Genetics and Molecular Research

Molecular characterization and phylogenetic analysis of porcine epidemic diarrhea virus samples obtained from farms in Gansu, China

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ABSTRACT. Porcine epidemic diarrhea poses significant sanitation problems in the porcine industry, and has negatively affected the economy in recent years. In this study, 48 fecal specimens were collected from piglets from four intensive swine farms located in the Gansu Province of China. The molecular diversity and phylogenetic relationships between porcine epidemic diarrhea viruses (PEDV) prevalent in Gansu were probed, and the resultant proteins were characterized. Sequence analysis of the spike protein (S) genes showed that each specimen had unique characteristics, and that the PEDV1/S/4 strain could be differentiated from the others via a unique mutation of the *S* gene. The phylogeny of S glycoprotein showed

that all strains were clustered into two major groups. The four Gansu PEDV field strains were characterized into different groups; this finding was consistent with the results of the protein characterization prediction. This analysis additionally revealed the unique characteristics of each specimen. The results of this study could be used to elucidate the prevalence of PEDV and contribute to the prevention of PEDV in Gansu.

Key words: Porcine epidemic diarrhea; Virus; Gene; Phylogenetic analysis

INTRODUCTION

Porcine epidemic diarrhea (PED) is a disease that is considered to be devastating for pig farmers with a rapid spread of infection (Wang et al., 2013; Alonso et al., 2014; Jung and Saif, 2015). The clinical symptoms of PED include severe enteritis, vomiting, and watery diarrhea, with high infectivity and lethality in piglets. Since the 2010s, there have been continuous outbreaks of this disease in swine farms in nearly all provinces in China (Chen et al., 2010; Sun et al., 2012; Wang et al., 2013; Zhang et al., 2013; Zhao et al., 2013; Jung and Saif, 2015; Song et al., 2015a). Specifically, during the initial prevalence of the disease in farrowing barns, the mortality of newborn piglets has been found to be approximately 100%, which is responsible for considerable financial losses faced by the swine industry (Shibata et al., 2000; Li et al., 2012; Jung and Saif, 2015).

The causative agent of PED, the PED virus (PEDV), is an enveloped virus possessing an approximately 28-kb, positive-sense, single-strand RNA genome with a 5'cap and a 3' polyadenylated tail. The genome comprises a 5' untranslated region (UTR), a 3'UTR, and at least seven open reading frames that encode 4 structural proteins: spike protein (S), envelope protein, membrane protein, and the nucleocapsid protein (Bosch et al., 2003; Park et al., 2011b; Bi et al., 2012; Song and Park, 2012; Kim et al., 2015; Song et al., 2015a).

The PEDV S protein, which is a type I glycoprotein composed of 1383 amino acids (aa), plays a pivotal role in regulating the interactions with specific host cell receptor glycoproteins in order to mediate the viral entry and stimulate the induction of neutralizing antibodies in the natural host (Park et al., 2011a; Shirato et al., 2011; Cho et al., 2014). Therefore, the S protein must be thoroughly researched to understand the genetic relationships and diversity of PEDV isolates.

In addition, the S glycoprotein could be used as a primary target for the development of effective vaccines against PEDV. Although most large-scale pig farms were vaccinated according to the proper immune program, immunized swine herds continue to be infected by PEDV (Li et al., 2012; Zhao et al., 2013; Jung and Saif, 2015; Song et al., 2015b) (Li et al., 2012; Zhao et al., 2013; Jung and Saif, 2015; Song et al., 2015b). Therefore, the genetic relationships between different strains must be elucidated to effectively control and prevent PEDV infection. In this study, we investigated the molecular epidemiology and analyzed the protein characteristics of the S protein obtained from Gansu PEDV field samples. PEDV S protein plays a vital role in viral function and higher variation; therefore, the study mainly focused on the *S* gene.

MATERIAL AND METHODS

Sample collection

The 48 fecal specimens were individually obtained from piglets grown in four different

intensive swine farms in the Gansu Province of China from August 2014 to May 2015. The piglets presented severe enteritis, vomiting, and watery diarrhea. All feces samples were homogenized with twice the amount of phosphate buffered saline. The suspensions were vortexed for 1 min and clarified by centrifugation for 10 min at 4000 *g*. The supernatants were stored at -80°C until further use.

RNA extraction

PEDV RNA was extracted from the supernatants of homogenized samples with MiniBEST Viral RNA/DNA Extraction Kit Ver.5.0 (Takara, Otsu, Japan) according to the manufacturer protocols. The RNA products were dissolved in 40 µL RNase free dH_2O and stored at -80°C.

Primers

One set of primers was designed and synthesized by the Beijing Genomic Institute (Beijing, China) to amplify the *S* genes based on the genome of PEDV CV777 (GenBank No.: AF353511.1): forward primer: 5'-ATGAGGTCTTTACTTCTGGTTG-3', reverse primer: 5'-TCACTGCACGTGGACCTT-3'.

RT-PCR, DNA cloning, and sequence analysis

RT-PCR was conducted to amplify the complete S genes from the isolated RNA, as described in the manual of the Primescript™ One step RT-PCR kit v.2 (TaKaRa) under the following conditions: reverse transcription at 50°C for 30 min, denaturation at 94°C for 2 min, and 32 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 1 min.

RT-PCR products were identified by electrophoresis on a 1% agarose gel, and cloned using a PMD19/T vector (TaKaRa). The recombinant vector was identified by PCR and enzyme digestion. The positive clones were sent to Beijing Genomics Institute for sequencing. All sequencing reactions were performed in triplicate. The PCR product sizes (4141-bp) were validated.

The nucleotide sequence and the deduced amino acid sequence of complete S genes of the four strains obtained from four different intensive swine farms (designated as *PEDV1*/S/4, *PEDV3*/S/1, *PEDV4*/S/3, and *PEDV2*/S/5), were aligned, and analyzed using the Megalin software (DNA Star). Phylogenetic trees were constructed using the Molecular Evolutionary Genetics Analysis (MEGA) software (version 6.0), by the neighbor-joining method based on the predicted amino acid sequence of the completed S proteins. The reference strains used for sequence alignment and phylogenetic analyses of the four PEDV strains are presented in Table 1.

Table 1. PEDV strains used for sequence alignment and phylogenetic analysis.

Isolate/strain	Accession No.	Origin
PEDV3/S/1	KT313037	In this study
PEDV4/S/3	KT313038	In this study
PEDV1/S/4	KT313039	In this study
PEDV2/S/5	KT313036	In this study
FJ/LY 2013	KJ646584.1	Southern China
HB/2012/2	JX435303.1	Southern China
BJ/2011/3	JX435298.1	Northern China
CH17/GZ	JQ979289.1	Southern China
FJ/QZ 2013	KJ646605.1	Southern China
CH/QTC/2015	KR296670.1	Southern China
JY5C	KF177254.1	Western China
CV777	JN599150.1	Europe
CH/HGC/01/2015	KR296667.1	Southern China

Protein characterization prediction

The aa sequences were characterized based on their predicted protein isoelectric points (pI), coiled coil regions, transmembrane helices in proteins, the presence and location of signal peptide cleavage sites in amino acid sequences, structure domain, threonine and tyrosine phosphorylation sites, and asparagine (N)-linked glycosylation sites (N-Glyc); these were determined using DNA star and the ExPASy - SIB bioinformatics resource portal online tools.

RESULTS

Sequence analysis of the complete *S* gene

The nucleotide sequences of the complete *S* genes of PEDV1/S/4 (GenBank accession No. KT313039), PEDV2/S/5 (GenBank accession No. KT313036), PEDV3/S/1 (GenBank accession No. KT313037), and PEDV4/S/3 (GenBank accession No. KT313038) were 4161, 4161, 4155, and 4155 bp long, respectively. S proteins of PEDV3/S/1 and PEDV4/S/3 were 1384 aa in length, with predicted Mrs of 151.5 and 151.4 kDa, respectively. S proteins of PEDV2/S/5 and PEDV1/S/4 were 1386 aa in length with predicted Mrs of 151.6 and 151.5 kDa, respectively. The *S* gene sequences of the four Gansu PEDV field samples were compared to those of chosen PEDV reference strains [excluding CV777 (GenBank accession No. JN599150.1) which was only 99% similar]. CV777 had unique mutations that were different from those seen in all other stains. The alignment analysis of the deduced amino acid sequences showed that most of the mutations were observed between positions 230 and 380.

PEDV3/S/1 and PEDV4/S/3displayed 7 common point mutations and deletion sites at the 380th position (Figure 1); moreover, PEDV3/S/1 and PEDV4/S/3 had unique point mutations at positions 1358 (C→G) and 1359 (C→R), respectively. In addition, PEDV2/S/5 and PEDV1/S/4 also displayed special mutations. Results of sequence identity based on the complete S gene of all PEDV strains are summarized in Table 2. In brief, the S gene of 4 Gansu PEDV field samples and chosen PEDV reference strains (except CV777) had very high homology; however, they also had their own characteristics.

Phylogenetic analysis

The phylogenetic relationships among the four Gansu samples, other PEDV strains isolated from various regions in China, and a CV777 strain were determined. A phylogenetic tree was constructed based on the deduced amino acid sequences of the complete *S* gene (Figure 2).

The phylogenetic relationships based on the S glycoprotein showed that all strains fell into two major groups. Group I consisted of 11 strains, while group II only contained CV777 and PEDV1/S/4. PEDV3/S/1 and PEDV4/S/3 formed a subgroup, and PEDV2/S/5 was closely related to PEDV3/S/1 and PEDV4/S/3, which were clustered into group I. The results were consistent with the findings from sequence analysis.

Protein characterizations

The S protein characterization of the four Gansu PEDV field strains is shown in Table 3. The structure of the S protein was similar across the Gansu field PEDV strains, and consisted of a signal peptide (1 to 20 aa), 2 conserved structure domains corona-S1 (234 to 736 aa) and corona-S2 (744 to 1385 aa), and a transmembrane region (1326 to 1348 aa), which corresponded

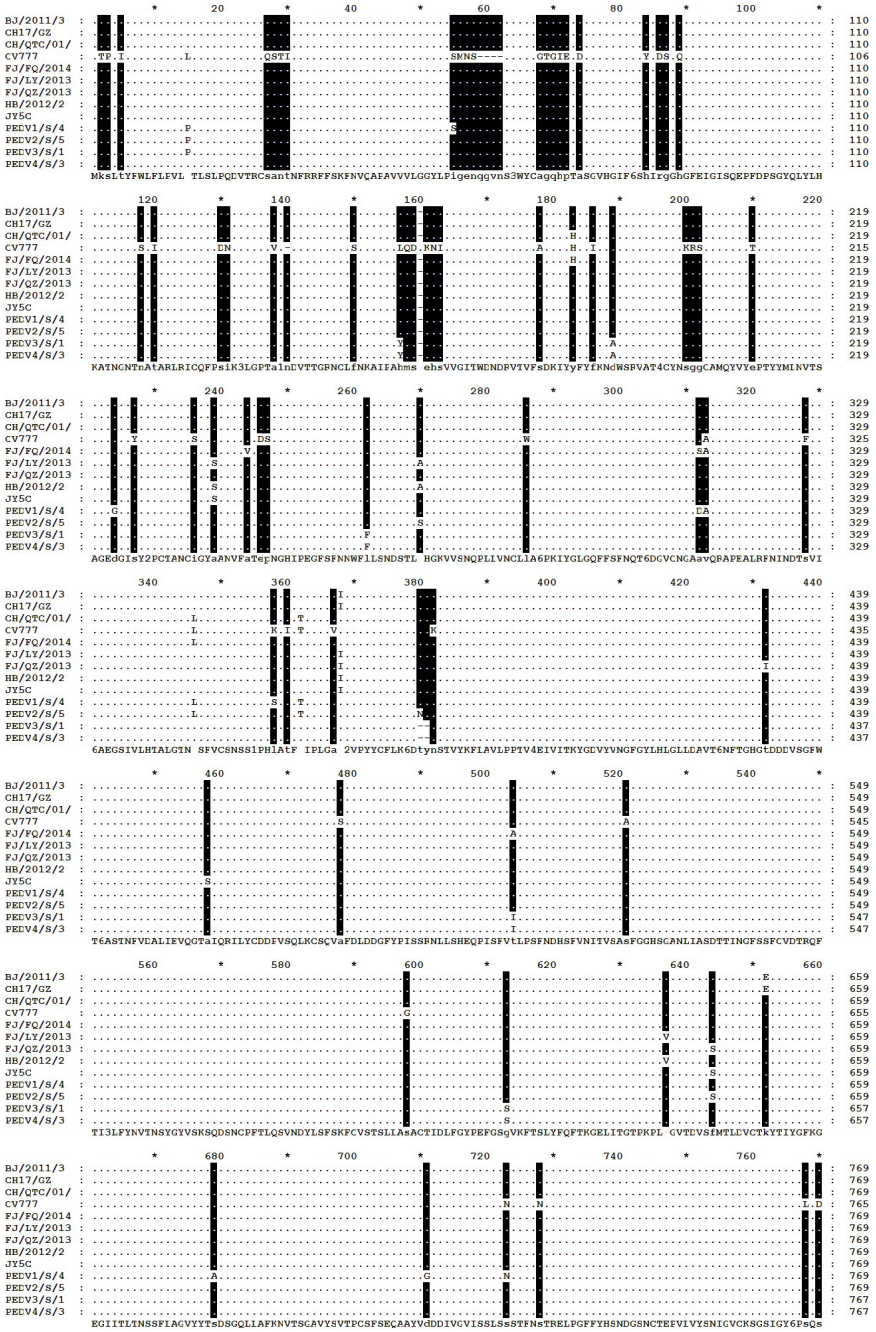

Figure 1. Amino acid sequence alignment of the S glycoprotein genes of Gansu PEDV isolates and PEDV strains. The dashes represent deleted amino acids. The shadows indicate the unique substitutions present within each strain.

Continued on next page

Figure 1. Continued.

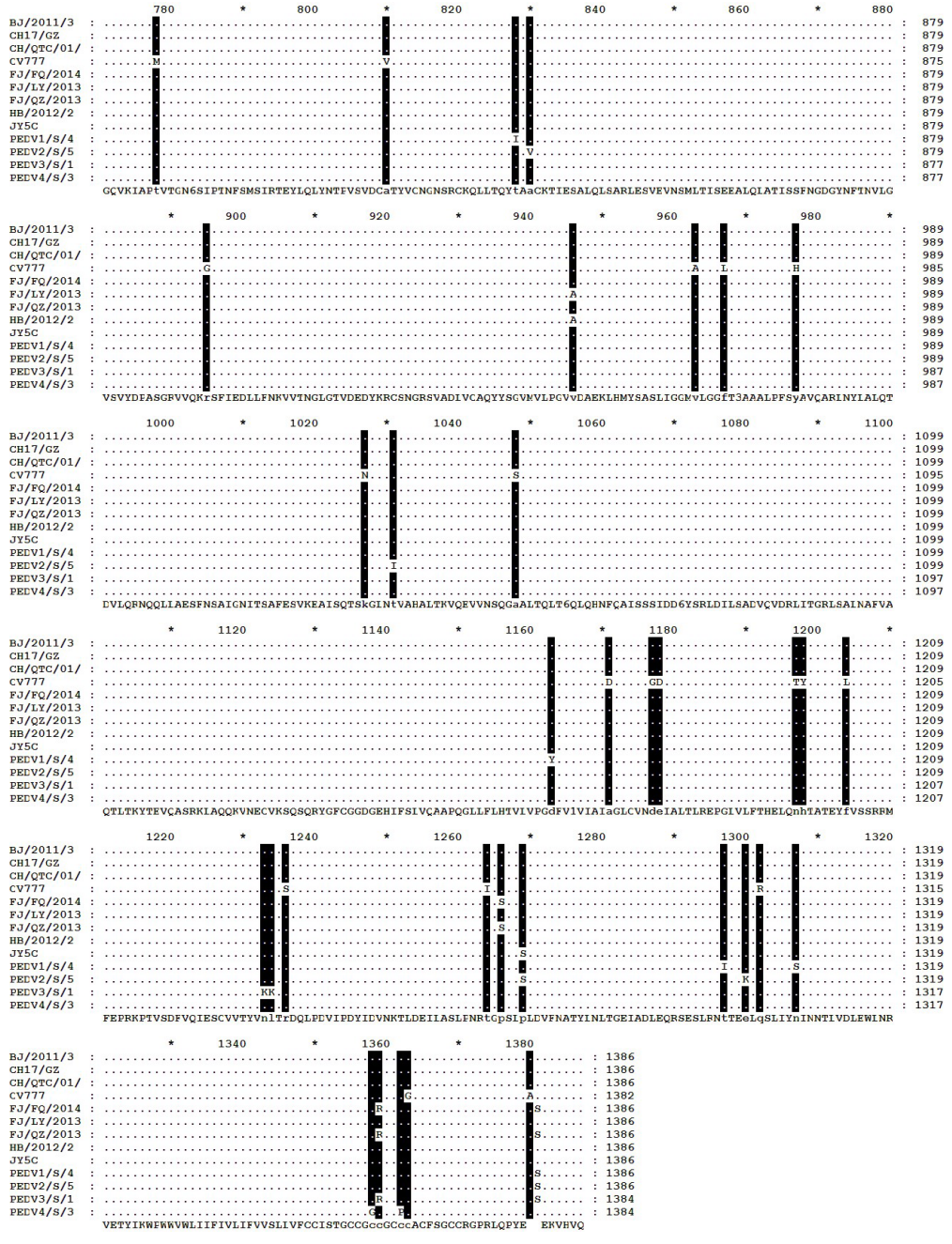

Table 2. Comparison of deduced amino acid sequences of S genes of Gansu PEDV strains and PEDV reference strains.

Strain/isolate	1	2	3	4	5	6	7	8	9	10	11	12	13
1 BJ/2011/3	-	99.7	99.4	92.8	99.1	99.6	99.4	99.6	99.4	98.4	98.9	98.8	99.1
2 CH17/GZ	0.3	-	99.2	93	98.9	99.4	99.3	99.4	99.3	98.3	98.8	98.8	98.9
3 CH/QTC/2015	0.7	0.8	-	93.1	99.1	99.2	99.1	99.2	99.1	98.6	99.1	98.8	99
4 CV777	7.4	7.2	7.1	-	92.9	92.8	92.5	92.8	92.7	92.5	92.6	92.2	92.3
5 FJ/FQ/2014	0.9	1.1	0.9	7.3	-	98.9	99.2	98.9	98.8	98.4	98.7	98.7	98.6
6 FJ/LY 2013	0.4	0.6	0.8	7.4	1.1	-	99.3	100	99.5	98.3	98.8	98.7	98.9
7 FJ/QZ 2013	0.6	0.7	0.9	7.7	0.8	0.7	-	99.3	99.3	98.3	98.9	98.8	98.8
8 HB/2012/2	0.4	0.6	0.8	7.4	1.1	0	0.7	-	99.5	98.3	98.8	98.7	98.9
9 JY5C	0.6	0.7	0.9	7.5	1.2	0.5	0.7	0.5	-	98.1	99	98.6	98.8
10 PEDV1/S/4	1.6	1.7	1.4	7.7	1.6	1.8	1.8	1.8	1.9	-	98.5	98.1	98
11 PEDV2/S/5	1.1	1.2	0.9	7.6	1.3	1.2	1.1	1.2	1	1.5	-	98.7	98.6
12 PEDV3/S/1	1.2	1.2	1.2	8	1.3	1.3	1.2	1.3	1.5	1.9	1.3	-	99.5
13 PEDV4/S/3	0.9	1.1	1	7.9	1.4	1.1	1.2	1.1	1.2	2	1.4	0.5	-

Percent similarity in upper triangle. Divergence in lower triangle.

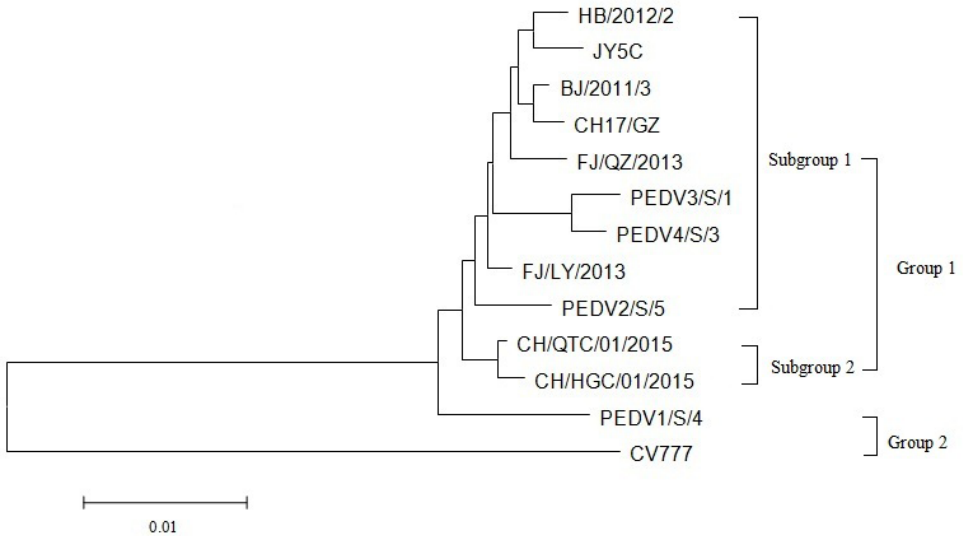

Figure 2. Phylogenetic relationships between Gansu PEDV isolates and other reference strains, based on the S glycoprotein amino acid sequences. The GenBank accession Nos. of these genes are listed in Table 1.

with the results of previous studies (Lee et al., 2010).

The prediction of coiled coil regions agreed with those of transmembrane regions; coiled coil regions existed between 1326 and 1348-aa in the 4 Gansu PEDV field strains.

The pI of S protein varied from 5.22 to 5.32 among the four Gansu PEDV field strains; PEDV2/S/5 and PEDV1/S/4 had the same pI value (5.28). The four Gansu PEDV field strains shared a similar epitope, except PEDV1/S/4. PEDV1/S/4 was short of an antigenic peptide position (1230 to 1237 aa) compared to the other PEDV field strains. We observed 22 potential N-Glyc in PEDV4/S/3, and 21 such sites in the other three Gansu PEDV field strains (Table 4). The identified potential phosphorylated sites showed the presence of strain-specific sites (16 in PEDV4/S/3, 14 in PEDV2/S/5, and 15 in PEDV1/S/4 and PEDV3/S/1; Table 4). These variations were attributed to mutations in the gene sequences of the PEDV field strains, consistent with the results of previous sequence analyses.

Table 3. Predicted protein characteristics of the deduced protein sequence of the complete S gene.

Strains	Predicted protein characterizations					
	pI value	Antigenic peptide		Transmembrane regions between positions	Coiled coil regions between positions	Signal peptide
		Number of hits	Position			
PEDV1/S/4	5.28	5	564-571 912-921 1125-1130 1206-1219 1285-1301	1326-1348	1326-1348	1-20
PEDV2/S/5	5.28	6	564-571 911-920 1123-1128 1203-1218 1230-1237 1284-1299	1326-1348	1326-1348	1-20
PEDV3/S/1	5.32	6	564-571 911-920 1123-1128 1203-1218 1230-1237 1284-1298	1326-1348	1326-1348	1-20
PEDV4/S/3	5.22	6	562-570 912-919 1124-1128 1230-1237 1203-1217 1284-1299	1326-1348	1326-1348	1-20

Table 4. Prediction of the post-transcriptional modification sites in the deduced protein sequence of the complete *S* gene.

Strains	Protein modification sites			
	No. of N-Glyc sites	No. of phosphorylated sites		
		Serine	Threonine	Tyrosine
PEDV1/S/4	21	8	3	4
PEDV2/S/5	21	7	3	4
PEDV3/S/1	21	7	4	4
PEDV4/S/3	22	8	4	4

DISCUSSION

As seen in previous studies with coronavirus S proteins, the glycoprotein of PEDV is known to play a pivotal role in the interaction of cell with the cellular receptor, mediating viral entry and inducing neutralizing antibodies in the natural host. Moreover, the *S* gene of PEDV is considered to be the most useful in revealing the genetic diversity of PEDV (Chang et al., 2002; Bosch et al., 2003; Nam and Lee, 2010; Park et al., 2011a; Shirato et al., 2011; Li et al., 2012). Therefore, the diversity of PEDV strains may be studied based on the *S* gene. However, most studies have only assessed the partial or full *S* genes, with the full S protein characterization being conducted rarely (Lee et al., 2010; Li et al., 2012; Gao et al., 2013). In this study, the complete nucleotide and deduced peptide sequences of S protein genes of the four Gansu PEDV field strains were compared to those of published PEDV reference strains, and the S protein characteristics were predicted. We demonstrated that the S glycoprotein genes of Gansu PEDV field strains were diverse, leading to variations in the predicted protein characteristics, similar to the results seen in previous studies.

Despite the presence of many mutations in the S protein gene between the four Gansu PEDV field strains and published reference strains, we observed that the variations were focused primarily on the N-terminal region of conserved structure domain corona-S1, which agreed with

the results of previous reports (Lee et al., 2010). The gene segment coding for the N-terminal conserved structure domain corona-S1 may therefore be an ideal molecular target for molecular epidemiology research.

Phylogenetic trees constructed based on deduced amino acid sequences indicated that PEDV1/S/4 was relatively close to CV777, but distantly related to group I. The other Gansu PEDV field strains were clustered into group I with PEDV3/S/1 and PEDV4/S/3 forming a subgroup. In all, the phylogenetic analysis suggested that there was high sequence variation among the Gansu PEDV field strains. The CV777 strain has been used to develop a PEDV vaccine in China; the results of this study indicate the need to determine if the range of PEDV1/S/4 infection is relatively smaller in Gansu.

The Gansu PEDV field strains presented similar functional domains, a signal peptide (1 to 20 aa) region, two conserved structural domains, and a transmembrane region; however, the protein characterization of these strains showed the presence of only one unique difference in the form of an amino acid substitution. *S* protein gene mutations may alter some functional sites and protein modification sites. The asparagine (N)-linked glycosylation sites and phosphorylated sites differed among the Gansu PEDV field strains, which was consistent with the results of a previous study (Lee et al., 2010; Chen et al., 2012). The prediction of epitopes, the pivotal functional sites closely associated with immunological recognition, should contribute to the design and development of epitope vaccines and antibodies (Chen et al., 2009; Pasick et al., 2014; Farrell and Gordon, 2015). PEDV1/S/4 had fewer epitopes than the other PEDV field strains, which further confirmed that PEDV1/S/4 was distantly related to the other three Gansu PEDV field strains.

In conclusion, the Gansu PEDV field strains underwent genetic diversity in their S glycoprotein genes and were clustered into different groups. PEDV2/S/5, PEDV3/S/1, and PEDV4/S/3 were closely related to the strains isolated from other Chinese provinces; however, they also displayed unique characteristics. PEDV1/S/4, on the other hand, was phylogenetically related to CV777. The results of this study indicated the importance of sequence analysis and protein characteristic prediction in understanding the genetic diversity among PEDV isolates, and its role in the development of an effective vaccine.

Conflicts of interest

The authors declare no conflict of interest.

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