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RESEARCH ARTICLE

Genetic Characterization of Porcine Epidemic Diarrhea Virus in China Between 2014 and 2018: Emergence of the G1c Subtype

Wenqiang Jiao[1, 2, 3, 4§], Guangxu Xing[2§], Yunchao Liu[2], Yindi Xu[1, 4], Zhifng Wang[1, 4], Dongliang Li[2, 3], Keling Wang[1, 4] and Gaiping Zhang[2, 3*]

[1]Institute of Animal Husbadnry and Veterinary Research, Henan Academy of Agricultural Sciences, Zhegnzhou 450002, China; [2]Henan Provincial Key Laboratory of Animal Immunology, Henan Academy of Agricultural Science, Zhenghzou 450002, China; [3]College of Animal Science and Veterinary Medicine, Henan Agricultural University, Zhengzhou 450002, China; [4]Henan Key Laboratory of Farm Animal Breeding and Nutritional Regulation, Zhengzhou 450002, China
[§]These authors contributed equally to this work.
*Corresponding author: zhanggaiping2003@163.com

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ABSTRACT

Porcine epidemic diarrhea virus (PEDV) has caused substantial economical loss to the Chinese swine industry. To illustrate the genetic characterization of PEDV circulating in China, 205 clinical samples between 2014 and 2018 were collected from 7 provinces in China. 93.17% (191 of 205) of the intestinal and fecal samples were positive for PEDV. 25 S1 amino acid (aa) together with 27 ORF3 genes from 8 provinces were sequenced and analyzed. The phylogenetic trees based on the S1 and ORF3 genes were constructed by the neighbor-joining method using MEGA 7 software. PEDV prevalence was 86.96% (40 of 46) of the swine farms in the 8 provinces and the PEDV positive rate was 93.17% (191 of 205) in the tested samples. Genetic analysis showed CH-JIANGXI-1-2016 CH-JIAGNXI-2-2016, CH-JIANGXI-3-2016 and CH-JIANGXI-2017 had three notable insertions or deletions occurred at aa 59-62, 160, and 139 (140) when compared to all of the strains in this study; moreover, phylogenetic analysis indicated that the four isolates formed a new branch significantly different from G1a, G1b and Indel subtype based on S1 gene: that is the G1c subtype. More research is needed to determine whether the insertions and deletions had biological influence on the virus. The results acquired in the present study showed the genetic diversity of PEDV circulating in 8 provinces, providing information for the development of new diagnostic methods and new vaccines.

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INTRODUCTION

Porcine epidemic diarrhea (PED), is an acute, highly contagious viral enteric disease caused by porcine epidemic diarrhea virus (PEDV) characterized by watery diarrhea and severe dehydration and is associated with a high death rate in suckling pigs (Madson *et al*., 2014; Lee and Lee., 2014; Wang *et al*., 2016). In 1971, the first reports were in the United Kingdom and Belgium, and subsequently in other European countries, Asia and more recently in America (Puranaveja *et al*., 2009; Wang *et al*., 2014). In China, the first PEDV isolate was confirmed in 1971. Since then and especially since 2010, when a severe PED epidemic occurred on the mainland investigations into the genetic nature of the PEDV circulating in China were initiated.

PEDV is an enveloped, single stranded RNA virus with a 28Kb genome with a 5' cap and a 3' polyadenylated tail (Tian *et al*., 2013), and belongs to the *Coronavirus* genus, *Coronaviridae* family. The genome of PEDV consists of a 5'untranslated region (UTR), a 3'UTR and at least seven open reading frames (ORFs) encoding spike (S), envelope (E), membrane (M), and nucleotide (N), and replicase 1a, 1b together with ORF3. The S glycoprotein is located at the surface of the virus and is deeply involved in the viral entry into cells by membrane fusion (Hou *et al.*, 2017*;* Lin *et al.*, 2017; Gillam *et al.*, 2018) The S protein contains at least four

neutralizing epitopes (aa 499－638, 748－755, 764－771 and 1368－1374) (Chang *et al*., 2002; Cruz *et al*., 2008; Sun *et al*., 2008), indicating that the S protein can stimulate the production of neutralizing antibodies against PEDV and suggesting it is an ideal candidate for the development of vaccine against PEDV. The gene ORF3 also contributes to the virulence of PEDV and it has been reported that the virulence is diminished by changes in the sequence of ORF3 (Park *et al*., 2007; Challika *et al.*, 2018; Zhang *et al.,* 2018; Challika *et al.*, 2019). Furthermore, deletions of amino acids between residues 82-99 makes ORF3 an ideal candidate gene for differentiating vaccine strains and field strains. Thus, together the S glycoprotein and ORF3 gene can be used to investigate the genetic characterization of PEDV.

The purpose of this study is to better examine the genetic diversity and molecular characteristics of PEDV isolates which have been circulating in China. We sequenced 25 S1 glycoprotein genes and 27 ORF3 genes of isolates from the provinces Henan, Hebei, Shanxi, Hubei, Shandong, Gansu, Jiangxi, Shanghai over the period 2014-2018, providing information for the development of PEDV control programs and a new type of vaccine.

MATERIALS AND METHODS

Intestinal and fecal samples were collected from piglets suffering from severe diarrhea between November 2014 and January 2018 in Henan, Hebei, Shandong, Shanxi, Gansu, Hubei, Jiangxi provinces and Shanghai China. The samples were diluted (1:5) in phosphate-buffered saline (PBS, pH 7.4), and frozen at -80°C until tested. After three times of freezing and thawing, the intestinal and fecal samples were homogenized and clarified by centrifugation at 13000g for 5min.

Total RNA was extracted from the supernatants using RNase Mini Plus Total RNA Extraction Kit (QIAGEN, Germany) according to the manufacturer's instructions, and the RNA immediately used for synthesizing cDNA. Then, PCR was performed using Primer star GXL (TAKARA, Japan) with cDNA as template, the primers were shown in Table 1. PCR products with the expected size were purified using a MiniBEST Agarose Gel DNA Extraction Kit (TAKARA, Japan), ligated to pMD 19-T vector (TAKARA, Japan), and then used to transform competent Escherichia coli JM109 cells (TAKARA, Japan). The positive samples were verified using PCR and commercial sequencing (Shanghai Sangon Biological Engineering Technology & Services).

The obtained sequences were further edited and assembled manually using EditSeq program (DNASTAR version 7.0). Phylogenetic analysis based on the S1 and ORF3 sequences was conducted by Neighbor-joining (NJ) algorithm with 1000 bootstrap replicates using the Molecular Evolutionary Genetics Analysis 7 (MEGA 7) program.

RESULTS

PEDV detection: PEDV was confirmed by reverse transcription polymerase chain reaction (RT-PCR) in 86.96% (40 of 46) of the swine farms in the 8 provinces, 93.17% (191 of 205) in the tested samples. All the sequences have been submitted to GenBank, the accession number was as follows: MH891846, MH909821, MH909797, MH909798, MH909799, MH909800, MH909801, MH909802, MH909803, MH909804, MH909805, MH909806, MH909807, MH909808, MH909809, MH909810, MH909811, MH909812, MH909813, MH909814, MH909815, MH909816, MH909817, MH909818, MH909819, MH909820, MH909821, MH922974, MH922975, MH922976, MH922977, MH922978, MH922979, MH922980, MH922981, MH922982, MH973704, MH973705, MH973706, MH973707, MH973708, MH973709, MH973710, MH973711, MH973712, MH973713, MH973714, MH973715, MH973716, MH973717, MH932662.

Phylogenetic analysis of the S1 gene: As shown in Fig. 2, all the isolates in this study were subtype G2 except CH-JIANGXI-1-2016, CH-JIANGXI-2-2016, CH-JIANGXI-3-2016, CH-JIANGXI-2017 and CH-HENPY-2017. In the G2 group, CH-HENNY-2015, CH-HENZMDBY-2016, CH-HENGY-2016 and CH-HENPY-2017 CH-HENYC-2015 were in the sub-group G2a; the remaining 16 isolates belonged to G2b group. It was interesting to find that CH-JIANGXI-1-2016, CH-JIANGXI-2-2016, CH-JIANGXI-3-2016, CH-JIANGXI-2017 and CH-HENPY-2017 clustered most closely to the G1 group, moreover, the four isolates formed a new branch significantly different from G1a, G1b and Indel subtype: that is the G1c subtype. The strains in this study were marked by blue triangle. The names of the strains, years, places of isolation, GenBank accession numbers were shown in Table 2.

The sequences obtained in this study shared 91.7-94.5% and 91.4-94.4% identity with CV777 and CV777 attenuated strains respectively at the nucleotide level and 88.9-93.6% and 89.7-94.0% identity at the amino acid level.

Table 1: Primers used in this study

No.	Sense	Sequence, 5'-3'	Size	Target gene
1	Forward	ATGAAGTCTTTAACTTACTTCT	2254	S1
2	Reverse	TATACACCAACACAGGCTCTG		
3	Forward	ATGTTTCTTGGACTTTTTCAAT	833	ORF3
4	Reverse	TCATTCACTAATTGTAGCAT		

Table 2: PEDV strains used for sequence alignment and phylogenetic analysis

Reference strain	Accession no.	Origin
CV777	AF353511	Belgium, 1978
CV777 attenuated	KT323979	China, 1998
CH/S	JN547228	China, 2011
DR13	JQ023162	South Korea, 2012
LZC	EF185992	China, 2007
SM98	GU937797	South Korea, 2011
AH2012	KC210145	China, 2012
CH/HNKF-3	KU977490	China, 2016
CH/HNNY-2	KU977493	China, 2016
CH/JX-1/2013	KF760557	China, 2013
IA1	KF468753	USA, 2013
USA/Colorado/2013	KF272920	USA, 2013
MN	KF468752	USA, 2013
CH/ZMDZY/11	KC196276	China, 2013
AJ1102	JX188454	China, 2012
AH2012	KC210145	China, 2013
914_2015_AUT	KT895908	Austria, 2016
1842/2016 ITA	KY111278	Italy, 2017
CH/HBQX/10	JX501318	China, 2013

Fig. 1: The amino acid sequences of the S1 protein, aa59-aa163, found in this study, with the reference CV777 and CV777 attenuated strain. All the strains in this study had two notable insertions at aa 59-62 and 160, respectively, except CH-JIANGXI-1-2016 CH-JIAGNXI-2-2016, CH-JIANGXI-3-2016 and CH-JIANGXI-2017. Two deletion at aa 139 in CH-JIANGXI-1-2016 CH-JIAGNXI-2-2016, CH-JIANGXI-3-2016 CH-JIANGXI-2017, was observed compared to CV777 and CV777 attenuated strain, and all of the strains except CH-JIANGXI-1-2016 CH-JIAGNXI-2-2016, CH-JIANGXI-3-2016 CH-JIANGXI-2017 had deletions at aa 159 and 160 compared to CV777.

Table 3: Analysis of amino acid mutations in epitopes domains of field strains and the CV777 attenuated vaccine strain (aa 20－30, aa 32－40, aa 64－75, aa 245－252, aa 499－638, aa 747－754)

Strains	24	27	28	29	64	68	69	70	71	72	248	502	522	523	526	528	530	536	541	543	547	554	568	581	588	610	613	617	624
CV777		Q	S	T	S	G	T	G	L	E	S	I	A		L	S					T		A		L	S			
CV777 attenuated		Q	S	T	S	G	T	G	L	E	S	I	A									T							
CH-HENGY-2016												T								L			A						
CH-HENPY-2015												I						I											
CH-HENHB-2016											L																		
CH-HNZMDBY-2016																													
CH-SHANXJC-2017											L																		
CH-SHANXCZ-2015											L																		
CH-HENPY-2017													A													L			
CH-HENYS-2017											L																		
CH-HENDF-2017						Y		E																					
CH-HENYC-2015				K								S									N		N	G	G	L	S		
CH-HENJY-2014											L																		
CH-HENLS-2014													A		R		D			F				D					
CH-HENXX-2015											L																		
CH-SHANGH-2017	A													L									N						
CH-JIANGXI-1-2016		Q	L	T	S	G	T	G	L	E		I																	
CH-JIANGXI-3-2016		Q	L	T	S	G	T	G	L	E		I																	
CH-JIANGXI-2017		Q	L	T	S	G	T	G	L	E		I																	
CH-JIANGXI-2-2016		Q	L	T	S	G	T	G	L	E		I																	
CH-HUB-1-2017																													
CH-HUB-2-2017			G									I			Y							L						
CH-HENNH-2015				K								S									N		N		G		G	L	S
CH-HEBHD-2015				K								S									N		N		G		G	L	
CH-HENZK-2017													A														L		
CH-HENKF-2015											L																		
CH-HENNY-2015													F						L				N						

Compared to CV777, all of the strains in this study had two notable insertions at aa 59-62 and 160 (as shown in Fig. 1), except for CH-JIANGXI-1-2016 CH-JIAGNXI-2-2016, CH-JIANGXI-3-2016 and CH-JIANGXI-2017. Moreover two deletions at aa 139 and 140 in CH-JIANGXI-1-2016 CH-JIAGNXI-2-2016, CH-JIANGXI-3-2016 CH-JIANGXI-2017, was observed compared to CV777 and the CV777 attenuated strain, whereas all of the other strains, except CH-JIANGXI-1-2016 CH-JIAGNXI-2-2016, CH-JIANGXI-3-2016 CH-JIANGXI-2017, had deletions at aa 159 and 160 compared to CV777.

Antigenic sites located at aa 20-30, 32-40, 64-75, 245-252, 499-638 and 747-754 within S1 were analyzed using Meg Align, DNA STAR. The antigenic sites at aa 32-40, 245-252, 747-754 were conserved between CV777 and CV777 attenuated strain; in contrast, antigenic sites at 20-30, 64-75, 499-638 were highly variable.

Phylogenetic analysis of the ORF3 gene: All 27 ORF3 sequences in this study were 675 nucleotides in length without insertions or deletions. The sequences obtained in the present study exhibited 95.7-96.9% nucleotide identity as well as 94.7-97.8% amino acid identity when compared with the CV777 strain, and 88.0-90.6% nucleotide identity and 84.8-88.0% aa identity with the CV777 attenuated strain. The phylogenetic analysis showed that CH-HENPY-2015 and CH-SHANXPD-2017 strains, together with CV777 were G2a subtype, whereas the remaining 25 strains were G2b subtype. The 25 G2b subtype strains clustered closely to MN and IA1 strains (Fig. 3). The

strains from this study are marked by blue triangles. The names of the strains, years, places of isolation, GenBank accession numbers are shown in Table 2.

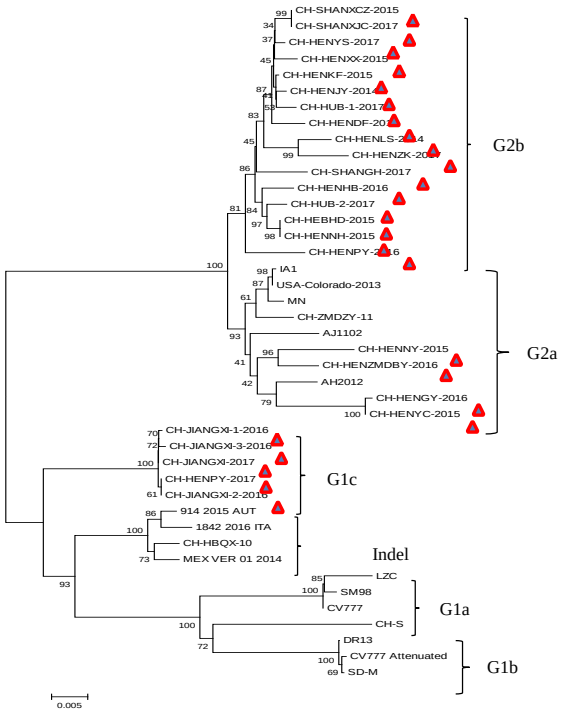

Fig. 2: Phylogenetic analysis of S1 nucleotide sequences of 25 PEDV isolates, including the reference. The tree was constructed by the neighbor-joining method in MEGA 5 software.

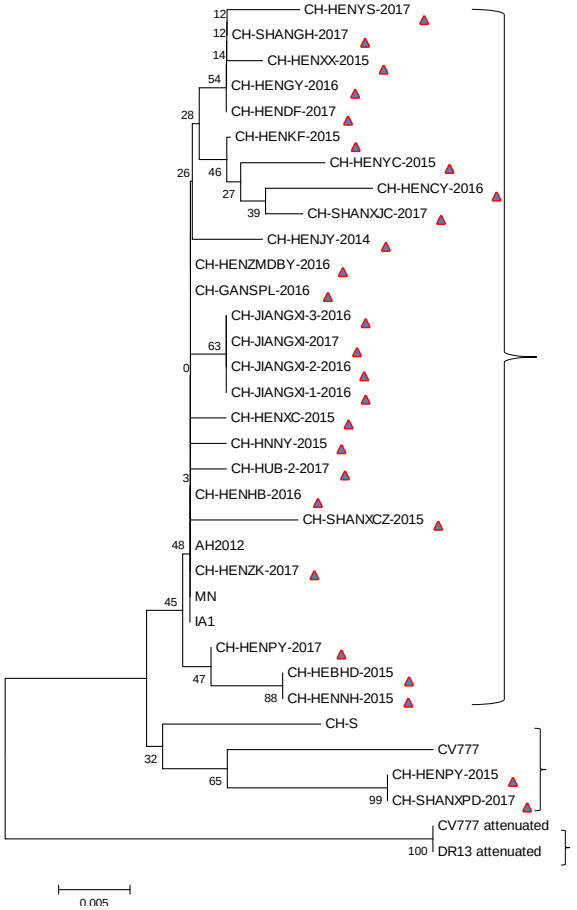

Fig. 3: Phylogenetic analysis of ORF3 nucleotide sequences of 27 PEDV isolates, including the reference.

DISCUSSION

Since 2010, porcine epidemic diarrhea virus has been associated with considerable financial loss in Chinese pig farming. Although strict biosecurity and compulsory immunization with vaccines based on the CV777 attenuated strain of PEDV were employed, some pig farms were still affected by PEDV. Although the preventative measures have worked to some extent they cannot prevent the spread of PEDV completely (Temeeyasen *et al*., 2014). Outbreaks of PED have made further investigation of the diversity of PEDV an urgent requirement since mutations at antigenic sites may be accounting for the failure of PEDV vaccination. Therefore, a more complete knowledge of the extent of the genetic diversity of PEDV may provide useful information for the development of a better control and prevention programs.

The PEDV positive identification rate in the present study was 93.17% (191 of 205) of the suspicious samples, and included 86.96% (40 of 46) of the pig farms involved from 8 provinces in China, compared to those of 92.25% and 94.03%, respectively, implicating PEDV as the predominant pathogen causing diarrhea in piglets in China (Su *et al.*, 2016).

The S protein is a type I transmembrane glycoprotein exposed on the surface of the virion and is responsible for the attachment of the virus to the cell surface and its entry into the cell, further it induces the production of a neutralizing antibody (Lee and Lee, 2014; Oh *et al*., 2014; Wang *et al.*, 2018). It has been reported that phylogenetic analysis based on the N terminal of PEDV S1 gave similar results to that based on the full length gene (Lee *et al*., 2010; Sun *et al*., 2015). Considering that the full length S gene was hard to acquire, in this study we partially cloned the S gene to genetically characterize the PEDVs circulating in 8 provinces in China. Comparison of the antigenic sites showed that aa 27-30, aa 64-75, and aa 499-638 were highly variable when compared to CV777 and CV777 attenuated strain (the substitutions are shown in Table 3). It remains to be determined as to whether the changes in the antigenic sites could affect the virulence of PEDV or not. Additional single amino acid substitutions at the antigenic sites were found in the present study, possibly indicating that the gene has been varying under immune pressure. Genetic analysis based on the S1 gene showed that CH-JIANGXI-1-2016, CH-JIANGXI-2-2016, CH-JIANGXI-3-2016, CH-JIANGXI-2017 and CH-HENPY-2017 were of the G1c subtype, CH-HENNY-2015, CH-HENZMDBY-2016, CH-HENGY-2016 and CH-HENYC-2015 were G2a subtype and the remaining 16 isolates were G2b subtype. Genetic analysis based on the ORF3 gene in this study showed CH-HENPY-2017 and CH-SHANXPD-2017 were G2a subtype, the rest belonged to G2b subtype. Taken together this study of PEDV circulating in China indicates that the virus has become increasingly complex, and its diversification from the vaccine strain could explain why the vaccine provides only partial protection.

Consistent with CV777 and CV777 attenuated strain, CH-JIANGXI-1-2016 CH-JIAGNXI-2-2016, CH-JIANGXI-3-2016 and CH-JIANGXI-2017 also had deletion at aa 59-62, 140 and 159-160 when compared to G2 subtype strains; however, CH-JIANGXI-1-2016 CH-

JIAGNXI-2-2016, CH-JIANGXI-3-2016 and CH-JIANGXI-2017 had a unique deletion at aa 139. It was interesting to note that the in-dels did not occur within the antigenic sites. Further research is needed to determine whether the changes are related to the pathogenicity or the biological functions of PEDV, or if the aa residues themselves were antigenic sites.

The ion channel protein encoded by the ORF3 gene is deeply involved in the virulence of PEDV (Song and park, 2012; Wang *et al*., 2012). Recent studies showed that porcine epidemic diarrhea virus ORF3 protein causes endoplasmic reticulum stress to facilitate autophagy (Zou *et al.*, 2019). Moreover, field and attenuated vaccine strains can be differentiated by comparing size of the RT-PCR products since vaccine strain-derived isolates had unique continuous deletions of 49 and 51 ORF3 nucleotides (Chen *et al*., 2010). Compared with CV777 and CV777 attenuated strains, the ORF3 sequences acquired in this study did not show the deletions of the CV777 vaccine strain. Interestingly, phylogenetic analysis based on the S1 gene showed that CH-JIANGXI-1-2016 CH-JIAGNXI-2-2016, CH-JIANGXI-3-2016 and CH-JIANGXI-2017 were G1c subtype, while phylogenetic analysis based on the ORF3 gene showed all the four strains to be of the G2b subtype. Recombination of the CV777 attenuated vaccine strain, and other vaccine strains, with field strains may explain how it came about that phylogenetic analysis based on different genes showed different results. It remains to be determined whether the putative recombination affects the biological functions or pathogenicity of the virus.

List of abbreviations: PEDV, porcine epidemic diarrhea virus; RT-PCR, reverse transcription polymerase chain reaction; ORF, open reading frame; MEGA, molecular evolutionary and genetics analysis.

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Authors contribution: WJ and GX collected the PEDV suspicious samples and drafted the manuscript; YL, YX, ZW and DL cloned the S1 and ORF3 genes; KW analyzed the S1 and ORF3 sequences; GZ conceived the project.

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