

Original Article

Molecular characterization and phylogenetic analysis of porcine epidemic diarrhea virus isolates in Eastern China

Chunyan Jiang¹, Xiaoju Zhang¹, Jianfeng Han², Haijian He¹, Chaoying Zhang¹, Hongbing Zhang¹, Jingjing Jin¹, Liang Wang³, Bingqian Ge¹, Yanli Wang¹, Yongjie Liu⁴

¹ Department of Animal Production, Faculty of Agriculture and Bioengineering, Jinhua Polytechnic, Jinhua, Zhejiang, China

² Feed Research Institute, Chinese Academy of Agricultural Sciences, Beijing, China

³ Department of Agriculture and Forestry Engineering, Shanxi Yuncheng Agricultural Vocational And Technical College, Yuncheng, Shanxi, China

⁴ College of Veterinary Medicine, Nanjing Agricultural University, Nanjing, Jiangsu, China

Abstract

Introduction: Porcine epidemic diarrhea virus (PEDV) is one of the most common viral pathogens causing swine diarrhea.

Methodology: We performed a genetic evolution analysis of the S1 gene of endemic PEDV strains in Eastern China. The S1 genes of 37 PEDV-positive samples were amplified and sequenced, and compared to the standard CV777 strain, 120 nucleotides were found to have mutations.

Results: The nucleotide and deduced amino acid homologies between the sequences and those of the CV777 strain were 90%–91% and 88.2%–90%, respectively, and their homologies to the vaccine strain were 88.6%–89.7% and 86.2%–87.8%, respectively. Genetic evolution and variation analyses indicated that the 37 PEDV strains belonged to genogroup 2-1, while the CV777 strain, vaccine strain, and earlier Chinese strains all belonged to genogroup 1-1.

Conclusions: The newly emerged clinical PEDV strains indicate that the PEDV CV777 vaccine currently used in China may not fully protect pigs from infection with recent epidemic strains, and will require the development of new vaccine strains.

Key words: Swine diarrhea; porcine epidemic diarrhea virus; genetic evolution analysis; S1 gene.

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Introduction

Diarrhea is one of the most significant diseases currently threatening the pig industry in China. The causes of diarrhea are complicated, and several pathogens can be involved. The common viral pathogens are porcine epidemic diarrhea virus (PEDV), porcine transmissible gastroenteritis virus (TGEV), and porcine rotavirus serotype A (GARV), all of which have similar clinical manifestations. PEDV has the highest infection and incidence rates among these pathogens, and causes acute enteritis and watery diarrhea in pigs. PEDV was first reported in 1978 in Belgium and the UK [1,2]. It was isolated from infected pigs for the first time in 1980 in China, and has since become quite common. Since October 2010, many provinces in China have witnessed outbreaks of diarrhea due to PEDV among pig herds [3], and PEDV has caused substantial economic losses. The pig industry in Eastern China has been greatly impacted by porcine diarrhea. PEDV is an RNA virus. The spike (S)

protein of PEDV is a glycosylated protein that is involved in viral pathogenesis, which contains distinct S1 and S2 domains. The S protein attaches to host cellular receptors, resulting in viral entry by membrane fusion [4-7] and stimulating the production of neutralizing antibodies [4-8]. Mutations in the PEDV S gene are associated with growth adaptation *in vitro* and the attenuation of virulence *in vivo* [9,10]. Mutations play an important role in the structure of the S gene, and in determining isolate diversity, the actual epidemiological characteristics of the infection, and the incidence of epidemics caused by mutated strains [11-13]. S gene sequence analysis is critical for assessing the effectiveness of existing vaccines and for developing additional vaccines. Nucleotide sequence comparison has demonstrated that the S1 domain has a higher mutation rate than the S2 domain and is characterized by higher diversity. In addition, the S1 subunit can bind to specific receptors [14]. According to phylogenetic analyses of the S gene, the PEDV

strains were divided into two distinct clusters, genogroup 1 (G1; classical) and genogroup 2 (G2; field epidemic or pandemic) [12,15], and S-INDEL strains [16]. Most epidemic strains are in the G2 group [15]. The present study was performed to analyze S1 gene variations in prevalent PEDV strains and discovered new clinical strains which warrant the development of new vaccine strain.

Methodology

Sources of samples and viruses

A total of 387 samples were used for the diarrhea virus analysis, including feces and small intestine tissue, were collected from 387 individual diarrheic pigs on 38 pig farms in 4 regions of Eastern China (Zhejiang, Jiangsu, Fujian Provinces, as well as Shanghai) from April 2013 to January 2016. PEDV-positive samples were obtained from the Animal Disease Control Center laboratory of Jinhua Polytechnic. All animal work was approved by Jinhua Polytechnic School Animal Care and Use Committee and the methods were carried out in accordance with the approved guidelines.

Reagents, instruments and primers

PrimeScript One-Step reverse transcription polymerase chain reaction (RT-PCR) Kit Ver.2, RNAiso Plus (total RNA extraction reagent), TaKaRa Ex Taq Hot Start, and DL2000 DNA markers were purchased from Takara Bio Biotechnology (Dalian, China). Agarose were purchased from Sangon Biotech (Shanghai, China). Chloroform, isopropanol, and ethanol were purchased from Shanghai Chemical Reagent (Shanghai, China). The S1000TM Thermal Cycler, Bio-Rad Gel DocTM XR+ UV gel imaging analysis system, and the nucleic acid electrophoresis instrument were purchased from Bio-Rad (Hercules, CA, USA).

Nucleotide sequences of the PCR primers of PEDV-S1 were designed by State Key Laboratory of Agricultural Microbiology in China as follows:

Forward: 5'-CCACCATGAAGTCTTTAACTTACTTCTGGT-3',
Reverse: 5'-GAAATTGGCTGTTCATGACTC-3'.
Correctly sized PCR products were 1497 bp, and were verified by agarose gel electrophoresis and DNA sequencing. The primers were synthesized by Suzhou Genewiz Bio Technology (Suzhou, China).

Sample pretreatment

The samples were collected from the carcasses of pigs that had died of known or suspected viral diarrhea.

Tissue segments of the small intestine (1–2 cm) were placed in homogenate tubes, treated with 250 μ L of phosphate-buffered saline, and subjected to high-speed homogenization. This was followed by freezing and thawing three times and centrifugation at 13,400 \times g for 5 minutes. Finally, 200 μ L of each supernatant was stored at -20°C until analyzed.

Each fecal sample was diluted 10-fold with 10 mM phosphate-buffered saline, vortexed, and centrifuged for 5 minutes at 13,400 \times g. The supernatants were stored at -20°C .

RNA extraction

All supplies used for RNA extraction, including deionized water, were treated with 0.1% diethyl pyrocarbonate (DEPC) to avoid RNase contamination. Each liquid sample (200 μ L) was combined with 1 mL RNAiso Plus and RNA was extracted according to the manufacturer's instructions.

One-step RT-PCR

The extraction of PEDV template RNA was performed following the PrimeScript One-Step RT-PCR kit instructions. The reaction included 2 μ L of PrimeScript One-Step enzyme mix (including PrimeScript reverse transcriptase, TaKaRa Ex Taq Hot Start, and RNase inhibitor), 25 μ L of 2 \times One-Step buffer (including reaction buffer and dNTP mixture (final concentration, 400 μ M)), 1 μ L each of the forward and reverse primers (10 μ M), 5 μ L template RNA, and DEPC water to 50 μ L.

The thermal cycling profile was as follows: 50 $^{\circ}\text{C}$ for 30 minutes; 94 $^{\circ}\text{C}$ for 2 minutes; 35 cycles of 94 $^{\circ}\text{C}$ for 30 seconds, 54 $^{\circ}\text{C}$ for 30 seconds, and 72 $^{\circ}\text{C}$ for 1.5 minutes; and 72 $^{\circ}\text{C}$ for 10 minutes. The PCR products were electrophoresed in 1.5% agarose gels.

Sequencing and sequence alignment of PCR products

The selection criteria for the studied samples: First of all, they must be PEDV-positive samples by rapid detection method [17] from 387 samples. Secondly, their PEDV S1 gene can be successfully amplified and sequenced. Thirdly, the regional representation was also considered. The PEDV S1-positive PCR products were sent to Suzhou Genewiz Bio Technology for sequencing, sequence alignment, and confirmation of the sequencing results using NCBI BLAST software. Gene sequences were analyzed with DNAMAN (LynnonBiosoft), MegAlign (DNASTAR, Inc.), Vector NTI 11.5 (Invitrogen Corporation), and MEGA (Molecular Evolutionary Genetics Analysis) 6.0 software.

Results

Amplification and analysis of S1 gene sequences

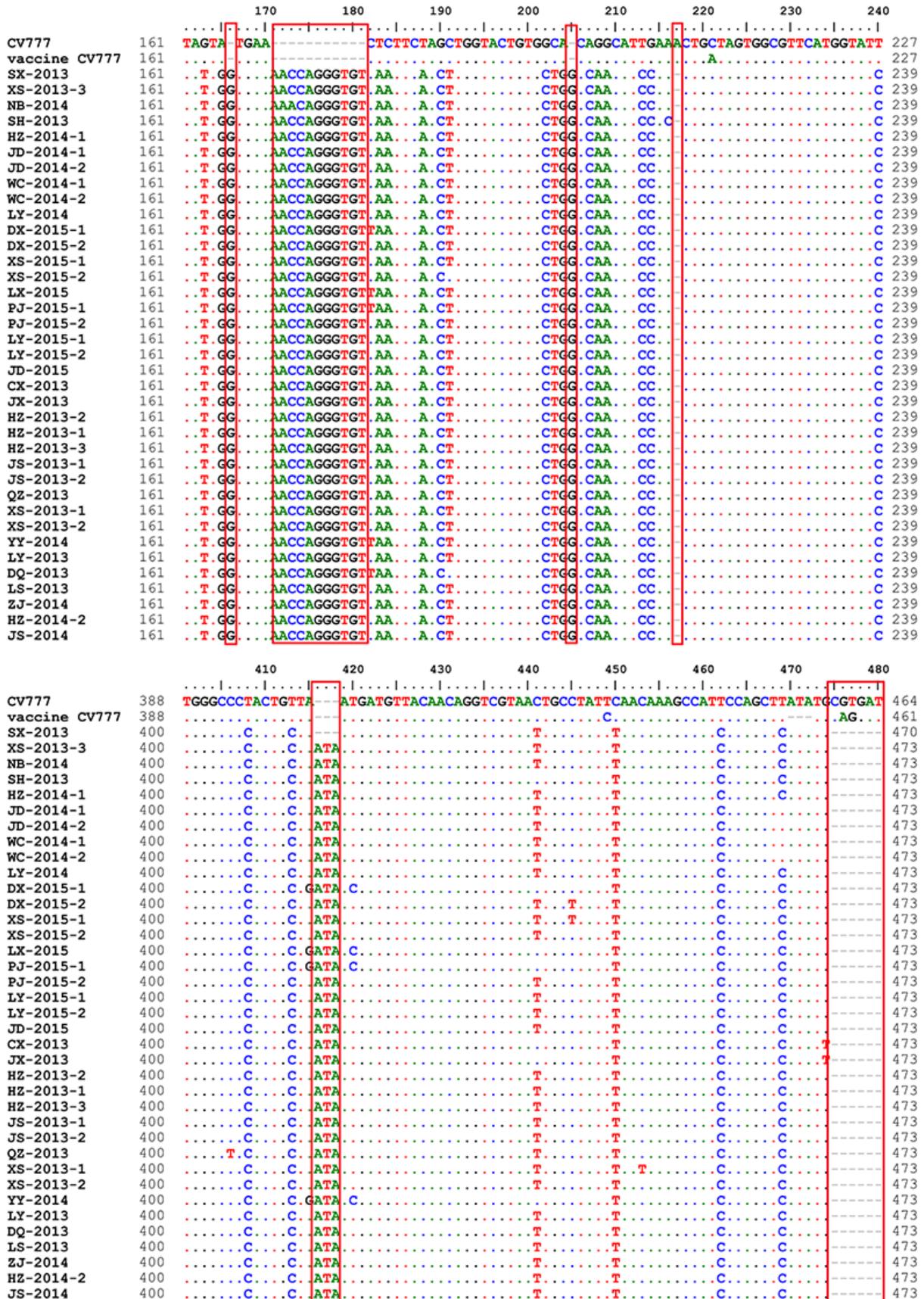
The PEDV-positive rate in the tested samples was 54.5% (211/387) by rapid detection method (Table 1). In this study, according to the selection criteria, 37 strains were finally established. The PEDV S1 genes from 37 selected PEDV-positive samples were amplified by RT-PCR. The amplified S1 sequences were 1494–1497 nucleotides (nt) long; these sequences encoded amino acids (aa) 1–497 and were positioned between nt 20634 and 22131 of the total PEDV S gene sequence. The S1 sequences were compared on the NCBI website, and the target sequence was confirmed.

The 37 epidemic strains from Eastern China were compared with the CV777 and vaccine strains using BioEdit software, and comparatively, nucleotide mutations, insertions, and deletions were observed in all epidemic strains. A 1-nt gene deletion occurred at 217 (A), and a 6-nt deletion occurred at 475–480 (CGTGAT). One-nucleotide gene insertions occurred at 166 (G) and 205 (G), and an 11-nt insertion occurred at 171–181 (AACCAGGGTGT). With the exception of strain SX-2013, all of the strains had a 3 nt (ATA) gene insertion at 416–418 (Figure 1).

Table 1. The PEDV-positive/negative sample number and 37 epidemic PEDV strains selected.

Farms No.	Farms Name	Samples number	Positive number	Negative number	Selected strain number	Selected virus strain name
1	LS	12	3	9	1	LS-2013
2	JX	12	10	2	1	JX-2013
3	JSA	12	4	8	2	JS-2013-1, JS-2013-2
4	YF	10	8	2	0	
5	HZA	13	5	8	3	HZ-2013-1, HZ-2013-2, HZ-2013-3
6	JK	10	4	6	0	
7	GL	9	2	7	0	
8	QZ	9	4	5	1	QZ-2013
9	XSA	10	8	2	3	XS-2013-1, XS-2013-2, XS-2013-3
10	JC	9	5	4	0	
11	ZM	10	6	4	0	
12	CX	11	9	2	1	CX-2013
13	DQ	9	7	2	1	DQ-2013
14	SX	14	6	8	1	SX-2013
15	SH	7	4	3	1	SH-2013
16	LYA	8	5	3	1	LY-2013
17	NB	8	6	2	1	NB-2014
18	HZB	10	10	0	2	HZ-2014-1, HZ-2014-2
19	QF	10	8	2	0	
20	YY	10	6	4	1	YY-2014
21	ZJ	11	7	4	1	ZJ-2014
22	JSB	11	6	5	1	JS-2014
23	JDA	8	6	2	2	JD-2014-1, JD-2014-2
24	ZQ	10	4	6	0	
25	WC	10	5	5	2	WC-2014-1, WC-2014-2
26	DJ	15	3	12	0	
27	LYB	8	4	4	1	LY-2014
28	DX	9	6	3	2	DX-2015-1, DX-2015-2
29	RM	10	4	6	0	
30	XSB	12	7	5	2	XS-2015-1, XS-2015-2
31	HF	10	7	3	0	
32	LX	10	6	4	1	LX-2015
33	PJ	12	5	7	2	PJ-2015-1, PJ-2015-2
34	LK	12	4	8	0	
35	LYC	11	5	6	2	LY-2015-1, LY-2015-2
36	JZ	8	5	3	0	
37	JDB	12	6	6	1	JD-2015
38	HH	5	1	4	0	
Total		387	211	176	37	

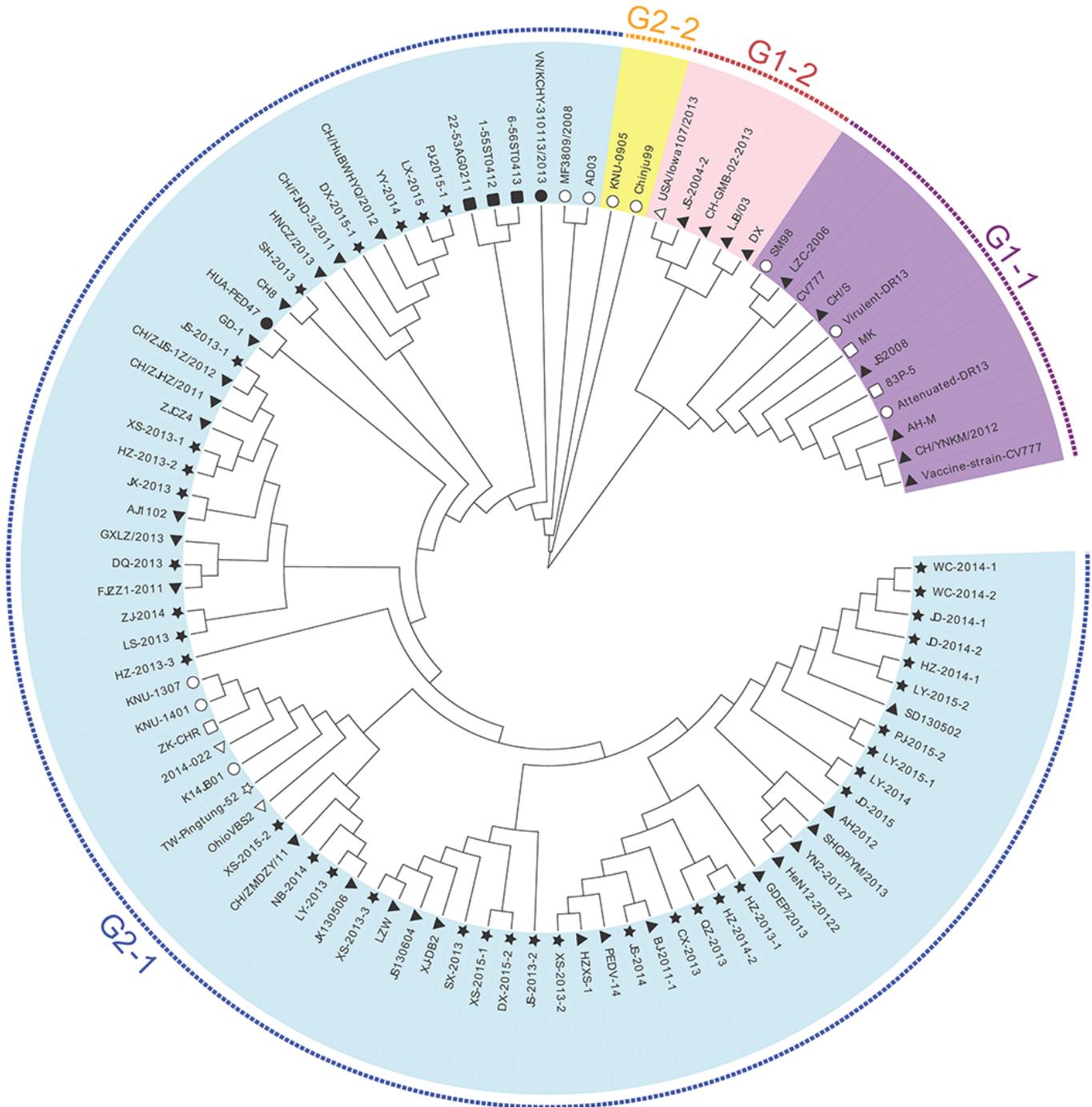
Figure 1. Analysis and comparison of the nucleotide sequences of the S1 genes of 37 epidemic strains.



Most of the strains contained the following mutations: ten bases from G to A (G4A, G80A, G208A, G209A, G256A, G388A, G481A, G590A, G1066A, and G1362A), 29 bases from T to C (T13C, T14C, T71C, T86C, T144C, T149C, T213C, T240C, T250C, T303C, T342C, T390C, T408C, T413C, T462C, T469C, T531C, T600C, T630C, T636C, T742C,

T753C, T1074C, T1082C, T1086C, T1107C, T1188C, T1245C, and T1458C), 30 bases from C to T (C42T, C50T, C78T, C92T, C137T, C140T, C191T, C203T, C246T, C252T, C255T, C336T, C386T, C450T, C550T, C559T, C603T, C642T, C678T, C710T, C801T, C903T, C921T, C987T, C1125T, C1320T, C1356T, C1464T, C1467T, and C1491T), four bases

Figure 2. Phylogenetic tree of the S1 genes of 37 epidemic and reference PEDV strains. The 37 endemic PEDV strains are marked by black stars, Vietnam strains by black circles, Thailand strains by black squares, Chinese local strains isolated in recent years by black triangles, Japanese strains by white squares, South Korea strains by white circles, Taiwan strains by white star, and American and Canadian strains by white triangles. Virus CV777 is the Chinese strain JN599150, attenuated by cell passage.



from A to C (A79C, A207C, A683C, and A900C), seven bases from T to G (T81G, T259G, T491G, T741G, T811G, T945G, and T1375G), eight bases from C to A (C85A, C134A, C184A, C601A, C632A, C840A, C909A, and C951A), 12 bases from A to G (A128G, A165G, A204G, A257G, A389G, A484G, A591G, A604G, A607G, A631G, A792G, and A822G), four bases from T to A (T143A, T183A, T188A, and T258A), four bases from G to T (G163T, G267T, G297T, and G535T), seven bases from G to C (G190C, G202C, G214C, G487C, G688C, G915C, and G943C), two bases from C to G (C244G and C260G), and three bases from A to T (A392T, A483T, and A666T).

Genetic evolution analysis of PEDV epidemic strains

Fifty-eight S1 genes were selected from the GenBank database as reference sequences for genetic evolution analysis. Mega6.0 software was used to construct a neighbor joining phylogenetic tree. Genogroup 2 (G2; field epidemic or pandemic) consisted of 77 strains. G2-1 included the 37 endemic strains, 24 strains isolated from China in the last five years, a strain from the USA (OhioVBS2), a Canadian strain (2014-022), a Japanese strain (ZK-CHR), five Korean strains (K14JB01, KNU-1401, KNU-1307, MF3809/2008, and AD03), two Vietnamese strains (VN-KCHY-310113-2013 and HUA-PED47), three Thai strains (22-53AG0211, 1-55ST0412, and 6-56ST0413), and one Taiwanese strain (TW-Pingtung-52). G2-2 consisted of two Korean strains, Chinju99 and KNU-0905.

Genogroup 1 (G1; classical) consisted of 17 reference strains. G1-1 was composed of two Japanese strains (MK, 83P-5), three Korean strains (SM98, virulent Korea DR13, and attenuated DR13), the standard PEDV strain (CV777), the vaccine strain CV777, and five strains isolated in China (LZC-2006, CH/S, JS2008, AH-M, and CH/YNKM/2012). G1-2 contained one American strain (Iowa107) and four Chinese strains (JS-2004-2, CH-GMB-02-2013, LJB/03 and DX; Figure 2).

Nucleotide and amino acid homology analyses of the PEDV partial S1 gene

Among the endemic and reference strains, the nucleotide sequence homology was 96.12% integrally. The homologies between the 37 endemic strains and the CV777 reference and vaccine strains were 90%–91% and 88.6%–89.7%, respectively. The 37 endemic strains had the highest homologies to the reference strains K14JB01 (Korea), TW-Pingtung-52 (Taiwan), 2014-022 (Canada), and OhioVBS2 (USA; all 99.3%).

However, homology was lowest with the vaccine CV777 strain, at only 88.6%–89.7%. The homology to the reference strain JS2008, which was isolated in China in 2008, was 88.9%–90.1%, while the homology between the three strains isolated from China more recently, AH2012, BJ-2011-1, and PEDV-14, was 97.1%–100%.

A similar phenomenon was observed at the amino acid level. The homology of the amino acid sequences was 95.71% as a whole. The 37 endemic strains had highest homologies to the reference strains K14JB01 (Korea) and OhioVBS2 (USA; both 99.8%), whereas homology to the vaccine CV777 strain was lower, at 86.2%–87.8%.

Analysis of the deduced amino acid sequences of the partial S1 gene

In this study, the amplified partial S1 gene was 1497 nt, encoding 495–497 aa. The PJ-2015-1, PJ-2015-2, and LY-2015-1 genes encoded 495 aa proteins, the SX-2013, XS-2015-2, LX-2015, LY-2015-2, JD-2015, and DX-2015-1 genes encoded 496 aa proteins, and all other strains encoded 497 aa proteins. DNAMAN software was used to analyze the aa results, and as shown in Figure 3, compared with the CV777 reference strain, the epidemic strains had a total of 44 mutation sites, including five aa insertions (NQGV inserted at 57–60 and N inserted at 143), and two aa deletions (at 166–167). The S1 gene had five hypervariable regions: at 26–28 (from QST to SAN), at 54–56 (from SMN to IGE), at 67–71 (from GTGIE to AGQHP), at 160–167 (from YMRDGKDI to HMSEHS--), and at 204–206 (from RRS to SGG); two two-aa mutations (at 133–134 from DN to SI, at 250–251 from DS to EP); and a number of single aa mutations (I4T, I123T, P14S, S61N, S63T, L81V, Y83H, D85R, V141A, A182S, H187Y, L190F, L350F, R200K, T214E, Y231S, T240I, D318Q, E373Q, and S462A). In addition, the 37 epidemic strains had different aa mutation sites and deletions. The deletion and insertion sites were similar to those of K14JB01 (the Korean strain) and OhioVBS2 (the American strain).

Prediction of S1 protein glycosylation sites and epitopes

The N-glycosylation sites of the S1 protein from the 37 strains were predicted using NetNGlyc 1.0 (CBS Prediction Servers) [18]. S1 protein of CV777 vaccine strain has 11 N-glycosylation sites (56NSSF, 111NTSA, 126NKTL, 211NVTS, 228NCSG, 259NDST, 295NQTM, 319NDTF, 339NLSF, 346NSSD, 420NFTG). Compared with the CV777

vaccine strain, in SX-2013 S1 protein, six aa mutations (N56E, S113N, N126I, S230I, M298I, and F322S) and one insertion (NQG V at 57–60) resulted in the destruction of the N-glycosylation site. And the mutation of six other aa (S61N, S63T, S117N, I119T, F326S, and K380N) increased the number of N-glycosylation sites. Figure 4 shows 10 predicted N-glycosylation sites (61NSTW, 117NATA, 214NVTS, 262NDST, 298NQT I, 322NDTS, 342NFSF, 349NSSN, 379NSTV, 423NFTG) of the SX-2013 S protein.

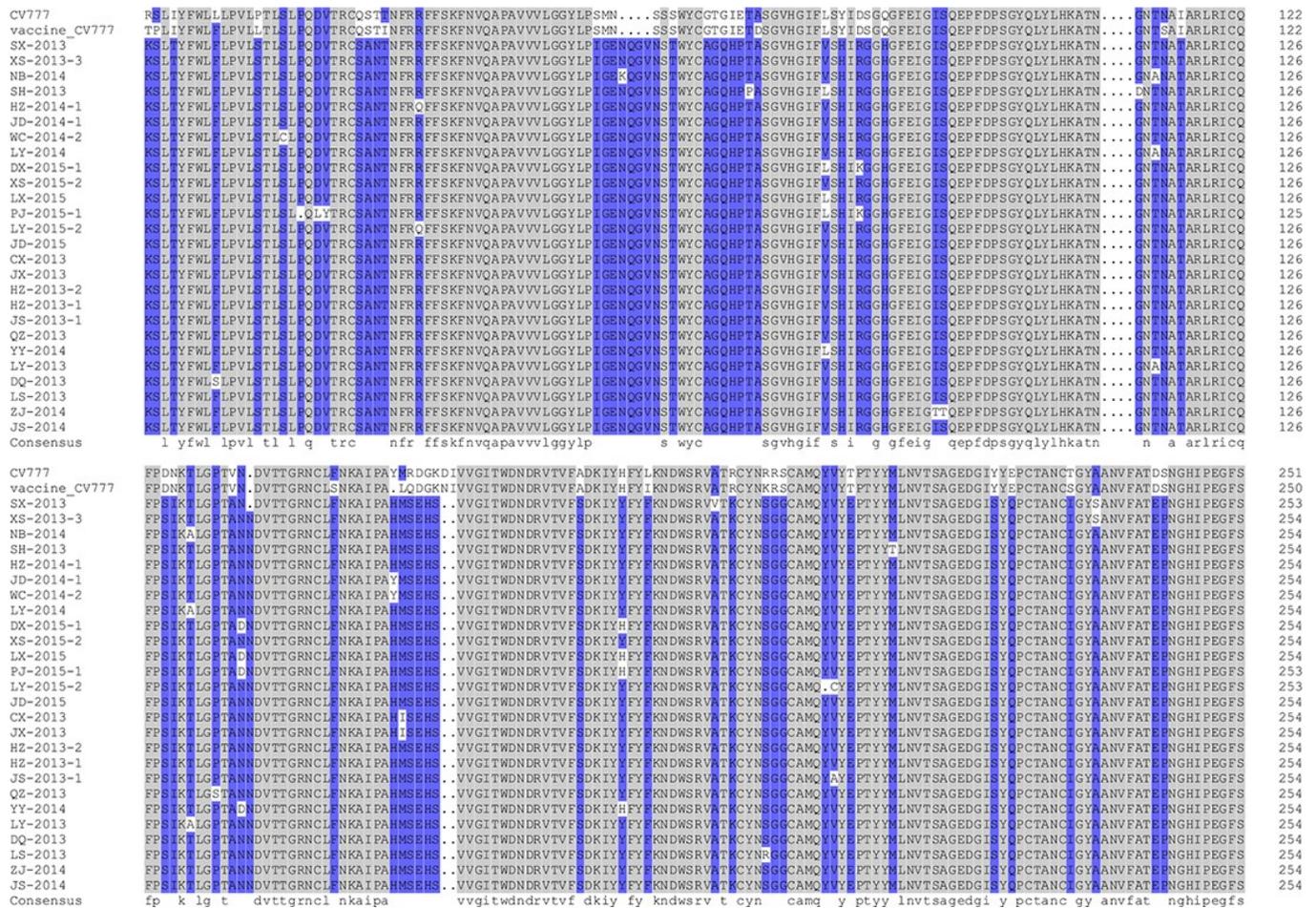
The signal peptide cleavage site and number were predicted to check whether signal peptide has been affected which is relevant to the protein’s targeting at virus surface using SignalP 4.1 (CBS Prediction Servers) [19] online signal peptide prediction software. The results showed that the 37 epidemic strains and the CV777 standard strain all contained only one signal peptide. The signal peptide of the PJ-2015-1 strain spanned aa 1–26, with the cleavage site at aa 26; the rest of the strains contained signal peptides from aa 1–19, with cleavage at aa 19.

Discussion

The major symptom of diarrheic piglets caused by PEDV is watery, gray-yellow, or gray diarrhea, which is sometimes preceded by vomiting. Dehydration in piglets is serious, with death occurring within 2–4 d, and a nearly 100% mortality rate. In a laboratory epidemiological survey, porcine epidemic diarrhea (PED) was much more prevalent from November to January in 2010–2012 [20]. The incidence of PEDV in piglets was 10%–100%, and the mortality rate reached 40%–92.58% on some PEDV-exposed pig farms. In the present study, the prevalence of PEDV was as high as 54.5%, and was the main viral pathogen for diarrhea in Eastern China, consistent with reports on PEDV in other provinces in the past five years [21–23].

In this study, G2 was divided into two subgroups including G2-1 and G2-2 by evolutionary tree software based on certain algorithms, reflecting the genetic distance and the branch of evolution. G2-2 consisted of two Korean strains, Chinju99 and KNU-0905. The 37 strains were concentrated in the G2-1 subgroup, and all contained mutations, deletions, and insertions (mainly

Figure 3. Analysis and comparison of the amino acid sequences of the S1 genes of 37 epidemic strains.

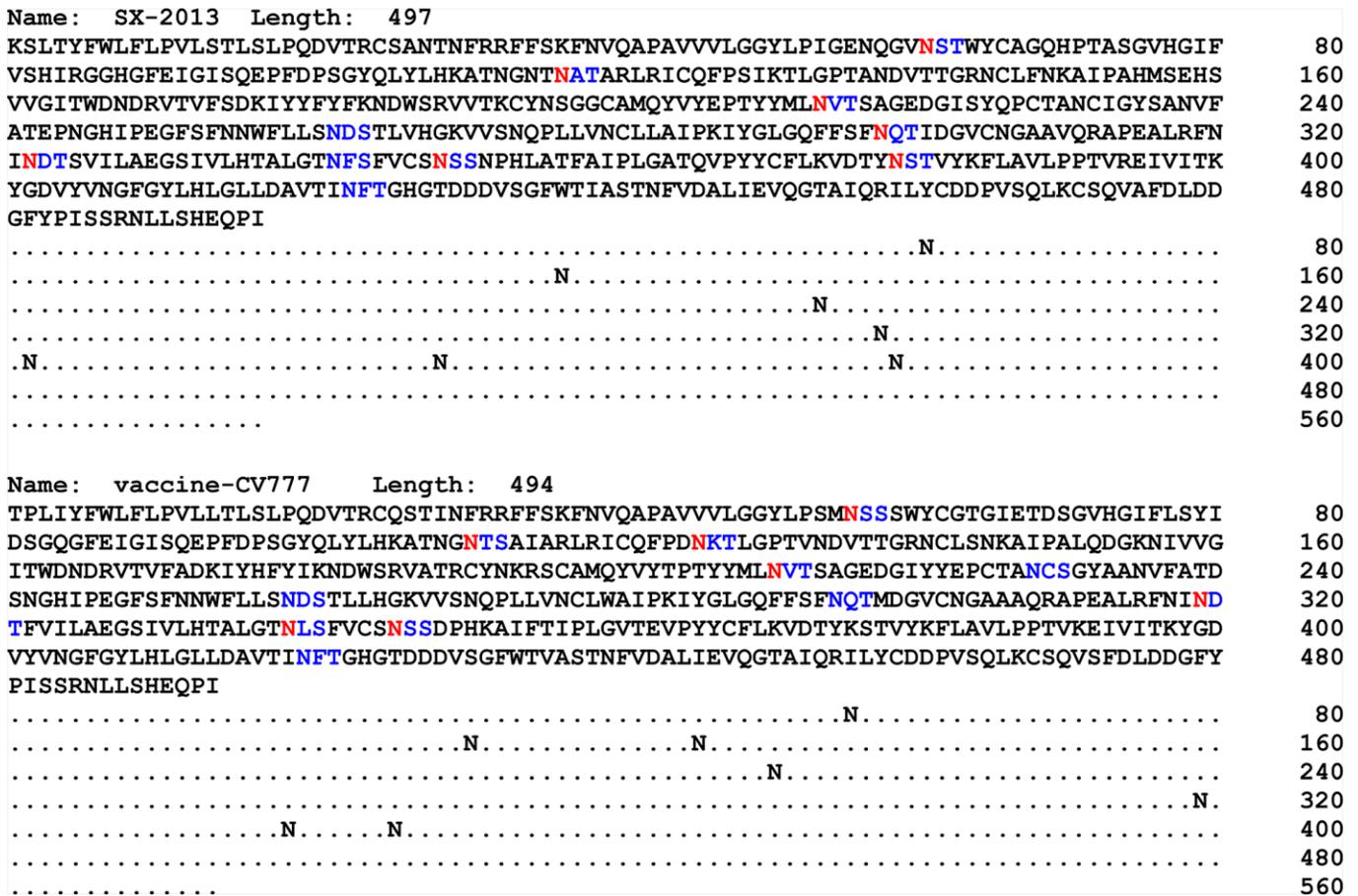


mutations and insertions) compared with the CV777 vaccine strain and older Chinese isolates. PEDV evolutionary strains were prevalent in China, as with the exception of earlier isolated strains such as the CH/S, LJB/03, DX, and JS-2004-2, the 37 strains obtained in this study were, in terms of homology and evolutionary relationships, more similar to strains isolated in recent years in South Korea (K14JB01, KNU-1401, and KNU-1307), Vietnam (VN-KCHY-310113-2013 and HUA-PED47), Thailand (22-53AG0211, 1-55ST0412, and 6-56ST0413), the USA (OhioVBS2), and Canada (2014-022). Thus, PEDV undergoes continuous variation, and the strains isolated after 2013 are different from earlier strains (such as JS-2004-2), consistent with similar reports [24,25]. Moreover, strains recently isolated in Asia, the USA [26-31], Canada [32], and Europe [16] display high homology. Although the route of transmission is not clear, it has been theorized that contamination of feed or feed ingredients [32,33] or equipment and vehicles used to transport pigs [27] are the main routes of

transmission, although further investigation and surveillance are required. However, the homology between the recent endemic strains and the PEDV vaccine strain currently in widespread use is not high, which may impact the protective effect of the vaccine.

The prediction of the N-glycosylation sites of the PEDV S protein has important biological significance [32]. A large number of aa mutations have been reported in the S1 region [28]. In this study, the S1 proteins of the 37 strains were all predicted to have ten glycosylation sites and 41 amino acid mutations compared with the CV777 vaccine strain which has eleven glycosylation sites. In contrast, the two strains from Vietnam had seven glycosylation sites, six strains (CV777, USA-Iowa107-2013, virulent DR13, attenuated DR13, vaccine strain CV777, and JS2008) had 11, and two strains from South Korea (SM98, 83P-5) had 12. Some amino acid mutations and insertions changed the N-glycosylation sites. Because the S1 domain is the major epitope region, these sites may alter the antigenicity and pathogenicity of the strains, and

Figure 4. Predicted glycosylation sites of the (SX-2013 and CV777 vaccine stains) S1 protein.



Asn-Xaa-Ser/Thr sequences in the sequence output below are highlighted in blue; Asparagines predicted to be N-glycosylated are highlighted in red.

further studies addressing this are required. However, signal peptide prediction results for the 37 strains were similar to the CV777 strain, indicating that the signal peptide and cleavage site were stable.

Conclusion

Given the increasing incidence of swine PEDV in China, PEDV coinfection with other viral pathogens will further increase the incidence of PED, which occurs widely in China. A diarrhea epidemic occurred despite immunization with attenuated or inactivated diarrhea triple vaccine (PEDV+TGEV+GARV) or bivalent vaccine (PEDV+TGEV) at 43.5% of pig sites [34], indicating that the protective effect of the current PEDV vaccine needs further improvement [35]. But the CV777 vaccine is G1 genogroup and samples are G2 genogroup, like Sato *et al.* [36] show partial protection against G2 genogroup with G1 genogroup vaccine. Because the vaccine protection can depend on vaccine strain and other factors such as coinfection with other virus, inactivation method of virus or vaccine administration way etc. There are large variations in the S1 genes of Eastern China PEDV strains compared with the Chinese vaccine strain CV777, indicating rapid gene mutation in epidemic PEDV strains. More effective surveillance and management strategies need to be developed for porcine diarrhea. Our study also highlights the need to improve current vaccines and simultaneously take steps to enhance the immunity of sows to control the spread of pathogens to piglets. Future work is needed to develop a new vaccine strain different from CV777 based on our present study such as mix of inactivated virus G1+G2 genogroup to enhance the vaccine efficacy against newly emerged PEDV.

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Authors' contributions

JCY, ZXJ and HJF carried out the studies, participated in collecting data, and drafted the manuscript. HHJ, ZCY, ZHB and JJJ performed the statistical analysis and participated in its design. WL, GBQ, WYL and LYJ helped to draft the manuscript. All authors read and approved the final manuscript.

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Corresponding author

Chunyan Jiang, PhD
Wuzhou street No. 1188, Wucheng district, Jinhua
Zhejiang Province, P.R. China 321007
Tel: 86-579-82230787
Fax: 86-579-82230787
Email: chyjiang@hotmail.com

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