Molecular Epidemiological Study of Porcine Epidemic Diarrhea from Winter 2020 to Spring 2021 in Jiangsu Province

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Abstract

Background: Porcine epidemic diarrhea (PED) is an acute and highly contagious infectious disease caused by the porcine epidemic diarrhea virus (PEDV) that occurs most frequently from winter to spring. It is associated with high morbidity and mortality rates, especially among piglets, and causes huge losses in the pig industry. The aim of this molecular epidemiological study was to identify the current strains of PEDV that are prevalent in Jiangsu Province, China.

Methods: From winter 2020 to spring 2021, 793 small intestine tissue, fecal, and anal swab samples were collected from 72 pig farms in 11 counties in the jurisdiction of 5 regions of Jiangsu Province (Yancheng, Suqian, Changzhou, Xuzhou, and Yangzhou). A highly variable region of the S gene was amplified and sequenced, and phylogenetic analysis was conducted to compare this sequence with corresponding sequences from reference strains deposited in GenBank.

Results: A total of 457 samples from 57 pig farms were positive for PEDV: this implies a positivity rate of 79% (57/72) for pig farms and a sample positivity rate of 57.6% (457/793). The positivity rates were 78% (107/137) in Yancheng, 53% (218/409) in Suqian, 48% (94/195) in Changzhou, 80% (16/20) in Xuzhou, and 88% (14/16) in Yangzhou. Seven representative samples were selected for sequencing, and phylogenetic analysis showed that the seven isolated strains exhibited 88.0%–100% nucleotide identity and 87.3%–99% amino acid identity. Additionally, our isolates exhibited 88.3%–99.7% nucleotide identity and 88%–98.5% amino acid identity with the reference PEDV strains. Phylogenetic tree analysis indicated that there were considerable difference in the sources of the variants.

Conclusions: PEDV had a high infection rate among pigs and is possibly the main pathogenic agent of pig diarrhea in Jiangsu province. Importantly, vaccines must be screened for their efficacy against the newly identified variants.

Background

Porcine epidemic diarrhea (PED) was first observed in the 1970s in Europe[1, 2], from where it spread to other parts of the globe and, currently, causes huge economic losses in the pig industry. Before 2010, there was no large-scale outbreak of PED in China, and it was mostly sporadic or in the form of local epidemics[3, 4]. However, since the end of 2010, diarrhea outbreaks among neonatal piglets characterized by severe vomiting, watery diarrhea, and high mortality were found to be mainly caused by porcine epidemic diarrhea virus (PEDV) mutant strains in pig farms in China; the outbreaks successively spread from the South to the North and from the East to the West[5, 6]. In particular, variant strains of this virus in China are characterized by sudden onset, rapid spread, a wide epidemic range, and a long epidemic time[7]. Transmission occurs via the fecal-oral route and nasal inhalation. Once inside piglets, the virus attacks the intestinal cells, which lose the ability for digestion and absorption of nutrients from breast milk and food[8]. Acute diarrhea, vomiting, dehydration, and death tend to occur in piglets, most of which are infected within 7 days after birth, and the fatality rate in young piglets is as high as 100%[9, 10].

Antibiotics have limited efficacy in the control of this disease, and the morbidity and mortality are currently very high[11] .Additionally, the genetic variations in PEDV strains in recent years have led to changes in the antigen epitopes of the virus, and the currently available inactivated and attenuated vaccines against PEDV do not offer adequate immunoprotection [12]. It is important to understand the genetic and epidemiological basis of this infection in order to develop strategies to curb and prevent its spread.

PEDV is a non-segmented single-stranded plus-stranded cystic RNA virus that belongs to Nidovirales, Coronaviridae, α-coronavirus[13]. As observed in other known coronaviruses, the genome is approximately 28 kb in size and has a cap structure at the 5' end and a poly A tail at the 3' end[14]. The full-length genome of PEDV contains a 5' uncoded region, a 3' uncoded region, and seven open reading frames. The seven open reading frames encode three non-structural proteins (PP1A, PP1B, and ORF3) and four structural proteins (S, E, M, and N)[15, 16]. Among viral proteins, glycoprotein S is considered to be the most antigenic protein; it contains 1383 amino acids and has a molecular weight of about 150 kDa[14, 17, 18]. Previous studies have shown that glycoprotein S plays an important role in promoting the fusion of the virus and cell membranes, virulence, induction of neutralizing antibodies, and adhesion of virus particles to receptors on host cells[19, 20]. This protein is commonly used to identify the genetic relationships between different PEDV strains and the epidemiological status of pig farms [21, 22]. With regard to PEDV strains in China, researchers such as Su, Wen *et al*. have carried out a series of studies on the pathogen. The results showed that the PEDV strains currently prevalent in China are new genotypes and, due to genetic variation, the prevention and control from some existing vaccines are not ideal. It is necessary to select vaccines with high antigen content that match with the circulating strains [23, 24]. Therefore, it is necessary to obtain comprehensive and accurate data on the prevalence and molecular characteristics of the currently circulating PEDV strains.

The present study aimed to investigate the molecular epidemiological and phylogenetic features of recent PEDV strains in China. To this end, seven strains of PEDV were isolated from 793 fecal and intestinal samples of piglets with diarrhea were collected from 72 pig farms in five regions of Jiangsu Province, China, from 2020 to 2021. The nucleotide sequences of the S1 gene of the seven isolated strains were analyzed and compared with PEDV reference strains from China, Japan, the United Kingdom, South Korea, Switzerland, and Spain. This study provides the latest information on the prevalence of PEDV in Jiangsu Province from 2020 to 2021. This information will help in the development of new strategies for PEDV prevention and control in China.

Methods

2.1 Collection and processing of clinical samples

From the winter of 2020 to the spring of 2021, a total of 793 clinical samples (intestinal tissue, feces, and anal swabs) were collected from piglets with diarrhea from 72 pig farms located in five regions (Yancheng, Suqian, Changzhou, Xuzhou, and Yangzhou) of Jiangsu Province with diarrhea

outbreak(Figure 1). These clinical samples were resuspended with sterile phosphate-buffered saline at a 1:4 dilution and centrifuged at 12000 rpm at 4°C for 10 min. The supernatant was transferred and stored at -80°C for subsequent RNA extraction.

2.2 RNA extraction and RT-PCR analysis

Viral RNA was extracted from the samples using Trizol reagent (Takara, China). Nucleic acid was eluted with 20 μL of RNase-free water according to the manufacturer's instructions (Takara, China). Reverse transcription was performed according to the manufacturer's instructions (Takara, China). The full genome sequence of PEDV was downloaded from the GenBank database (GenBank accession no. LT906620.1), and the MegAlign program of the DNAStar software (version 8.4; DNAStar Inc., Madison, WI, USA) was used for comparison. Primer Premier 5.0 (Palo Alto, CA, USA) was used to design primers against the PEDV COE gene, which is highly conserved:

forward primer (PEDV-COE F), 5'–CGGGATCCTTCTAGAAACCTTCTGAGTC–3';

reverse primer (PEDV-COE R), 5'–CGGAATTCATACTTGGTACACACAT)–3'.

The PCR reaction mixture consisted of cDNA (1 μL), 2× Green Mix (Vazyme, China) (12.5 μL), RNase-free water (9.5 μL), and the primers PEDV-COE F (10 μM, 1 μL) and PEDV-COE R (10 μM, 1 μL). The total volume was 25 μL. The amplification protocol was as follows: 94°C for 5 min; followed by 30 cycles of 94°C for 30 s, 61°C for 30 s, and 72°C for 32 s; and a final extension step at 72°C for 10 min. The expected size of the PCR product was 509 bp(Figure 1), and the product was detected in 1% agarose gel electrophoresis.

2.3 Partial sequence analysis of the S1 gene

In this study, 7 PEDV strains that were identified from the isolates obtained from the pig farms in Jiangsu Province were selected, and part of their S1 gene was sequenced. The following primers against PEDV S1 were designed for amplification of some cDNA fragments in the highly variable region of the gene: PEDV-S1 (forward), 5'–GGTAAGTTGCTAGTGCGTAA–3'; PEDV-S1 (reverse), 5'–GCAGTATGAAGTACAATTGAGCC–3'. The amplification products were detected by 1% agarose gel electrophoresis. The size of the target fragment was 1075 bp. PCR products matching the expected size were purified using a QIAquick Gel Extraction Kit (QIAGEN) according to the manufacturer's instructions and cloned into the pGEM-T Easy vector (Takara, China) overnight at 16℃. The plasmid was transformed into *Escherichia coli* DH-5α cells (Takara, China) according to the manufacturer's instructions. Positive clones were identified by β-galactoside blue and white spot screening and isolated. Positive plasmids were sequenced by Nanjing Tsingke.

2.4 Sequence alignment and phylogenetic analysis

The MegAlign program of DNAStar was used to perform sequence alignment and phylogenetic analysis of the partial S1 gene sequences from the 7 isolated strains and other PEDV reference strains in GenBank. The sources, dates, and accession numbers of the reference strains are provided in Table 1. In

the molecular evolutionary genetic analysis software Mega (version 8.4), a phylogenetic tree of the partial sequences of the S1 gene was constructed with 1000 Bootstrap replicas by using the neighborhood linkage method and P-distance model.

Table 1
Reference strains used in this study

Isolate (reference)	Accession number	Origin	Isolate (reference)	Accession number	Origin
Br1	LT906582.1	Germany,2018	PEDV-H3-Barcelona-Vic	MN692792.1	Spain,2019
KH	AB548622	Japan,2011	Spk1	AF500215	South Korea,2005
MK	AB548624	Japan,2011	DR13-attenuated	DQ462404	South Korea,2006
DR13	DQ862099	South Korea,2006	CV777	AF353511	Switzerland,2001
KNU-0801	GU180142	South Korea,2008	LZC	EF185992	China,2006
CHGD-01	JN980698	China,2011	ZL29	KU847996	China,2016
JS-A	MH748550.1	China,2017	YN144	KT021232	China,2015
JSCF-2018	MT294131.1	China,2018	FL2013	KP765609	China,2013
GJL	KF294256	China,2012	CH-HNQX-3-14	KR095279	China,2016
Attenuated CV777	KT323979	China,2015	AJ1102	JX188454	China,2012
GDsg12	MW478771.1	China,2018	SX-YC-2019	MT090140.1	China,2019
GDjx16	MN368689.1	China,2018	GDsg18-2	MN368722.1	China,2018

Results

3.1 Prevalence of PEDV in the clinical specimens of pigs with diarrhea

The data showed that 57.6% of the 793 specimens collected were positive for PEDV. With regard to regional differences, Yangzhou had the highest positivity rate at 88%, while Changzhou had the lowest

positivity rate at 48%. Further, all the pig farms (that is, 100%) tested positive in Xuzhou, Yancheng, and Yangzhou, and the majority of the farms tested positive in Suqian (84%) and Changzhou (66%) (Table 2).

Table 2

Number of samples and PEDV positivity rates in different regions in Jiangsu Province, China

Samples Source	Positive Rate	Positive rate of pig farms
1:Xuzhou	80%(16/20)	100% (1/1)
2:Suqian	53%(218/409)	84% (16/19)
3:Yancheng	78%(107/137)	100% (16/16)
4:Yangzhou	88%(14/16)	100%(1/1)
5:Changzhou	48%(94/195)	66%(23/35)

3.2 Homology of nucleotide and amino acid sequences of the PEDV S1 gene

The designed primers were used to amplify part of the S1 gene of the 7 isolated strains. According to the sequences of the S1 genes determined by the MEGA7 software, the 7 isolated strains exhibited 88.0–100% nucleotide identity and 87.3–99% amino acid identity. Further, the 7 isolated strains exhibited 88.3–99.7% nucleotide identity and 88–98.5% amino acid identity with the representative PEDV strains deposited in GenBank.

3.3 Phylogenetic analysis of PEDV strains in Jiangsu Province

In order to analyze the genetic evolutionary relationship between the endemic strains in Jiangsu and the reference strains, a genetic evolutionary tree was established based on the nucleotide sequences of the S1 gene of the 7 PEDV strains identified in this study and the S1 gene of the reference strains from GenBank (Figure 3). According to the constructed phylogenetic tree, PEDV could be divided into two large groups (GI and GII), of which 4 strains (CH-SQJS-3, CH-YCJS-1, CH-SQJS-1, and CH-SQJS-2) were in the GI group. The domestic reference strains (JSCF-2018, SX-YC-2019, GDSG18-2, GDSG12, GDJX16, YN144, AJ1102, GJL, CHGD-01, JS-A, FL2013, and CH-HNQX-3-14) matched the PEDV strains currently prevalent in China. The clade also included the Japanese reference strain (KH) and Korean reference strain (SPK1, KNU-0801). These results indicate that the four PEDV strains from the GI group may have evolved from one PEDV strain in China after 2010. Three strains in this study were in the GII group (CH-YZJS-1, CH-CZJS-1, and CH-XZJS-1). The clade also included domestic reference strains (ZL29, LZC, and Attenuated CV777), a Spanish reference strain (PEDV-H3-Barcelona-Vic), a Korean reference strain (DR13, DR13-

attenvoxel), a German reference strain (BR1), a Japanese reference strain (MK), and a Swiss reference strain (CV777). These results indicate that the genetic diversity of the PEDV strains in Jiangsu Province could be explained by differences in their sources.

Discussion

The present study provides molecular epidemiological data on the newly emerging strains of PEDV on pig farms in China. We analyzed 793 small intestine tissue, feces, and anal swab samples from 72 pig farms in 11 counties in the jurisdiction of 5 regions of Jiangsu Province (Yancheng, Suqian, Changzhou, Xuzhou, and Yangzhou). Recent research shows that winter and spring (November to March) are the most common PED seasons[25, 26], so we chose this period to analyze the prevalence of PED in Jiangsu Province. The results showed that 457 samples from 57 pig farms were positive: this is equivalent to a positivity rate of pig farms of 79% (57/72) and a sample positivity rate of 57.6% (457/793). The data are consistent with the survey results of Du in 2004 and Bi in 2012[4, 27], which reported positivity rates higher than 50%. In an epidemiological survey undertaken from February 2011 to March 2014, PEDV epidemics were reported in 29 provinces of China, with the exception of Tibet and Hainan: the percentage of PEDV-positive samples was 61.10–78.49%, and the percentage of PEDV-positive pig farms was 71.43–83.47%[28, 29]. Our results are consistent with the findings of this survey, too, and confirm that PEDV plays a dominant role in the current porcine diarrhea epidemics. Therefore, more strategies that target the prevention and control of this virus are required in the future. The present results indicate that there are differences in the prevalence of PEDV among different regions in Jiangsu Province, with the positivity rate ranging from 88% in Yangzhou to 48% in Changzhou. This means that the pathogenicity and transmission routes of PEDV need to be studied closely in order to develop appropriate strategies for the prevention of transmission. Importantly, some recent studies have shown that PEDV can be transmitted through feed and farm appliances[30]. Thus, the difference in prevalence across regions and farms could be attributable to differences in feed sources, rearing conditions, and climate.

Phylogenetic analysis of the 7 strains selected in this study showed that their nucleotide identity was 88.0–100% and amino acid identity was 87.3–99%. Additionally, the 7 PEDV strains exhibited 88.3–99.7% nucleotide identity and 88–98.5% amino acid identity with reference PEDV strains deposited in GenBank. A phylogenetic tree constructed based on genotyping of the S1 gene showed that the PEDV isolates could be divided into two large groups (GI and GII), of which 4 strains (CH-SQJS-3, CH-YCJS-1, CH-SQJS-1, and CH-SQJS-2) were in the GI group and 3 strains (CH-YZJS-1, CH-CZJS-1, and CH-XZJS-1) were in the GII group. CH-SQJS-1, CH-SQJS-2, and CH-SQJS-3 were isolated from different pig farms in Suqian. Further, although they are in the same GI group, they are not very closely related. This is probably because different pig farms introduce breeding pigs from different places and buy feed and other products from different sources, leading to the transmission of different PEDV strains[31]. Overall, the present results demonstrate the genetic diversity of the PEDV strains in Jiangsu Province and indicate that the strains may be from different sources. This has posed a challenge in the prevention and control of PED in Jiangsu Province, especially in terms of the immunoefficacy of vaccines. For example, CH-SQJS-3, CH-YCJS-1, CH-SQJS-1, and CH-SQJS-2 are classified in the GI group, but the DR13-attenuated

and attenuated CV777 vaccines provide protection against strains from the GII group. This explains the high PED incidence in this region and points to the need for preliminary screening of the vaccines against various strains in order to prevent and control the current PED outbreak.

Conclusions

The results of our study indicate that PEDV is one of the major pathogens responsible for the outbreak of clinical diarrhea in pig farms in China. Importantly, the PEDV strains isolated in our study are epidemic variants with genetic diversity. The recombination, insertion, and deletion of the S gene may contribute to the genetic diversity of PEDV and may also be related to the failure of vaccination and the emergence of new PEDV variants. Thus, our findings highlight the urgent need to develop vaccines based on recent PEDV variants in order to tackle the current PED epidemic in China and the need to further explore the pathogenesis and immunogenic strains of PEDV. We expect that the results of this study will be highly useful for the prevention and control of PEDV infections more effectively in Jiangsu Province.

Abbreviations

PED: Porcine epidemic diarrhea; PEDV: Porcine epidemic diarrhea virus; S: Spike protein; COE: Collagenase equivalent; RNA: Ribonucleic acid; ORF: Open reading frame; E: Envelope protein; N: Nucleocapsid protein; M: Membrane protein; MEGA: Molecular Evolutionary Genetics Analysis.

Declarations

Ethics approval and consent to participate

This study was approved by the Institutional Review Board of Yangzhou University. All methods were carried out in accordance with relevant guidelines and regulations and the study was carried out in compliance with the ARRIVE guidelines.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used during the current study are available from the corresponding author upon reasonable request. The datasets generated during the current study are available in the GenBank repository.

Competing interests

All authors declare that they have no competing interests.

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Authors' contributions

JWJ: study design, data analysis and discussion. ZTF, QJH and SZJ: Finished the experiments, data analysis and discussion and drafting the manuscript. SHX, QK, QAJ: revising the manuscript. All authors read and approved the final manuscript submitted for publication.

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Figures

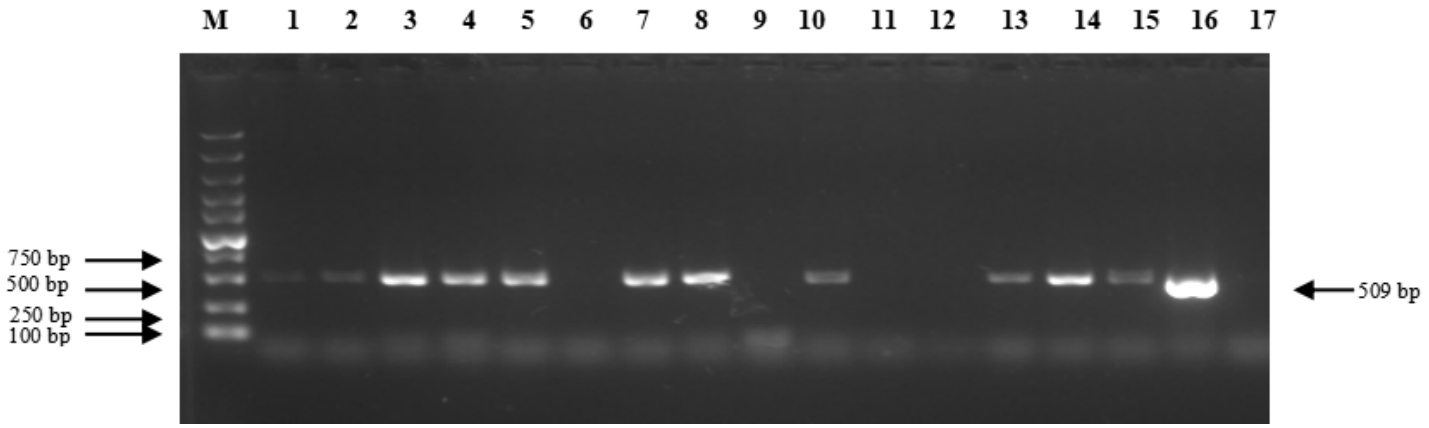

Figure 1

RT-PCR results of amplification of the partial S gene from PEDV isolated from the pig farms M: Super DNA10000 bp Marker, Lanes 1–15: predicted product (509 bp) of the PEDV COE gene; Lane 16: positive control (509 bp); Lane 17: negative control

Figure 2

Regions that were positive for PEDV from winter 2020 to spring 2021 in Jiangsu Province

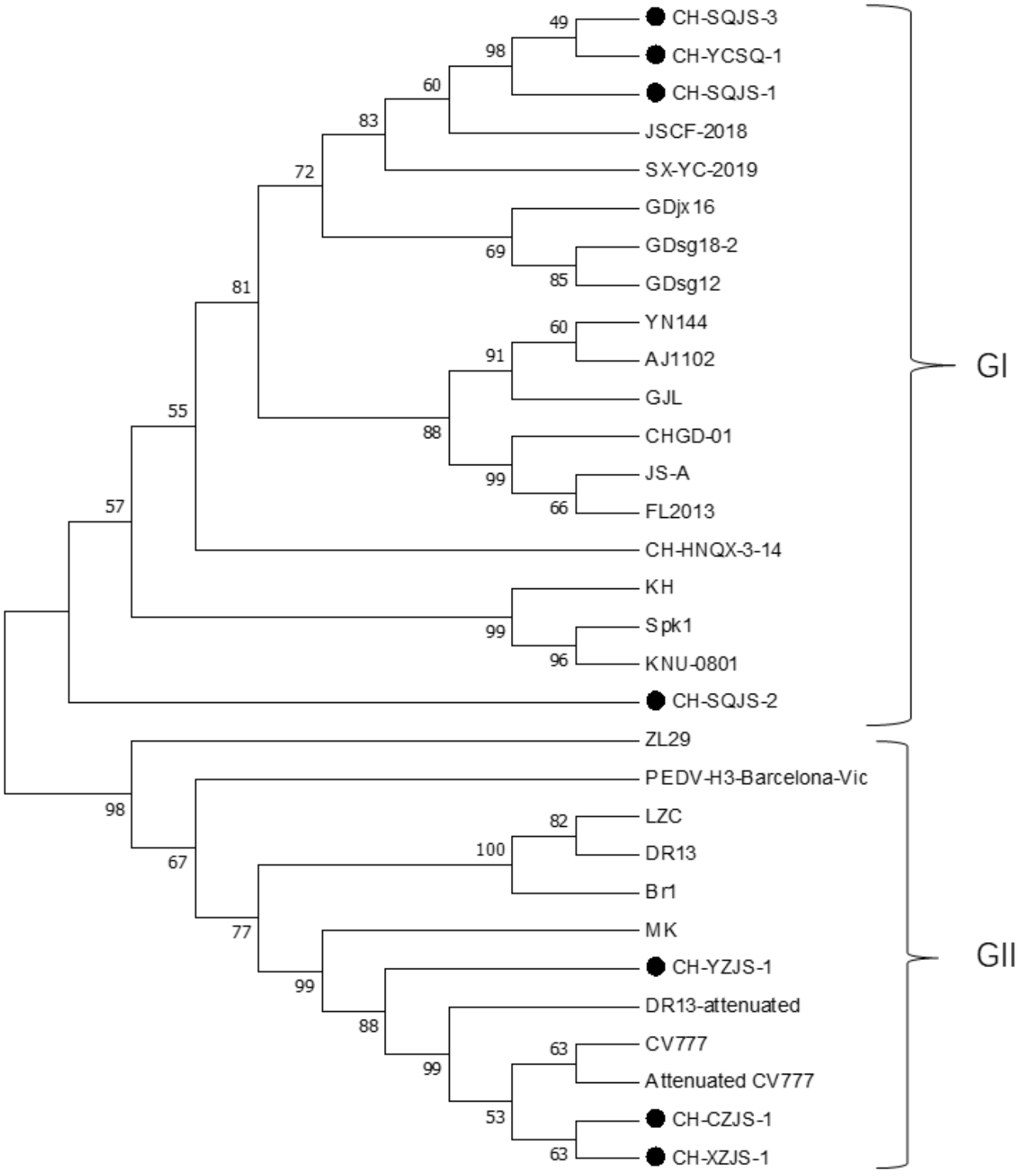

Figure 3

Phylogenetic analysis of the S1 nucleotide sequences of the 7 PEDV strains, including the reference strains