the genotypes of these isolates. These isolates and a total of 314 available and background-clear Chinese PEDV strains were used for phylogenetic and phylogeographic analysis. Four representative PEDV strains were subjected to antigenic index and hydrophilicity plots analysis, combining with our former genomic comparison study (Chen et al., 2015) and multiple nucleotide alignment of 405 Chinese mainland PEDV strains in this study, the nucleotide sequences of 404–472 bp of *S* gene contain three genetic markers that can be used to differentiate classical and variant virulent parental/attenuated PEDV strains.

2. Materials and methods

2.1. Clinical samples collection and preparation

A total of 603 samples (feces and intestines) were collected from 39 farms in the Hebei, Henan, Beijing, Hubei, Fujian, Zhejiang, Guangdong, Anhui, and Heilongjiang areas of China during the January 2014 to December 2015. These samples were individually collected and the samples were homogenized and diluted 1:10 with phosphate-buffered saline (PBS; 0.1 M, pH 7.4). The homogenate was freeze and thawed three times to release virus and further vortexed for 10 min and centrifuged at 10,000 rpm (Eppendorf, Germany) for 10 min at 4 °C. The supernatants were used for RNA extraction immediately or stored at −20 °C refrigerator for further usage.

2.2. RT-PCR and S1 N-terminal region and ORF3 gene sequence

The clarified supernatants of 200 μL were collected into sterile centrifuged tubes for total RNA extraction according to the instructions of Trizol reagent (Invitrogen, US). Reverse transcription (RT) was performed by using 1 μg of total RNA, PrimeScript™ RT Master Mix (TaKaRa, Japan) according to the manufacturer's instructions. PCR was conducted to amplify each cDNA fragment from RT products by using TaKaRa Ex Taq DNA polymerase (TaKaRa, Japan) according to the manufacturer's protocol. Multiplex RT-PCR method was used for detection of PEDV, porcine transmissible gastroenteritis virus (TGEV) and porcine group A Rotavirus (RVA) (Zhang and He, 2010). The detection of Deltacoronavirus was performed using the method we reported before (Wu et al., 2016). Two sets of primers based on published sequences were used to amplify the N-terminal region (1–1323 bp) of *S* gene and complete *ORF3* gene (Table 1). The amplicons were gel-purified, cloned into pMD®18-T Vector (Takara, Japan), and sequenced in both directions at GenScript company (Nanjing, China).

2.3. Phylogenetic and phylogeographic analysis of Chinese PEDV strains

A total of 314 available and background-clear Chinese PEDV strains were downloaded from GenBank (see Table S1 in the Supplemental material). The multiple sequence alignments were generated with ClustalX 2.0 program (Thompson et al., 1997). The multiple nucleotide and deduced aa sequences of these strains were aligned and analyzed with MEGA Version 6.06 (K. Tamura, Arizona State University, Phoenix, AZ, US) (Tamura et al., 2007) and MegAlign Version 5.01 software application. The phylogenetic trees of *S1* N-terminal regions 1–1323 bp and 1–750 bp with neighbor-joining method (bootstrap analysis with 1000 replicates) were constructed by the MEGA Version 6.06 software. The geographical dissection of the Chinese PEDV strains was presented based on the phylogenetic analysis. The isolated provinces of these isolates are shown on the map in Fig. 3. On the map, five figures were used to represent five different subgroups. The numbers behind different figures represent the number of the reported PEDV isolates of that subgroup.

2.4. Characterization (similarity, antigenic index and hydrophilicity plots) of PEDV strains and genetic markers identification

Similarity plots analysis by sliding window was performed as implemented in the SimPlot, v. 3.5.1 package (Lole et al., 1999). The similarity plot of 405 Chinese PEDV strains was drawn and the CV777 was set as query strain. Four representative classical and variant virulent parental/attenuated PEDV strains (CV777, CV777 vaccine, YN1, and YN144) were subjected to antigenic index and hydrophilicity analysis, based on the N-terminal of the S protein (1–441 aa). The antigenic index and hydrophilicity plots were obtained with the Lasergene package, Protean program (DNAStar Inc., Madison, WI, US) using the Jameson-Wolf and Kyte-Doolittle algorithms (Ie et al., 2010; Jameson and Wolf, 1988; Kyte and Doolittle, 1982), respectively. The comparative genomic study of classical and variant virulent parental/attenuated PEDV strains has been reported before. The genetic markers of classical and variant virulent parental/attenuated PEDV strains from genome level were also clear. On the basis of former study, in this study, we tried to find out the genetic markers of classical and variant virulent parental/attenuated PEDV strains on the N-terminal of *S* gene. The nucleotides information of N-terminal of *S* of the reference strains are presented in the Table S1 (Supplementary material).

Table 1
List of primers utilized in this study.

Primer name	Nucleotide sequence (5′-3′)	Purpose	Primer location (nt)[a]
S1-F	ATGAAGTCTTTAACCTACTTCTGGT	Sequencing	20,638–20,662
S1-R	GAAATTGGCTGTTCATGAC	Sequencing	22,109–22,127
ORF3J-F	GTTGTTGTAGGGGTCCTAGACT	Sequencing	24,728–24,729
ORF3J-R	GCCAAAGTATAACATTAACTACT	Sequencing	25,473–25,497

[a] Numbers correspond to positions within the CV777 genome.

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