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Epidemiology and phylogeny of spike gene of porcine epidemic diarrhea virus from Yunnan, China

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ABSTRACT

Porcine epidemic diarrhea (PED) causes acute enteric disease and yellowish watery diarrhea, making piglets fast dehydration to death. PED threatens pig industry and leads to substantial economic losses. After the first reports, PED in Yunnan province, China was again identified in 2013 during an epidemiological survey, with follow-up data showing an overall positive rate of 17.47% during 2013–2017, lower than that in other provinces in China. The complete S gene of porcine epidemic diarrhea virus (PEDV) is 4149–4158 bp long. Phylogenetic analysis of S gene was performed using 9 new isolates from Yunnan province, China, together with 225 full-length S genes available in GenBank. The nine Yunnan isolates were clustered into classical G1b and pandemic G2a groups, indicating new variants have been emerging in Yunnan province. When taking the previously submitted 3 isolates from China into consideration, all the 12 isolates were clustered into 4 groups, i.e., G1a, G1b, G2a and G2b, suggesting that a highly diverse and complex clustering might result from co-infections in more than 13 provinces in China, as well in South Korea, Japan, Vietnam, Thai and USA. Identification of new types of PEDV strains would stimulate the development of effective vaccines for the prevention and control of PED in a more precise manner.

1. Introduction

Porcine epidemic diarrhea virus (PEDV) is an enveloped, single stranded, positive-sense RNA virus, belonging to the order *Nidovirales*, family *Coronaviridae* and genus *Alphacoronavirus*. PEDV, first identified in England and Belgium in 1980s (Oldham, 1972; Pensaert and de Bouck, 1978), causes porcine epidemic diarrhea (PED), which is characterized by acute watery diarrhea in feeder pigs, fatteners and sows. Sporadic epidemics occurred around the world during 1990–2009. However, acute and severe outbreaks happened in many countries, causing death of several millions of neonatal piglets due to yellow watery diarrhea, dehydration, weight loss and high mortality in pigs < 10 days old since 2010, and thus resulting in huge economic losses to pig industry, with a mortality rate of 80–100% and a mobility rate of 100% (Song and Park, 2012; Li et al., 2012; Lee, 2015; Lin et al., 2016; Pensaert and Martelli, 2016; Choudhury et al., 2016; Wang et al., 2016a; Sun et al., 2016).

The genome of PEDV is about 28 kb in length, consisting of seven open reading frames (ORFs). Accumulating genomes of PEDV isolates were documented to reveal their origins, genetic relationships and evolutions (Li et al., 2012; Lin et al., 2016; Kocherhans et al., 2001; Chen et al., 2012; Vui et al., 2014; Theuns et al., 2015; Cheun-Arom et al., 2016; Gao et al., 2016; Sun et al., 2015; Cheun-Arom et al., 2015; Park et al., 2014; Fan et al., 2017a, 2017b; Lee and Lee, 2017; Tao et al., 2016; Liu et al., 2017; Li et al., 2017). A number of new prevailing variants with large INDELs (insertions and deletions) in the genome of PEDV have evolved recently and escaped from the prevention of prototypes CV777, SM98 and DR13 vaccines, consequently causing pandemic PED outbreaks due to their high virulence (Fan et al., 2017a,b; Lee and Lee, 2017; Diep et al., 2017a; Suzuki et al., 2016). The spike glycoprotein gene (S gene) was considered as a primary target for the development of a vaccine against PED, with the encoded protein

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most antigenic. Analysis of highly polymorphic S gene could divide the PEDV isolates into two clusters (G1 and G2), to reveal the molecular characteristics, epidemiology and phylogeny of PEDV strains (Song and Park, 2012; Li et al., 2012; Lee, 2015; Lin et al., 2016; Pensaert and Martelli, 2016; Choudhury et al., 2016; Wang et al., 2016a, 2016b; Gao et al., 2016; Park et al., 2007, 2013; Chen et al., 2016; Zeng et al., 2017; Cima, 2013; Huang et al., 2013; Stevenson et al., 2013). In addition, the re-emerging and quick spread of PED was reported in the United States in April 2013 (Cima, 2013; Huang et al., 2013; Stevenson et al., 2013). So far, PED has deteriorated the whole pig industry around the world with substantial economic losses due to the death of millions of sulking pigs. To reveal the prevailing variants of PEDV in Yunnan, China, the genetic characteristics and their relationships with other strains, the epidemiology of PED in Yunnan were investigated and the corresponding isolates were phylogenetically analyzed using the sequences of S genes, in order to shed light on the control and prevention of PED by developing more specific and effective vaccines.

2. Material and methods

2.1. Sampling

Four hundred and thirty-five samples were collected from small intestine tissues, diarrhea feces or blood from suckling piglets in both small-holding and large-scale pig farms in Yunnan province of China during June 2012–September 2017. The piglets were characterized by vomiting, severe watery diarrhea, and dehydration, therefore suspected to be infected with PEDV.

The diarrhea feces were obtained with medical swabs and re-suspended in 1 ml 0.9% sodium chloride solution in 1.5 mL Eppendorf tubes. After centrifuge at 10,000 × *g* for 10 min, 200 μL supernatants were transferred into new tubes for −80 °C storage till RNA extraction.

2.2. RNA extraction, cloning and sequencing

Total RNAs were extracted from the collected samples from small intestine, diarrhea feces or blood, according to the instructions of RNA extraction kit (RNAiso Plus, Cat. No. AA6702-1, TaKaRa), followed by reverse transcription EasyScript RT/RI Enzyme Mix, Cat. No. K21105, TransGen Biotech) and PCR (2 x TransHiFi Super Mix, Cat. No. L10602, TransGen Biotech). All the 435 samples were subjected to reverse transcription polymerase chain reaction (RT-PCR) to identify whether the piglets were PEDV infected. A primer pair (forward primer 5′-TTT ATTCTGTCACGCCATGT-3′ and reverse primer 5′-CCAGATTTACARA-CACCTATG-3′) was designed according to the sequence under GenBank Accession No. AF353511.1 to amplify a partial region of S gene with a length of 199 bp. The PCR program consisted of an initial denaturation at 94 °C for 5 min, followed by 25 amplification cycles of denaturation at 94 °C for 1 min, annealing at 53 °C for 1 min, and extension at 72 °C for 90 s, with a final extension step at 72 °C for 7 min.

The complete S gene of PEDV were amplified using five pairs of universal primers (Table 1), which were also designed according to the reference sequences KR873435.1 and KR610994.1. The PCR products were subjected to 1.5% agarose gel electrophoresis for detecting the presence of PEDV gene fragment by visualization under ultraviolet light after staining with 1.0 μg/ml ethidium bromide (EB). The positive PCR products were electrophoresed on 3.0% agarose gel for the purification of amplified S gene according to the instruction (Bio Teke Corparaion, Beijing). The purified PCR products were ligated into pMD18-T vectors (Sangon Biological Engineering Co. Ltd., Shanghai). The plasmids were then transformed into *E. coli* DH5a cells (Takara Biotech Co. Ltd., Dalian) for 16–18 h culture, followed by plasmid DNA extraction and sequencing (Sangon Biological Engineering Co. Ltd).

Table 1
Five pairs of PCR primers for the amplification of complete S gene of PEDV.

Primer names	Nucleotides sequence (5' → 3')	Tm (°C)	Amplicon (bp)
PEDV_20566 PEDV_21610	F1: GGTAAGTTGCTAGTGCGTAA R1: TTCAGCAAGAATGACAGAGG	53	1064
PEDV_21547 PEDV_22510	F2: GTGTTTGTAATGGAGCTGCTGT R2: TAGGCGTGCCAGTAATCAACT	55	984
PEDV_22467 PEDV_23522	F3: GGTGTTAAGTTTACGTCCCT R3: CTGCAGAAGTAAAACCTCCT	53	1075
PEDV_23506 PEDV_24503	F4: TCGGTGGTATGGTGCTAG R4: GACTCTGAACGSTGCTKT	55	1000
PEDV_24408 PEDV_24976	F5: CTCTGCCCAATAGAACTG R5: CGCCTCAAAGAAGACGCT	53	588

Note: All the primer sequences were designed based on the sequence of GenBank KR873435.1 (KNU-141112-P10 strain, Thailand). The numbers included in the primer names indicate the positions of the primers in the PEDV genome.

2.3. Sequence processing and phylogenetic analysis

The resulting raw sequences were aligned using DNAStar 6.0 (DNAStar Inc., Madison, WI). The complete S gene (4143–4170 bp) were identified by comparison with PEDV genome as references from GenBank database, Accession No. AF353511.1 (CV777 vaccine) and KR873435.1 (KNU-141112-P10 isolate). The phylogenetic trees were constructed with a total of 225 sequences of S gene from GenBank (Table S1) using Neighbor-Joining method of MEGA software, Version 4 (Tamura et al., 2007). The phylogeny was tested using bootstrap methods with 1000 replications and the evolutionary distances were computed using the p-distance method. Polymorphic sites were determined using MEGA 4 and DnaSP software, Version 5.10.01 (Librado and Rozas, 2009).

3. Results

3.1. Epidemiology of PEDV in Yunnan between June 2012 and September 2017

Seventy-six positive samples were identified from 435 suspected samples during the period of June 2012 to September 2017, with an overall positive rate of 17.47% (Table 2). Nine PEDV isolates were identified from Lanping county (YnP1-2, March 2016; YnP7, March 2017), Luliang county (YnP3, April 2016), Honghe county (YnP4-6, December 2016), Mangshi city (YnP8, April 2017), and Chuxiong city (YnP9, April 2017) in Yunnan province, respectively. The viruses were subsequently propagated for cloning and sequencing.

In our study, the group of Chuxiong city containing a population of 226 sows and a nursery unit for 1225 piglets, started to develop PED symptoms on March 4, 2016, with all the ages of pigs affected within 10 days. The weaning piglets showed significantly reduced feed intake and diarrhea, while the suckling piglets showed diarrheic and lethargy. One to two days after the onset of the first diarrhea, the piglets began to lay

Table 2
The positive rates of PEDV during June 2012- September 2017 in Yunnan.

Year	Sample numbers	Sample types			Positive numbers	Positive rate (%)
		Feces	Intestine	Blood		
2012	1	0	1	0	0	0
2013	162	153	9	0	12	7.41
2014	108	105	3	0	10	9.26
2015	41	29	3	9	7	17.07
2016	17	6	7	4	5	29.41
2017	106	47	36	23	42	39.62
Total	435	340	59	36	76	17.47