RESEARCH ARTICLE

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

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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

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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, Hebei, 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 Technology & Services).

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: 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-HENZMBBY-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.

| Accession no. | Origin        | Group |
|---------------|---------------|-------|
| MH909797      | CH-CHINA      |       |
| MH909798      | CH-CHINA      |       |
| MH909799      | CH-CHINA      |       |
| MH909800      | CH-CHINA      |       |
| MH909801      | CH-CHINA      |       |
| MH909802      | CH-CHINA      |       |
| MH909803      | CH-CHINA      |       |
| MH909804      | CH-CHINA      |       |
| MH909805      | CH-CHINA      |       |
| MH909806      | CH-CHINA      |       |
| MH909807      | CH-CHINA      |       |
| MH909808      | CH-CHINA      |       |
| MH909809      | CH-CHINA      |       |
| MH909810      | CH-CHINA      |       |
| MH909811      | CH-CHINA      |       |
| MH909812      | CH-CHINA      |       |
| MH909813      | CH-CHINA      |       |
| MH909814      | CH-CHINA      |       |
| MH909815      | CH-CHINA      |       |
| MH909816      | CH-CHINA      |       |
| MH909817      | CH-CHINA      |       |
| MH909818      | CH-CHINA      |       |
| MH909819      | CH-CHINA      |       |
| MH909820      | CH-CHINA      |       |
| MH909821      | CH-CHINA      |       |
| MH922974      | CH-CHINA      |       |
| MH922975      | CH-CHINA      |       |
| MH922976      | CH-CHINA      |       |
| MH922977      | CH-CHINA      |       |
| MH922978      | CH-CHINA      |       |
| MH922980      | CH-CHINA      |       |
| MH922981      | CH-CHINA      |       |
| MH973704      | CH-CHINA      |       |
| MH973705      | CH-CHINA      |       |
| MH973706      | CH-CHINA      |       |
| MH973707      | CH-CHINA      |       |
| MH973708      | CH-CHINA      |       |
| MH973709      | CH-CHINA      |       |
| MH973710      | CH-CHINA      |       |
| MH973711      | CH-CHINA      |       |
| MH973712      | CH-CHINA      |       |
| MH973713      | CH-CHINA      |       |
| MH973714      | CH-CHINA      |       |
| MH973715      | CH-CHINA      |       |
| MH973716      | CH-CHINA      |       |
| MH973717      | CH-CHINA      |       |
| MH932662      | CH-CHINA      |       |
Table 3: Analysis of amino acid mutations in epitopes domains of field strains and the CV777 attenuated vaccine strain (aa 20 – 30, 32 – 40, aa 64 – 75, aa 245 – 252, aa 499 – 638, aa 747 – 754)

| Strains          | 24  | 27  | 28  | 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   | S    | A   | L   | S   | T   | A   | L   | S   | A   | T   | L   | A   | L   | A   | L   | A   | L   | A   | L   | A   |
| CV777 attenuated|     |     |     |     |     |     |     |     |      |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| CH-HENGY-2016   | I   | I   | I   | I   | I   | I   | I   | I   | I    | I   | I   | I   | I   | I   | I   | I   | I   | I   | I   | I   | I   | I   | I   | I   | I   | I   | I   | I   |
| CH-HENPY-2015   | L   | L   | L   | L   | L   | L   | L   | L   | L    | L   | L   | L   | L   | L   | L   | L   | L   | L   | L   | L   | L   | L   | L   | L   | L   | L   | L   | L   |
| CH-HENHIB-2016  | K   | K   | K   | K   | K   | K   | K   | K   | K    | K   | K   | K   | K   | K   | K   | K   | K   | K   | K   | K   | K   | K   | K   | K   | K   | K   | K   | K   |
| CH-HENNY-2015   | Y   | H   | H   | H   | H   | H   | H   | H   | H    | H   | H   | H   | H   | H   | H   | H   | H   | H   | H   | H   | H   | H   | H   | H   | H   | H   | H   | H   |
| CH-HENLS-2014   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| CH-HENXX-2015   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| CH-SHANGE-2017  |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| CH-SHANGX-2017  |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| CH-HUB-2017     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| CH-HUB-2017     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| CH-SHAINX-2015  |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| CH-HENNY-2015   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| CH-HENNY-2015   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |

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-JIANGXI-2-2016, CH-JIANGXI-3-2016 and CH-JIANGXI-2017. Moreover two deletions at aa 139 and 140 in CH-JIANGXI-1-2016 CH-JIANGXI-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-JIANGXI-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 MegAlign, 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).
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.

**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-4-2016 were of the G1c subtype, CH-HENNY-2015, CH-HENMZMBY-2016, CH-HENGY-2016 and CH-HENCY-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-SHANXP-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-JIANGXI-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-
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