

## ORIGINAL ARTICLE

# Two Distinct Genotypes of Porcine Epidemic Diarrhoea Virus in Vaccinated Pig Flocks in Shandong Province of China, 2012–2015

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porcine epidemic diarrhoea virus; S gene; genotype; mutation; Gd subgenotype

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**Summary**

As one of the top pork producers in China, Shandong Province suffered frequent outbreaks of porcine epidemic diarrhoea virus (PEDV) on pig farms from January 2012 to July 2015, resulting in significant economic losses. To better understand the prevalence situation, we conducted molecular epidemiological analyses of 38 PEDV strains isolated from 13 cities in Shandong Province. The detection rate of PEDV was 71.2% (146/205) by reverse transcription polymerase chain reaction (RT-PCR). The S genes of the 38 isolated samples were 4146 to 4161 nt in length and shared high levels of sequence identity (93.3–99.6% nt, 92.1–99.4% aa) with those of the 41 reference strains. Among the 38 strains, 31 strains that occupied 12 cities were classed into G3 genotype, while the other seven that only existed in four cities were classed into G2 genotype. In addition, the strains CH-SDLY-2-2014 and CH-SDLY-3-2014 isolated from Linyi were classed into the Gd subgenotype. Notably, there were multiple insertions or deletions in the S genes and several mutations in the neutralizing epitopes of the PEDV S protein. Overall, the results revealed that G2 and G3 are the predominant PEDV genotypes circulating in Shandong Province during 2012–2015, and Gd subgenotype in G3 group had already spread towards northern China in 2014.

**Introduction**

Porcine epidemic diarrhoea virus (PEDV) is an enveloped, single-stranded RNA virus belonging to the genus *Alphacoronavirus*, subfamily *Coronavirinae*, family *Coronaviridae* (Pensaert and de Bouck, 1978). The PEDV genome with a 5' cap and a 3' polyadenylated tail is approximately 28 kb in length and contains seven open reading frames (ORFs), which encode replicase 1a and 1b, the spike (S) protein, ORF3 protein, envelope (E) protein, membrane (M) protein and nucleoprotein (N), arranged in the order 5'-replicase 1a/1b-S-ORF3-E-M-N-3' (Murphy et al., 1999). The PEDV S protein is a glycoprotein peplomer (surface antigen) on the viral surface and plays a pivotal role in induction of neutralizing antibodies in natural hosts (Chang et al., 2002; Temeeyasen et al., 2014).

Porcine epidemic diarrhoea virus was first reported in Belgium and the United Kingdom in 1978 (Pensaert and de Bouck, 1978). Within two decades, it swept over continents of Europe, Asia and North America (Song and Park, 2012; Huang et al., 2013; Jung et al., 2014; Vlasova et al., 2014; Sun et al., 2015).

In China, PED has been reported in many provinces, with mortality up to 100%, mainly involving breeding piglets within 10 days of age, usually 2–3 days after birth (Li et al., 2012). As a major swine-producing province in China, Shandong has suffered great economic losses from outbreaks of PED, even with vaccination of the flocks. Since 2012, PED outbreaks have remarkably increased in Shandong Province (Zhao et al., 2012). To investigate the molecular diversity and epidemiology of the virus in Shandong, we sequenced the full-length S gene of 38 PEDV-positive samples collected from January 2012 to July 2015.

Strain	City	Isolated year	S gene (nt/aa)	Accession numbers
CH-SDCQ-2014	Jinan	2014	4149/1382	KU133234
CH-SDQF-1-2014	Jining	2014	4152/1383	KU133256
CH-SDQF-2-2014	Jining	2014	4152/1383	KU133257
CH-SDQF-3-2014	Jining	2014	4152/1383	KU133258
CH-SDRZ-2013	Rizhao	2013	4152/1383	KU133262
CH-SDZB-2012	Zibo	2012	4146/1381	KU133267
CH-SDZC-2012	Zibo	2012	4161/1386	KU133268
CH-SDBZ-1-2015	Binzhou	2015	4161/1386	KU133232
CH-SDBZ-2-2015	Binzhou	2015	4161/1386	KU133233
CH-SDCQ-2015	Jinan	2015	4161/1386	KU133235
CH-SDDP-1-2014	Taian	2014	4161/1386	KU133236
CH-SDDP-2-2014	Taian	2014	4161/1386	KU133237
CH-SDDP-3-2014	Taian	2014	4161/1386	KU133238
CH-SDDY-2012	Dongying	2012	4161/1386	KU133239
CH-SDDZ-2012	Dezhou	2012	4161/1386	KU133240
CH-SDHZ-1-2013	Heze	2013	4161/1386	KU133241
CH-SDHZ-2-2013	Heze	2013	4161/1386	KU133242
CH-SDHZ-3-2013	Heze	2013	4161/1386	KU133243
CH-SDHZ-4-2013	Heze	2013	4161/1386	KU133244
CH-SDJY-2012	Jinan	2012	4161/1386	KU133245
CH-SDLQ-2015	Liaocheng	2015	4161/1386	KU133246
CH-SDLS-1-2014	Jining	2014	4161/1386	KU133247
CH-SDLS-2-2014	Jining	2014	4161/1386	KU133248
CH-SDLY-1-2012	Linyi	2012	4161/1386	KU133249
CH-SDLY-1-2013	Linyi	2013	4161/1386	KU133250
CH-SDLY-1-2014	Linyi	2014	4161/1386	KU133251
CH-SDLY-2-2012	Linyi	2012	4161/1386	KU133252
CH-SDLY-2-2013	Linyi	2013	4161/1386	KU133253
CH-SDLY-2-2014	Linyi	2014	4158/1385	KU133254
CH-SDLY-3-2014	Linyi	2014	4158/1385	KU133255
CH-SDQF-4-2014	Jining	2014	4161/1386	KU133259
CH-SDQF-5-2014	Jining	2014	4161/1386	KU133260
CH-SDQH-2014	Dezhou	2014	4161/1386	KU133261
CH-SDTA-2012	Taian	2012	4161/1386	KU133263
CH-SDWF-2012	Weifang	2012	4161/1386	KU133264
CH-SDWF-2015	Weifang	2015	4161/1386	KU133265
CH-SDYT-2012	Yantai	2012	4161/1386	KU133266
CH-SDZC-2015	Zibo	2015	4158/1385	KU133269

**Table 1.** The porcine epidemic diarrhoea virus strains isolated from Shandong Province, China, in this study

## Materials and Methods

### Specimens

A total of 205 intestinal and faecal samples were collected from 2-day-old to 7-day-old dead piglets with typical symptoms of PEDV infection from 13 cities in Shandong Province of China from 20 January 2012 to 15 July 2015. The morbidity within infected herds was near 100%, a total mortality rate was usually over 50% in suckling piglets, and the sick piglets died in 2–3 days with typical watery diarrhoea, dehydration and vomiting. According to the manufacturer's instructions, total RNA was extracted from the samples using TriZol reagent (Invitrogen, Carlsbad, CA, USA). Then, the samples were detected by reverse transcription polymerase chain reaction (RT-PCR) with the primers targeted at PEDV M gene: Mf, 5'-CCC CAG TAC

TGT TAT TGA CGT ATA AAC-3' and Mr, 5'-GTT TAG ACT AAA TGA AGC ACT TTC-3'. A total of 38 PEDV-positive samples were randomly selected for amplification of the S gene and sequence analysis (Table 1).

### Amplification of S genes

The primers for PCR and sequencing the S genes were as follows: fragment A (S1f: 5'-ATG AGG TCT TTA ACC T-3' and S1r: 5'-CTG TTC GTG ACT CAG AAG-3'), fragment B (S2f: 5'-TAC CTT CTA CTG TCA GGG-3' and S2r: 5'-CTC GCT TGA ACA GCA TAG-3') and fragment C (S3f: 5'-ACT CTG GTG TCA TGG TAC-3' and S3r: 5'-TCA CTG CAC GTG GAC TTT-3'). According to the manufacturer's instructions, S genes were amplified from the PEDV-positive samples with Quant One Step RT-PCR Kit

**Table 2.** The 41 reference porcine epidemic diarrhoea virus strains used in this study

Field strain	Regions	Isolated		Accession numbers
		year	S gene (nt/aa)	
JS-HZ2012	Jiangsu/China	2012	4161/1386	KC210147
CH-ZJJS-2Z-2012	Zhejiang/China	2012	4161/1386	KF840546
GD-B	Guangdong/China	2012	4161/1386	JX088695
SHOP-YM-2013	Shanghai/China	2013	4161/1386	KJ196348
CH-ZJHZ-1C-2012	Zhejiang/China	2012	4161/1386	KF840553
BJ-2011-1	Beijing/China	2011	4146/1381	JN825712
AH2012	Anhui/China	2012	4161/1386	KC210145
CH-SDZD-1-2012	Shandong/China	2012	4161/1386	KF840550
CH-SDZD-2-2012	Shandong/China	2012	4161/1386	KF840549
CH-JXZS-3H-2012	Jiangxi/China	2012	4161/1386	KF840541
LC	Guangdong/China	2012	4158/1385	JX489155
CH-GDGZ-2012	Guangdong/China	2012	4158/1385	KF384500
GD-1	Guangdong/China	2012	4158/1385	JX647847
GD-A	Guangdong/China	2012	4158/1385	JX112709
CH-ZMDZY-11	Henan/China	2011	4161/1386	KC196276
IA2	USA	2013	4161/1386	KF468754
IA1	USA	2013	4161/1386	KF468753
USA-Ohio69-2013	USA	2013	4161/1386	KJ645665
PC21A	USA	2013	4161/1386	KF272920
KUIDL-PED-2014-007	Korea	2014	4161/1386	KJ588064
KUN-141112-feces	Korea	2014	4161/1386	KR873431
KUIDL-PED-2014-002	Korea	2014	4161/1386	KJ588063
MN	USA	2013	4161/1386	KF468752
CH-HuBWHYQ-2012	Hubei/China	2012	4161/1386	KF840551
CH-FJND-3-2011	Fujian/China	2011	4161/1386	JQ282909
OH851	USA	2013	4152/1383	KJ399978
USA-Lowa-106-2013	USA	2013	4152/1383	KJ645695
KUN-1406-01	Korea	2014	4152/1383	KM403155
USA-M52-2013	USA	2013	4152/1383	KJ645704
FR-001-2014	France	2014	4152/1383	KR011756
15V010-BEL-2015	Belgium	2015	4152/1383	KR003452
GER-L00719-2014	German	2014	4152/1383	LM645058
CH-S	Shanghai/China	1986	4152/1383	JN547228
Attenuated Chinese PEDV	Hubei/China	2012	4149/1382	KC189944
Attenuated DR13	Korea	2003	4149/1382	JQ023162
JS2008	Jiangsu/China	2008	4161/1386	KC109141
MK	Japan	1996	4152/1383	AB548624
Virulent DR13	Korea	1999	4152/1383	JQ023161
SM98	Korea	1998	4143/1380	GU937797
CV777	Belgium	1978	4152/1383	AF353511
LZC	Gansu/China	2006	4152/1383	EF185992

(Tiangen Biotech, Beijing, China). The Quant One Step reaction was carried out under the following conditions: reverse transcription at 50°C for 30 min, initial denaturation at 94°C for 2 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 30 s, extension at 65°C for 1 min and a final elongation step at 65°C for 10 min. Following purification by AxyPrep DNA Gel Extraction kit (Axygen, Union City, CA, USA), the PCR products were ligated into pGEM-T easy vector (Promega, Madison, WI, USA). The nucleotide (nt) sequences of the positive clones were determined from a commercial service (Shanghai

Sangon Biological Engineering Technology & Service Co., Ltd). At least five individual clones were sequenced for each sample.

### Sequences analysis

The S gene sequences of 38 PEDV strains isolated from 13 cities in Shandong Province were obtained for phylogenetic analysis (Table 1). A total of 41 PEDV reference strains from other provinces of China and other countries were selected from the GenBank database for comparison

(Table 2). Multiple protein sequences were aligned using the MegAlign program by the Clustal W method in the DNASTAR software package (Burland, 2000). A phylogenetic tree was constructed by the neighbour-joining method using the Mega 6.0 software (Tamura et al., 2013).

## Results

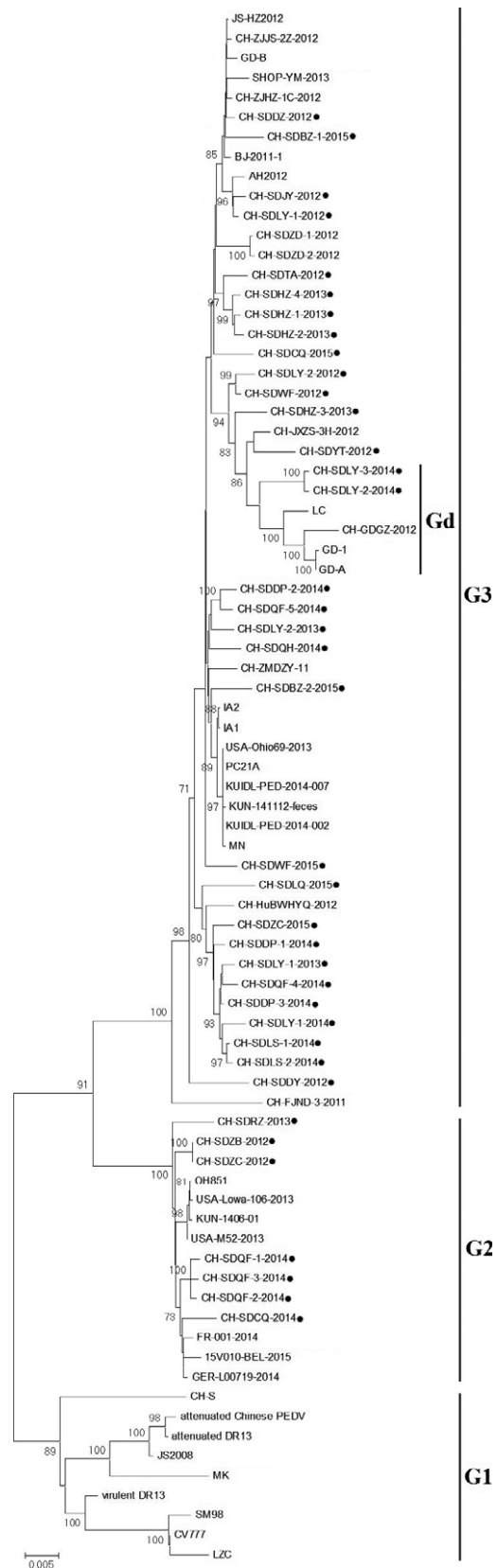
### Sequence properties of the S genes

Among the 205 intestinal and faecal samples from 13 cities in Shandong Province of China, 146 were positive for PEDV by RT-PCR, and the detection rate was 71.2% (146/205). The S gene sequences of the 38 PEDV samples were submitted to GenBank and assigned accession Nos: KU133232–KU133269 (Table 1). The S genes of the 38 PEDV samples were 4146 to 4161 nt in length, encoding the S proteins of 1381 to 1386 amino acids, respectively. Among the 38 samples, 29 were 4161 nt in length and encoded a protein of 1386 amino acids, while the other nine samples were 4152 nt (CH-SDQF-1-2014, CH-SDQF-2-2014, CH-SDQF-3-2014 and CH-SDRZ-2013), 4158 nt (CH-SDLY-2-2014, CH-SDLY-3-2014 and CH-SDZC-2015), 4149 nt (CH-SDCQ-2014) and 4146 nt (CH-SDZB-2012) in length, respectively (Table 1). Multiple sequence alignment showed that the S genes above shared 95.0–99.9% nucleotide sequence identity and 94.3–99.8% amino acid sequence identity with each other, while shared 93.3–99.6% nucleotide similarity and 92.1–99.4% amino acid similarity with those of the 41 PEDV reference strains.

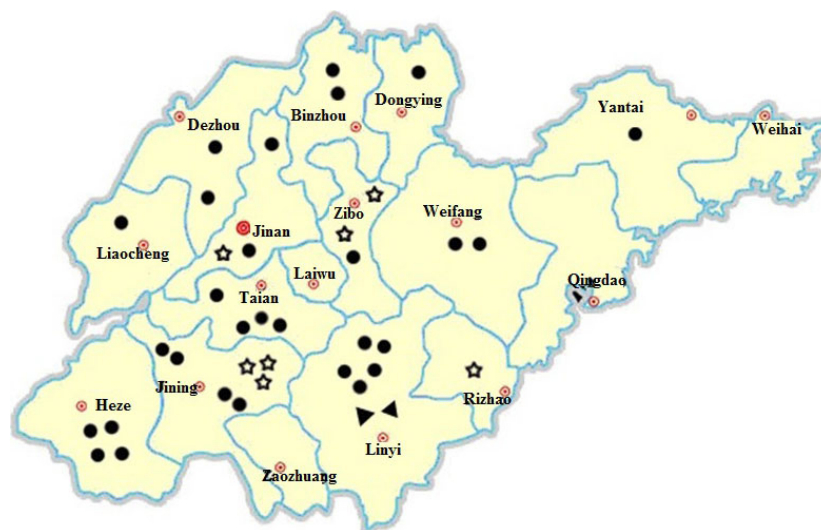
### Phylogenetic analysis of the S gene

Phylogenetic analysis based on the Shandong PEDV field isolates (Table 1) and PEDV reference strains (Table 2) was performed. The results showed that the 79 PEDV strains could be divided into three genotypes: G1, G2 and G3 (Fig. 1). Nine reference strains belonged to G1, namely previous Chinese (JS2008, LZC and CH-S), Korean (SM98 and virulent DR13), Japanese (MK) and European (CV777) strains and two vaccine strains (attenuated Chinese PEDV and attenuated DR13). None of the strains isolated in this study were included in G1.

Seven of the 38 sequenced samples were clustered in G2, together with three current European strains (15V010-BEL-2015, GER-L00719-2014, FR-001-2014), three recent



**Fig. 1.** The rooted phylogenetic tree of 38 field strains homologous and other representative S gene constructed by the neighbour-joining method within the Mega 6.0 software. Bootstrap majority consensus values on 1000 replicates are indicated at each branch point in per cent. Black solid circles indicate the 38 porcine epidemic diarrhoea virus strains isolated in this study. Bootstrap values are represented at key nodes. Scale bar represents nucleotide substitutions per site.



**Fig. 2.** Map representing the 17 cities of Shandong Province in China. The 38 porcine epidemic diarrhoea virus (PEDV) strains were collected from 13 cities in Shandong. Black dot represents the PEDV strains in G3 genotype; black triangled dot represents the PEDV strains in Gd subgenotype; star represents the PEDV strains in G2 genotype. [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

emergent S INDEL strains from the US (OH851, USA-Iowa-106-2013, USA-M52-2013) and one current Korean strain (KUN-1406-01). The majority of the S genes were consistent in length in G2 (4152 nt), except for CH-SDZB-2012 (4146 nt) and CH-SDCQ-2014 (4149 nt). The seven strains were isolated from four cities in Shandong Province (Fig. 2), sharing 98.8–99.8% nucleotide identity with each other and 98.9–99.5% with the other reference strains from G2.

Fifty-six strains were classified to G3, including the rest 31 strains isolated in this study, 17 recent Chinese field strains, three Korean strains and five US strains. The 17 reference strains from China were originated from ten provinces in eastern China. The five US strains were reported to belong to North American clades I and II, following the PEDV outbreak in April 2013 (Vlasova et al., 2014). The three Korean strains are thought to be co-circulating in Korea and showed high homology with the five US strains (Choi et al., 2014; Lee et al., 2015). The 31 isolated strains occupied all the 13 cities, suggesting that G3 was the most prevalent genotype in Shandong Province (Fig. 2). In group G3, the 31 strains isolated in this study shared 97.3–99.9% nucleotide identity with each other and 96.9–99.5% with other reference strains. Interestingly, phylogenetic analysis showed that G3 strains predominantly displayed a 4161-nt S gene sequence with the exception of Gd subgenotype (4158 nt). CH-SDLY-2-2014 and CH-SDLY-3-2014 both belonged to the Gd subgenotype, which is a newly described variant subgenotype in 2012.

Till now, four neutralizing epitopes of the PEDV S protein have been reported, that is COE (CO-26K equivalent) (499–638 aa), SS2 (748–755 aa), SS6 (764–771 aa) and 2C10 (1368–1374 aa) (Chang et al., 2002; Cruz et al., 2008; Sun et al., 2008). According to the alignment results of

selected sequences from the phylogenetic tree, SS2 and 2C10 were found to be conserved in all field strains, while epitope SS6 displayed 2-aa or 3-aa mutations in most field strains. Compared with strain CV777, amino acid changes in COE domain existed in all of the aligned sequences (Fig. 3).

## Discussion

Since 2012, PEDV has re-emerged and rapidly disseminated all over the world (Stevenson et al., 2013). In our study, the positive rate of suspected samples was 71.2% (146/205) in Shandong Province of China, indicating the high prevalence of PEDV among the pig populations. The S gene is important in the phylogenetic and epidemiological analysis of coronaviruses in the field (Stevenson et al., 2013). According to the phylogenetic analysis of S genes, the 38 representative isolates from this study were clustered into two distinct and evolutionarily distant groups (Fig. 1). Among the 38 strains from 13 cities, 31 (G3) occupied 12 cities and the other 7 (G2) only exist in four cities (Fig. 2), which revealed that the genotypes G2 and G3 are circulating in Shandong from 2012 to 2015, and G3 was the predominant genotype of PEDV.

Subgroup Gd strains were first identified in Guangdong Province during 2011 to 2013 and then rapidly spread to nearby areas (Chen et al., 2012; Fan et al., 2012; Wei et al., 2012; Tian et al., 2013). The CH-SDLY-2-2014 and CH-SDLY-3-2014 strains were first identified from Linyi, Shandong Province in 2014, which were further verified to be subgenotype Gd. This is the first report of Gd subgenotype strains in Northern China. Considering the frequent animal trades between Linyi and South China, the current data indicated that the transportation of animals might be a

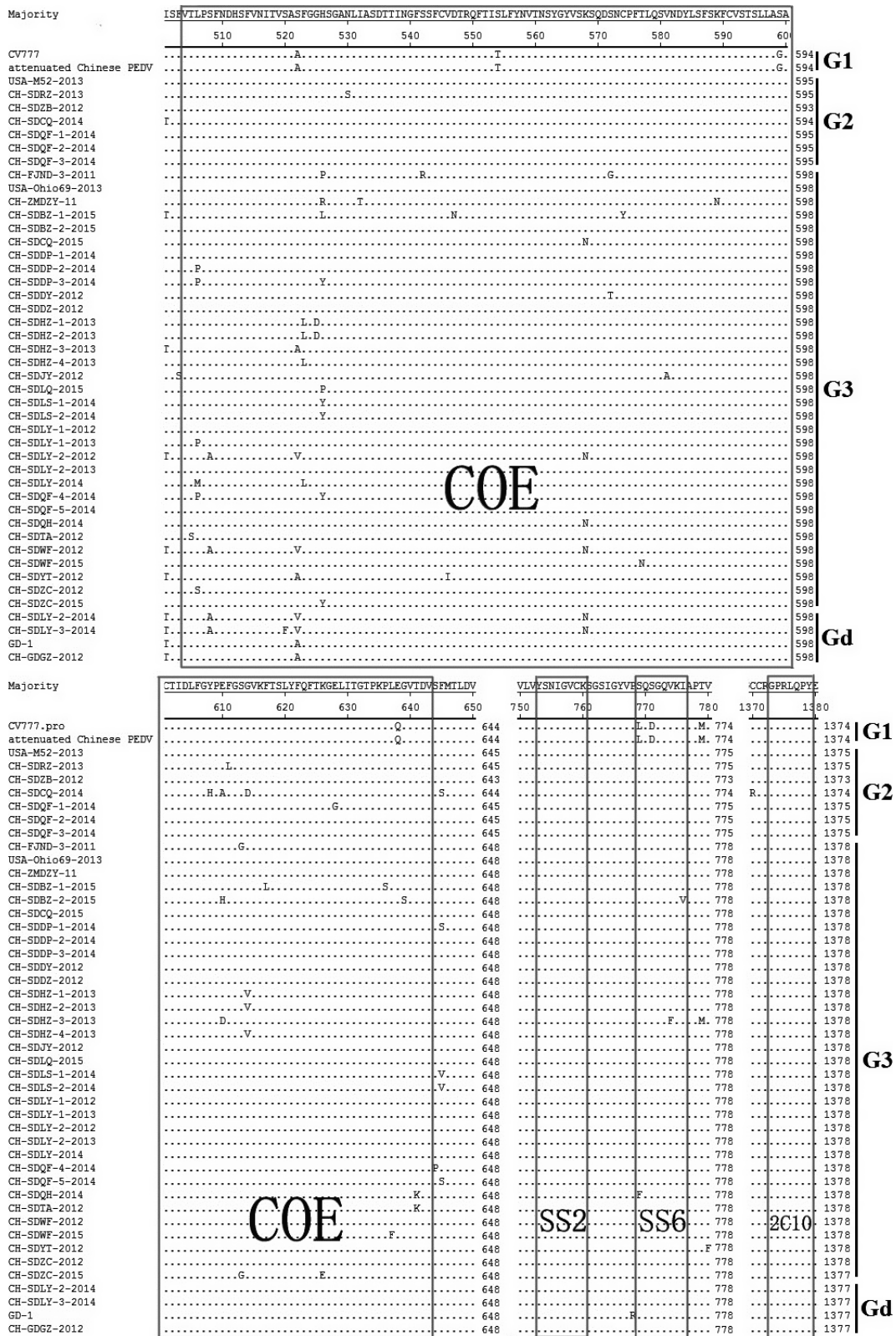


Fig. 3. Comparison of the antigen epitopes of S proteins of strains and reference strains. Ellipses represent the consensus amino acids. COE, SS6, SS2 and 2C10 are in the box. Strains in this study are marked with asterisks (\*).

source of PEDV transmission, and this can provide instructive information for the prevention strategy of exotic PEDV genotype invasion.

The PEDV S protein is an important glycoprotein peplomer on the viral surface, and mutations related to virulence in the S gene antigenic regions may confer increased pathogenicity and antigenicity to the new PEDV variants, thereby reducing the protective efficacy of the vaccination (Park et al., 2007; Stevenson et al., 2013). Multiple alignment analysis showed that many amino acid mutations existed in the neutralizing epitopes of the PEDV S protein, which might be the most important reason for the frequent PEDV outbreaks in Shandong Province during 2012–2015.

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### Conflicts of Interest

The authors declare no conflict of interests.

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