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Isolation and characterization of Chinese porcine epidemic diarrhea virus with novel mutations and deletions in the S gene

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ABSTRACT

Porcine epidemic diarrhea (PEDV) has raised growing concerns in the pig-breeding industry because it has caused significant economic losses. To better understand the molecular epidemiology and genetic diversity of PEDV field isolates, in this study, the complete spike (S) and ORF3 genes of 17 PEDV variants in Zhejiang, China during 2014 to 2017, were characterized and analyzed. Phylogenetic analysis based on the S gene and ORF3 gene of these 17 novel PEDV strains and PEDV reference strains indicated that all the PEDV strains fell into two groups designated G1 and G2. Notably, the strains identified in 2014–2015 were in G2, while the other five strains identified from 2016 to 2017 were in G1. Sequencing and phylogenetic analyses showed that recently prevalent Chinese PEDV field strains shared higher identities with United States strains than with South Korean strains. Compared with classical vaccine strains, a series of deletions and frequently occurring mutations were observed in the receptor binding domains of our PEDV strains. Besides, we successfully isolated and reported the genetic characterization two novel PEDV strains, PEDV-LA1 and PEDV-LY4-98, found on the Chinese mainland, which had significant variations in the S gene. Meanwhile, the virulence of the new mutants may be changed, the PEDV-LY4-98 strain, which has multiple mutations in the signal peptide-encoding fragment of the S gene showed delayed cytopathic effects and smaller plaque size compared with strain PEDV-LA1, which lacks these mutations. Three unique amino acid substitutions (L7, G8, and V9) were identified in the SP-encoding fragment of the S1 N-terminal domain of the PEDV-LY4-98 S protein compared with the S proteins of all the previous PEDV strains. The animal experiment revealed that these two novel strains were high pathogenic to neonatal pigs. Whether these amino acids substitutions and the N-glycosylation site substitutions influence the antigenicity and pathogenicity of PEDV remains to be investigated. Meanwhile, amino acid substitutions in the neutralizing epitopes may have conferred the capacity for immune evasion in these PEDV field strains. This study improves our understanding of ongoing PEDV outbreaks in China, and it will guide further efforts to develop effective measures to control this virus.

1. Introduction

Porcine epidemic diarrhea (PED), which is caused by porcine epidemic diarrhea virus (PEDV), is an acute, highly contagious, and devastating swine disease that is characterized by enteritis and lethal watery diarrhea that results in dehydration, which frequently leads to high mortality in piglets (Coussement et al., 1982; Wood, 1977). PED was first reported in Belgium and the United Kingdom in 1978 (Kocherhans et al., 2001; Pensaert and de Bouck, 1978) and then emerged in a number of other European countries. Since 2010, more variant PEDV strains whose sequences differ from that of the classic European strain (CV777) have appeared recently in many Asian countries, including China (Bi et al., 2012; Li et al., 2012a; Pan et al., 2012; Song et al., 2015; Sun et al., 2015), South Korea, Japan, and Vietnam (Lee et al., 2010; Park et al., 2011). PEDV was first identified in the 1980 s in China, and since then, PED outbreaks have increased

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markedly and spread rapidly across countries. In October 2010, a large-scale outbreak of PED caused by a PEDV variant occurred in China, resulting in tremendous economic losses. New outbreaks associated with a novel PEDV strain that is genetically distant from the prototype PEDV strain, CV777, have been reported in China (Chen et al., 2012; Li et al., 2012a, b). PED is recognized worldwide for the dramatic changes observed in its epidemic character and pathogenic molecules. Since its initial recognition in May 2013, PEDV has rapidly spread across the United States (U.S.), resulting in high mortality in piglets in more than 17 states to date (Chen et al., 2014; Huang et al., 2013; Pasma et al., 2016).

PEDV is an enveloped, single-stranded, positive-sense RNA virus belonging to the order Nidovirales, the family Coronaviridae, the subfamily Coronavirinae, and the genus Alphacoronavirus (Huang et al., 2013). The approximately 28-kb genome contains a 5′ untranslated region (UTR), a 3′ UTR, at least seven open reading frames (ORFs), which encode four structural proteins, namely the spike (S), envelope (E), membrane (M), and nucleocapsid (N), and three non-structural proteins, designated as replicases 1a and 1ab and ORF3, which are arranged in the order of 5′ UTR-ORF1a/1b-S-ORF3-E-M-N-3′ UTR (Brian and Baric, 2005). Two long ORFs, ORF1a and ORF1b, occupy two-thirds of the genome and encode two nonstructural polyproteins (pp1a and pp1b) that direct genome replication, transcription and viral polyprotein processing.

The PEDV S protein is a key factor that mediates the entry of PEDV into host cells by binding to aminopeptidase N and/or glycan receptors through an unknown mechanism, thereby initiating the fusion of the virus envelope with the host cell membrane and the subsequent infection cycle (He et al., 2005; Ye et al., 2015). Thus, the S glycoprotein is the primary target for the development of vaccines against PEDV, as well as for understanding the genetic relationships between different strains and the epidemiological status of PEDV in the field. The PEDV S protein is a type I glycoprotein that consists of three segments - an ectodomain, a single-pass transmembrane anchor, and a short intracellular tail (Li, 2012). The ectodomain is often cleaved into a receptor-binding S1 subunit and a membrane-fusion S2 subunit, and the amino acid sequences of S1 subunits diverge across different genera, but are relatively conserved within each genus (Deng et al., 2016; Zheng et al., 2006). S1 can recognize a variety of host receptors, including proteins and sugars (Makadiya et al., 2016; Wang et al., 2016). induce most of the host immune responses, and may serve as subunit vaccines against coronavirus infections. The S protein is also the major envelope glycoprotein, contains four neutralizing epitopes named COE, SS2, SS6, and 2C10, and the regions of the aligned sequences that correspond to these regions are amino acids 499–638, 748–755, 764–771, and 1368–1374 (Chang et al., 2002; Li, 2015; Sun et al., 2008). Mutations, deletions and/or insertions in the S protein may change the pathogenicity and antigenicity. A previous report suggested that a field CHN/FL2013 strain with a 7 aa-earlier termination of S protein showed reduced virulence in cell culture (Zhang et al., 2015). Park et al. reported that Korean strain MF3809/2008 was identified with a large deletion (204 aa) at amino acid positions 713–916 of the S protein reduced its virulence and changed its tissue tropism from intestinal (Park et al., 2014). The highly virulent PEDV strains from China had an increased number of substitutions within the S1 domain compared with the highly virulent American strains, probably due to the longer circulation time of PEDV in pigs in China. N terminal domain. bioinformatics predicted that the S protein of highly virulent PEDV strains changed in primary/secondary structures, high-specificity N-glycosylation sites, potential phosphorylation sites, and palmitoylation sites (Chiou et al., 2017; Hao et al., 2014) These changes may affect viral antigenicity and change viral neutralizing activity. In recent years, new S mutant strains have been constantly found and this may be related to the immune pressure of the vaccines that are used, including those from vaccine strains CV777 and DR13.

Unlike the structural proteins, little is known about the functions of the accessory proteins. The ORF3 gene, which is the only accessory gene, encodes an ion channel protein that regulates virus production (Wang et al., 2012), and it has been suggested to be an important virulence determinant (Ye et al., 2015). A previous report suggested that piglets that were inoculated orally with the wild-type CV777 strain became sick and developed severe diarrhea. However, piglets inoculated with strain KPEDV-9, which was serially passaged in Vero cells, showed reduced disease and fewer lesions (Kweon et al., 1999). Different ORF3 variants could serve as markers of vaccine- and wild-type PEDVs, and they could be a valuable tool for studying the molecular epidemiology of PEDV (Kim et al., 2016; Li et al., 2016).

In this study, we found two novel PEDV field strain PEDV-LA1 and PEDV-LY4-98 with amino acid mutations and deletions in the protein encoded by the S gene. The animal experiment revealed that PEDV-LY4-98 strain were high pathogenic to neonatal pigs. This study showed that a PEDV strain with the new molecular characterizations and phylogenies was found in southern China. This will be useful to take into consideration in the control and prevention of this disease. Thereby, a comprehensive study is necessary to better understand the genetic variations and relationships between different strains, and would be helpful to find out the reason of the continuously outbreak of PEDV and develop new strategy to control and prevent PEDV infection.

2. Materials and methods

2.1. Sample collection

In this study, a total of Eighty-five porcine samples, consisting of feces or intestine tissues, were collected from 8 farms in Zhejiang provinces during 2014 to 2017. All samples were taken from pigs that exhibited severe watery diarrhea, vomiting, and dehydration. Seventeen of the 85 samples were confirmed as positive for PEDV, as determined by a PEDV N gene-based real-time RT-PCR. PEDV-positive intestine tissues were used to generate a 10% (wt/vol) homogenate in phosphate-buffered saline (0.1 M, pH 7.2). The suspension was centrifuged at 4500 × *g* for 10 min at 4 °C, filtered through a 0.22-μm-pore-size syringe filter and used as an inoculum for virus isolation. One-tenth gram of feces was suspended in 1 ml phosphate-buffered saline(PBS), vortexed for 5 min, and then centrifuged at 4500 × *g* for 5 min.

2.2. PEDV N gene-based real-time RT-PCR

Viral RNA extraction was performed with 50 μl of the small intestine homogenates using the Nucleic Acid Co-prep Kit (GeneReach Biotechnology Corp., Taichung, Taiwan) according to the manufacturer's instructions. The concentrations of the extracted RNAs were measured by a Synergy H1 microplate reader (Bio Tek, Winooski, VT, USA), and then eluted into 90 μl of elution buffer. Primers and probes for real-time RT- PCR were designed and synthesized to target conserved regions of the PEDV N gene. RT-PCR was conducted using 50–200 ng of extracted RNA, a forward primer (5′–TTGGTGGTAATGT GGCTGTTCGTG–3′), a reverse primer (5′–ATCCACCTGTGAAACAAGA AGCTCAAC–3′), and the One-Step RT-PCR Kit (TaKaRa, Shiga, Japan) according to the manufacturer's protocol.

2.3. PCR amplification and sequencing

Viral RNAs were extracted from PEDV-positive sample suspensions as described above. RT was conducted using random hexamer primers, and first-strand cDNA was synthesized using the ReverTra Ace® qPCR RT Kit (Toyobo, Tokyo, Japan) at 42 °C for 60 min and then at 98 °C for 5 min to inactivate the Moloney murine leukemia virus reverse transcriptase, followed by incubation 4 °C for 5 min. The cDNA was immediately used for amplification or stored at −20 °C. The S and ORF3 genes were amplified by RT-PCR using KOD-Plus Neo (Toyobo, Tokyo, Japan) DNA polymerase with primers targeting sequences that are