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Short communication

Two novel porcine epidemic diarrhea virus (PEDV) recombinants from a natural recombinant and distinct subtypes of PEDV variants

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ABSTRACT

Porcine epidemic diarrhea virus (PEDV) causes devastating impact on global pig-breeding industry and current vaccines have become not effective against the circulating PEDV variants since 2011. During the up-to-date investigation of PEDV prevalence in Fujian China 2016, PEDV was identified in vaccinated pig farms suffering severe diarrhea while other common diarrhea-associated pathogens were not detected. Complete genomes of two PEDV representatives (XM1-2 and XM2-4) were determined. Genomic comparison showed that these two viruses share the highest nucleotide identities (99.10% and 98.79%) with the 2011 ZMDZY strain, but only 96.65% and 96.50% nucleotide identities with the attenuated CV777 strain. Amino acid alignment of spike (S) proteins indicated that they have the similar mutation, insertion and deletion pattern as other Chinese PEDV variants but also contain several unique substitutions. Phylogenetic analysis showed that 2016 PEDV variants belong to the cluster of recombination strains but form a new branch. Recombination detection suggested that both XM1-2 and XM2-4 are inter-subgroup recombinants with breakpoints within ORF1b. Remarkably, the natural recombinant HNQX-3 isolate serves as a parental virus for both natural recombinants identified in this study. This up-to-date investigation provides the direct evidence that natural recombinants may serve as parental viruses to generate recombined PEDV progenies that are probably associated with the vaccination failure.

Porcine epidemic diarrhea (PED) is an acute and highly contagious enteric disease characterized by diarrhea, vomiting, dehydration, and weight loss (Have et al., 1992; Wang et al., 2016a). The etiologic agent is PED virus (PEDV), an enveloped, positive-sense, single-stranded RNA virus belonging to the order *Nidovirales*, family *Coronaviridae*, subfamily *Coronavirinae*, and genus *Alphacoronavirus* (Brian and Baric, 2005; Chasey and Cartwright, 1978; Pensaert and de Bouck, 1978). PEDV can infect pigs of any age, and the mortality may be up to 100% in piglets (Sun et al., 2012). Vaccination has been used as a major strategy to control PED for many years, but both inactivated and live-attenuated CV777-based vaccines have become insufficiently protective, especially since the emergence of new variants in China in October 2010 (Li et al., 2014; Li et al., 2012; Sun et al., 2012). Furthermore, highly homological PEDV variants also emerged in the United States in April 2013, causing high mortality in piglets and huge economic losses (Huang et al., 2013; Stevenson et al., 2013; Vlasova et al., 2014).

The PEDV genome is approximately 28 kb in length. It contains 5′ and 3′ untranslated regions (UTR) and at least seven open reading frames (ORFs), encoding two replicase polyproteins (pp1a and pp1ab), four structural proteins (spike (S), envelope (E), membrane (M), and nucleocapsid (N)), and one hypothetical accessory protein (Kocherhans et al., 2001; Li et al., 2016). The S glycoprotein is on the surface of virion and interacts with host cellular receptor to mediate viral entry (Li, 2015), which contains at least four neutralizing epitopes (Chang et al., 2002; Kocherhans et al., 2001; Liu et al., 2017; Sun et al., 2008). In addition, the S gene shows a high degree of genetic diversity (Lee et al., 2010). Therefore, it's often used to analyze PEDV genetic variations (Chen et al., 2013a; Park et al., 2007; Puranaveja et al., 2009). However, to get a comprehensive understanding of PEDV evolution and transmission, full-length genome is generally considered a better choice for the analysis (Chen et al., 2014; Sun et al., 2015).

In this study, to investigate the up-to-date prevalence and evolution of porcine diarrheal pathogens in China, a panel of RT-PCR assays modified from previous studies (Chen et al., 2009; Lee et al., 2016; Liu et al., 2011; Zhao et al., 2013) for differential detection of six diarrhea-associated swine pathogens, including PEDV, transmissible

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gastroenteritis virus (TGEV), porcine deltacoronavirus (PDCoV), group A rotavirus (GARV), classical swine fever virus (CSFV) and porcine reproductive and respiratory syndrome virus (PRRSV), were routinely performed in our laboratory on clinical samples. Only PEDV was detected in two 2016 outbreaks among piglets suffering severe diarrhea, in which farms sows had been immunized with commercial attenuated and inactivated bivalent (PEDV and TGEV) vaccines. In order to characterize the 2016 PEDV strains, the complete genomes of one representative virus from each pig herd were determined. In addition, whole genome-based phylogenetic tree was constructed and recombination detection was performed to detail the generation and evolution of 2016 Chinese PEDV variants.

The samples were collected from two pig farms in Xiamen city, Fujian province in China that suffered severe diarrhea and had high morbidity and mortality in piglets. Pig farm 1 is a commercial swine farm containing ~400 sows and > 6000 pigs. From January to April 2016, piglets within one week old suffered from watery diarrhea. The morbidity was ~90% and the mortality of infected piglets was ~100%. All sows had been vaccinated with the commercial attenuated bivalent PEDV (CV777 strain) and TGEV (H strain) vaccine. After the outbreak, commercial inactivated bivalent PEDV and TGEV vaccine was also used to immunize the sows. Pig farm 2 has ~800 sows and > 14,000 pigs. From March to August 2016, piglets (≤1 week old) were affected and showed severe diarrhea. The morbidity was ~70% and the mortality of infected piglets was ~80%. Sows had also been immunized with both attenuated and inactivated bivalent vaccines. In addition, antibiotics (ciprofloxacin or mequindox) were selectively used to piglets. However, the outbreaks were not effectively controlled. Three and seven dead piglets (within one week of age) from farms 1 and 2 were frozen and sent to our laboratory in late March and early August 2016 for differential detection. PEDV was detected in all three intestine samples from pig farm 1 and four out of seven intestines from farm 2, while the other five diarrhea-associated pathogens (TGEV, PDCoV, GARV, CSFV, and PRRSV) were not detected in any samples (Data no shown).

The full-length S genes of all seven PEDV positive samples and the complete genomes of one representative virus from each farm were determined using primers shown in Table S1. In detail, total RNAs were extracted from 100 mg of intestines homogenized in 1 ml TRIzol® Reagent (Invitrogen) as previously described (Chen et al., 2016). cDNAs were generated by reverse transcription (RT) using random hexamer primers and the First Strand cDNA Synthesis Kit (TIANDZ, China) according to the manufacturer's instructions. The PCR reaction system and amplification condition were set as follow: the 50 μl PCR reaction contained 25 μl of 2 × EasyTaq® PCR SuperMix (TransGen, China), 1 μl of cDNA, 1 μl of both forward and reverse primers (10 μM), and 22 μl of ddH_2O. The amplification parameters were 94 °C for 2 min, followed by 35 PCR cycles (94 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min), and an elongation step at 72 °C for 10 min. The products were analyzed by 1% agarose gel electrophoresis. The amplicons were purified with Gel Extraction Kit (TIANDZ, China), cloned into SuperTOPO-Amp vector using SuperTOPO TA Cloning Kit (TIANDZ, China), and transformed into TOP10 chemically competent cell (TransGen, China). Recombinant clones were identified by colony PCR using M13 primer pairs and at least three positive clones were sent out for sequencing at Genewiz, China. The DNAMAN software (http://www.lynnon.com/) was used to align and assemble the obtained sequences to generate seven full-length S genes and two complete genomes. The complete genomes (XM1-2 and XM2-4) were deposited in the GenBank database with the accession numbers KX812523 and KX812524, respectively.

To analyze the sequence similarities of 2016 PEDV variants and CV777 vaccine strain (GenBank accession no. KT323979), the identities of each gene and encoded protein among VAC-CV777, XM1-2 and XM2-4 genomes were determined using DNAMAN. In addition, seven S proteins identified in this study were compared and aligned with the S proteins of six representative PEDV strains (the attenuated CV777 strain and AH2012, ZMDZY, CHYJ130330, PEDV-LYG, HNQX-3 variants) using ClustalX 2.0 (Larkin et al., 2007) and MEGA 6.06 (Tamura et al., 2013).

To evaluate the genetic relationships of different PEDV strains, the XM1-2 and XM2-4 genomes, together with other 68 PEDV complete genomes, including 2 attenuated vaccine strains, 11 global isolates and 55 Chinese isolates, were used for the phylogenetic analysis (Table S2). Multiplex sequence alignment was generated using ClustalX 2.0 (Larkin et al., 2007). Phylogentic analysis was performed using MEGA 6.06 program (Tamura et al., 2013). Phylogenetic tree was constructed from aligned complete genomes using the neighbor-joining method as previously described (Chen et al., 2011). The robustness of the phylogenetic tree was evaluated by bootstrapping using 1000 replicates.

Recombination detection program (RDP4) was used to detect whether the two 2016 PEDV variants were potential recombinants from field isolates (Martin and Rybicki, 2000). Seven methods embedded in RDP4 software package, including RDP, GENECONV, BootScan, Maxchi, Chimaera, SiScan, and 3Seq, were utilized to detect recombination events and breakpoints (Chen et al., 2017). The default settings were used for all methods and the highest acceptable P value cut-off was set at 0.01 as previously reported (Chen et al., 2013b). The detected recombination events were further evaluated by SimPlot 3.5.1 (Lole et al., 1999).

All seven full-length S genes of viruses from the positive samples were sequenced. The three S genes from pig farm 1 shared > 99.9% nucleotide and amino acid identity and the four S genes from pig farm 2 were exactly identical (Fig. S1). The piglet 2 from Xiamen pig farm 1 (denominated XM1-2) and piglet 4 from pig farm 2 (XM2-4) were selected as the representatives for complete genome sequencing. Both genomes were 28,038 nucleotides in length and shared 98.67% nucleotide identity. When comparing with the CV777 vaccine strain, the genomic identities were 96.65% and 96.50%, respectively. For each gene/protein, the lowest identities were observed in S gene/protein, which were only 93.28% and 93.49% (nucleotide) and 92.57% and 92.65% (amino acid), respectively (Table S3).

The multiple alignment of S proteins showed that 2016 PEDV variants exhibited several genetic markers identical to other non S-INDEL PEDV variants (Chiou et al., 2017; Vlasova et al., 2014; Wang et al., 2013), including a four-amino-acid "QGVN" insertion at positions 58–62, a one-amino-acid "N" insertion at position 140, and a one-amino-acid "G" deletion at position 160 of the S protein, when compared with the CV777 vaccine strain (Fig. S1). In line with other Chinese virulent variants (Li et al., 2012), these insertion, deletion and mutation found in the S proteins probably contribute to strong pathogenicities (≧80% mortality) of 2016 PEDV variants. In addition, three serine substitutions ($A_{521}S$, $T_{553}S$, and $G_{598}S$) in the neutralizing epitope COE and three serine mutations ($N_{728}S$, $L_{768}S$ and $D_{770}S$) in the neutralizing epitope S1D were also identified in 2016 PEDV variants. The serine substitutions in neutralizing epitopes might also change the immunogenicity of spike protein and consequently result in the immunization failure of current commercial vaccines based on classical attenuated CV777 strain (Li et al., 2016; Wang et al., 2013). Besides the consistent substitutions, 2016 PEDV variants had one unique $A_{1100}S$ mutation in the S protein that could differentiate them from the CV777 vaccine strain and non-INDEL variants, which might serve as a genetic marker of our 2016 variants. Moreover, our 2016 PEDV variants in pig farm 1 had eight unique substitutions ($L_{17}F$, $T_{24}A$, $I_{167}V$, $A_{194}V$, $V_{454}A$, $F_{644}S$, $P_{804}L$, and $F_{1366}L$) and in pig farm 2 had five unique substitutions ($T_{232}I$, $L_{505}M$, $A_{673}V$, $L_{999}M$, and $P_{1269}S$) that could distinguish themself (Fig. S1). The influence of unique substitutions dispersed in S proteins of our 2016 PEDV variants on pathogenicity and/or immunogenicity would be interesting to be investigated.

Consistence with previous studies (Chen et al., 2014; Huang et al., 2013; Wang et al., 2016a), our genome-based phylogenetic tree showed that the PEDV isolates could be divided into two major groups, G1 and G2. And the groups could be further divided into subgroups: G1-a, G1-b, R (only CH-S strain), G2-a, G2-b, and recombinant (Fig. 1).