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RESEARCH ARTICLE

Dynamic Investigation of Porcine Epidemic Diarrhea and Analysis Sequence of Spike Gene in South China over the Past Five Years

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ABSTRACT

The study executed a large-scale molecular epidemiological investigation of porcine epidemic diarrhea (PED) and five-yearlong monitoring of nine swine herds with the outbreak of diarrhea in south China. Porcine epidemic diarrhea virus (PEDV) exists all year round with varying degrees of mortality to suckling piglets, the highest mortality was in 2011 and followed by 2012, the lowest mortality was in 2013, with the mortality showed an increasing trend year by year during 2014 to 2015. It was noteworthy that the pregnant sows assumed the highest morbidity, followed by nursing sow while the nursed piglets and the growing pigs had lower morbidity. The current epidemic strains in south China were divided into two groups, Group 1 and Group 2 by generating the phylogenetic tree of thirty-five spike (S) genes during 2011-2015. Group 1 had 6.92-7.21% mutation rate while Group 2 owned 4.18% mutation rate when compared with CV777. The strains in Group 2 felled into the same branch with the previous Chinese isolates from 2004 while the strains in Group 1 had a close relationship with the United States strain. Interestingly, clinical morbidity caused by two types of PEDV strains were quite different, strains in G2-2 caused relatively minor mild clinical manifestations, presenting a smaller mortality and shorter onset period, while strains in G1-2 and G1-3 is caused by an outbreak of acute infectious diseases, showed a larger mortality and longer period especially PEDV strains in G1-3. It was also indicated that the effect way to prevent PEDV should focus on reduce the morbidity of pregnant sows.

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INTRODUCTION

Porcine epidemic diarrhea (PED) was caused by porcine epidemic diarrhea virus (PEDV) which was characterized by watery diarrhea, vomiting, dehydration, and a high mortality rate among swine (Vui, 2014; Alvarez *et al*., 2016). PEDV is an enveloped, single-stranded RNA virus, belongs to the family Coronaviridae. The genome contained seven open reading frames (ORFs) that encode four structural proteins: spike (S), envelop (E), membrane (M), and nucleocapsid (N) respectively (Luo *et al*., 2012;Wei *et al*., 2012). Spike (S) was believed as an important determinant for PEDV biological properties (Sun M *et al*., 2015) and was essential for ascertaining the genetic relationships among PEDV isolates, as well as the association between PEDV viral function and genetic mutations (Chen *et al*., 2013; Jung *et al*., 2015).

PEDV was reported in United States in April 2013 firstly (Stevenson *et al.*, 2013) and it has since been confirmed in Canada, Mexico, Germany, France, Switzerland, Hungary, Italy, and Vietnam (Song *et al*., 2006; Puranaveja *et al*., 2009; Chen *et al*., 2010; Park *et al*., 2011; Vui, 2014; Opriessnig, 2015; Scott *et al*., 2015; Alvarez *et al*., 2016). In China, PED was occurred before 2008(Chen *et al*., 2008). However, variant strains of PEDV associated with rapidly spread and large-scale outbreaks of diarrhea have emerged in China since Oct 2010, affecting pigs characterized by high mortality risks

among sucking piglets within 7 days and resulting in the death of >1,000,000 piglets (Sun *et al*., 2012), resulting a serious and continuous concern for the swine industry and significant economic losses in China. Most of the affected swine farms lost all of their newborn piglets which was distinguished from the previous ones which had been reported (Puranaveja *et al*., 2009). However, it was found relatively uncommon among weaners and growers, few of them showed any clinical signs during the outbreaks, this was the similar popular features with those in Korea and Thailand(Puranaveja *et al*., 2009; Song *et al*., 2013). Many reports had described the epidemiologic feature of porcine epidemic diarrhea in China. Sun RQ reported that the PEDV positive rate was 82.0% (105/128) in fecal and intestinal samples (Sun *et al*., 2012). The findings suggested that porcine epidemic diarrhea was very serious in China during Oct 2010 to 2012(Chen *et al*., 2012; Li *et al*., 2012; Li *et al*., 2012; Pan *et al*., 2012; Yang *et al*., 2013) . However, the performance of PED became less severity with reduced morbidity and mortality in the clinical since May 2013. Most pig farms did not appear with acute outbreaks of PED no longer, clinical signs became less and less. The reason why there was a huge conversion with the clinical of PED is not clear.

Even though popular features of PEDV had been investigated in China, there is a lack of knowledge about the dynamics of disease spread. In the study, a large scale dynamic investigation and five yearlong monitoring of 9 swine herds with the outbreak of diarrhea located in geographically separate regions in south China were conducted. The morbidity and mortality on affected farms were mainly determined. In addition, the full-length S gene of thirty-five PEDV field strains were determined to obtain the relationships and genetic diversity of PEDVs in south China over the past five years.

MATERIALS AND METHODS

Sampling: Porcine intestinal and fecal samples were collected between January 2011 and Jul 2015 from 18 swine farms in five provinces (Guangdong province, Guangxi province, Fujian province, Sichuan province, Jiangsu province) in south China with the piglets younger than 7 day old that showed watery diarrhea and dehydration. Each farm had more than 3000 breeding sows, in which management practices and hygienic conditions are generally satisfactory, most of the farms adopt an all-in-all-out system. The mortality of nine PEDV infection farms from January 2011 to Jul 2015 in south China were collected, which included suckling piglets, pregnant sows, nursing sows, nursed piglets and the growing pigs (Table 1).

Diagnosis of porcine epidemic diarrhea virus: The morbidity and mortality of the nine selected farms was accurate recorded by Office Software 2010. The disease was diagnosed by clinical symptoms first, and then the intestinal and fecal samples were sent to laboratory to confirm the presence of virus using duplex RT-PCR assay.

Determination of S genes: RNA was extracted from the supernatant using TRIzol Reagent (Invitrogen Corp, Carlsbad, CA, USA) following the manufacturer's instructions. The primers were shown in Table 2. The S gene of PEDV-positive strains were analyzed and submitted to GenBank.

Table 1: The information of pig farms infected by PEDV, China, 2011-2015

Herd name	Geographic origin	Herd size	Use commercial vaccines ([TGEV H] + [CV777])	Management mode
No.1	Guangdong province	5000	Yes	all-in-all-out system
No.2	Guangdong province	4000	Yes	all-in-all-out system
No.3	Sichuan province	5000	Yes	all-in-all-out system
No.4	Guangxi province	5000	Yes	all-in-all-out system
No.5	Jiangsu province	4000	Yes	all-in-all-out system
No.6	Guangdong province	5000	Yes	all-in-all-out system
No.7	Guangxi province	5000	Yes	all-in-all-out system
No.8	Fujian province	5000	Yes	all-in-all-out system
No.9	Guangdong province	4000	Yes	all-in-all-out system

Table 2: The primers used in our research

Primers	Sequence	Size and aim fregent
SF1	5'-CATTTGTGGCTTTTCTAATC-3'	S gene (4152-4161 bp)
SR1	5'-AGCACCACTAGTGACATTCTT-3'	
SF2	5'-GATTCTGGACAGTTGTTAGC-3'	
SR2	5'-CTTCGAGACATCTTTGACAAC-3'	

Sequence analysis and phylogenetic analysis of S genes: Nucleotide sequences were analyzed using the CLUSTALX v1.83, Bioedit v7.0.5.2 programs, MegAlign software (MEGA, version5.0) and DNAstar for alignment and sequence analysis. Phylogenetic tree was constructed using molecular evolutionary genetics analysis (MEGA, version5.0) with the neighbor-joining (NJ) method. Bootstrap values were estimated for 1,000 replicates. The reference strains used for phylogenetic analysis with PEDV strains were described in Table 3.

RESULTS

Statistics of the mortality and morbidity in infected pig farms: The recorded date in Office Software 2010 showed that there was 50-100% mortality rate in suckling piglets in 2011 and 0-30% mortality rate during 2012 to 2015. PEDV exists all year round with varying degrees of mortality to suckling piglets, the highest mortality was in 2011 and followed by 2012, the lowest mortality was in 2013, with the mortality showed an increasing trend year by year during 2014 to2015. The curve range of morbidity in 2011 was consistent with the range 2012, 2013 and 2014. (Fig. 1). The performance of the nine farms among suckling piglets in 2011 was listed separately due to the high mortality. It was shown that PEDV outbreak among the whole year with the highest mortality in February that arised in No.7 farm and No.1farm respectively. The mortality of the PEDV maintained in a high level until the August 2011 (Fig. 2).

In contrary, the outbreak of the PED brought mild impact on the nursing sows, nursed piglets and the growing pigs with morbidity ranged from 20-80%. The clinical signs of PEDV infected gilts and sows including anorexia, depression, agalactia, transient distaste, and watery diarrhea. The morbidity of pregnant sows, nursing sows, nursed piglets and the growing pigs were shown in Fig. 3. The pregnant sows assumed the highest morbidity, followed by nursing sow while the nursed piglets and the growing pigs had lower morbidity. It was noteworthy that the diarrhea epidemic caused the most significant impact on the pregnant sows with nearly one thousand sick pigs at No.7 farm in Aril 2012. It is interesting that morbidity of the pregnant sows was very low in February while the mortality of the piglets was very high in February.

Table 3: Details of the strains and the reference strains used in this study

Reference strains	Geographic origin	Accession no.	Recently Chinese PEDV field strains	Geographic origin	Accession no.
AH2012	China,2012	KC210145	CH-HKC-08-2011	China, 2011	JX242462
BJ-2011-03	China, 2006	DQ985739	CH-HYC-08-2011	China, 2011	JX242454
Brl-87	France, 1993	Z254483	CH-HYC-11-2011	China, 2011	JX242456
CH-FJND-3-2011	China, 2011	JN381492	CH-HYC-10-2011	China, 2011	JX242455
CNU-091222-01	Korean, 2011	JN184634	CH-LC-10-2011	China, 2011	JX242458
CV777	England, 2001	JN599150.1	CH-HYC-12-2011	China, 2011	JX242457
DR13	Korean, 2006	DQ862099	CH-ZWC-12-2011	China, 2011	JX242461
GDEP-2013	China, 2013	KF601200.1	CH-YHC-12-2011	China, 2011	JX242460
GXHZ-2013	China, 2013	KF601199.1	CH-SONGB-12-2011	China, 2011	JX242459
GXLZ-2013	China, 2013	KF601195.1	CH-SHT-12-2011	China, 2011	JX242464
GXNN-2013	China, 2013	KF601201.1	CH-LC-12-2011	China, 2011	JX242463
HNCZ-2013	China, 2013	KF601197.1	CH-GGC-11-2012	China, 2012	KC787538
IA1	USA,2013	KF468754	CH-LNC-01-2012	China, 2012	KC787541
JS-2004-2	China, 2004	AY653204	CH-STC-12-2012	China, 2012	KC787543
JXJA-2013	China, 2013	KF601198	CH-YGC-12-2012	China, 2012	KC787544
LJB-03	China, 2003	DQ985739.1	CH-YXC-01-2013	China, 2013	KC787545
LZC	China, 2006	EF185992	CH-CCC-01-2013	China, 2013	KC787536
MEX-104-2013	USA,2013	KJ645708	CH-DLC-01-2013	China, 2013	KC787537
OH851	USA,2013	KJ399978	CH-GMB-02-2013	China, 2013	KC787539
PC21A	USA,2014	KM392225.1	CH-HGC-01-2013	China, 2013	KC787540
SM98	Korean, 2010	GU937797	CH-SBC-03-2013	China, 2013	KC787542
TW-Chiayi-24	Taiwan,2015	KP276244	CH-CCC-2013	China, 2013	KT388421
TW-Chiayi-32	Taiwan,2015	KP276246.1	CH-GLC-2013	China, 2013	KT388420
TW-Pingtung-63	Taiwan,2015	KP276250	CH-HSY-2013	China, 2013	KT388419
TW-Yunlin-71	Taiwan,2015	KP276249	CH-LXC-2014	China, 2014	KT388418
TW-Yunlin-91	Taiwan,2015	KP276248.1	CH-SBC-2013	China, 2013	KT388417
USA-Colorado30-2013	USA,2013	KJ645638	CH-STC-2014	China, 2014	KT388416
USA-Iowa-18984-2013	USA,2013	KJ645694	CH-STNC-2014	China, 2014	KT388409
USA-Minnesota84-2013	USA,2013	KJ645707.1	CH-TPC-2014	China, 2014	KT388415
USA-Texas128-2013	USA,2013	KJ645697	CH-XDC2-2015	China, 2015	KT388414
			CH-XDC-2015	China, 2015	KT388413
			CH-XNC-2014	China, 2014	KT388412
			CH-XWC-2014	China, 2014	KT388411
			CH-YYC-2015	China, 2015	KT388410
			CH-CWC-2013	China, 2013	KT948011

Genetic analysis of the S genes: Nucleotide and deduced amino acid sequences of the S genes of PEDVs isolated in south China were determined and submitted to Genbank (Table 3). Sequencing result displayed that the S gene consisted of 4152-4161 nucleotides, encoded a 1384-1387 amino acid (aa)-long peptide. Four strains (CH-SHT-12-2011, CH-STC-12-2012, CH-HKC-08-2011 and CH-GMB-02-2013) have unique characteristics, different from other south Chinese strains and the reference strains. They have 1-aa (N) insertions at position 163 and 58 amino acids different when compared with CV777 and the mutation rate was 4.18%. All S genes (Except four strains above) of south Chinese strains had 4-aa (QGVN) and 1-aa (N) insertions between positions 59-62 and 140 when compared with CV777 (The result was showed in Table 3). All S genes (Except four strains above) of south China strains had 96 amino acids different when compared with CV777 and the mutation rate was 6.92%, the variations of amino acids were identical to the United States strains (PC21A, IA1, MEX-104-2013 and USA-Colorado30-2013) and Taiwan strains (TW-Chiayi-32). In addition, the variations of amino acids in south Chinese strains were similar to Korean strains isolated in 2011 (CNU-091222-01 and CNU-091222-02).

Phylogenetic analysis of the S gene: Thirty-five S genes which were determined during 2011 to 2015 and thirty reference strains were selected to construct the phylogenetic tree on the basis of nucleotide and deduced amino acid sequences (Fig. 5). The result showed that all PEDV strains could be divided into two groups, Group1 and Group 2. Group1 have four subgroups, G1-1, G1-2, G1-3 and G1-4. Subgroup G1-1 comprises seven United States strains, five Taiwan strains and two south China strains; G1-4 comprises one Korean strain and one Chinese strain. Twenty-five south China strains isolated during 2011 to 2015, eight Chinese strains (isolated in 2012-2013) and one United States strain formed subgroup G1-2 and G1-3. It is remarkably that the subgroup G1-2 comprises most of the strains isolated in 2012-2015 while the subgroup G1-3 comprises most of the strains isolated in 2011. Group2 have two subgroups, G2-1 and G2-2. Subgroup G2-1 contains PEDV isolates that included three previous strains and vaccine strain; Subgroup G2-2 comprises nine Chinese strains, one of them isolated in 2004 (JS-2004-2) and eight else isolated 2011-2015 in our study. It was also found that among thirty-five south China strains, twenty-five of them have a close relationship with the United States strain (OH851-5), two of them have a close relationship with the United States strains and Taiwan strains while eight else were felled into the same branch with the previous Chinese isolates from 2004.

Sequence homology analysis of the S gene: Sequence homology results showed that the south China strains shared 93.0-100% nucleotide sequence identity with each other and 92.4-99.0% with the thirty reference strains reported in GenBank respectively. PEDVs in Group1 have 97.5-100% nucleotide sequence identity with each other, and they have 93.7-95.9% sequence identity with the strains in Group 2. More precisely, eight south China

strains in G2-2 have lower (94.8-95.7%) sequence identities with other twenty-seven Chinese field PEDV strains in subgroup G1-2 and G1-3. The subgroups G1-2 and G1-2 have 97.9% -98.7% homologies with each other. According to the south China strains property analysis, eight strains in G2-2 had lower identity to other south China strains in subgroup G1-2 and G1-3, whereas the strains had higher sequence identity with early reference strain isolated in 2004 (JS-2004-2). These results indicated that there were at least two types of PEDV strains existing in south China. Interestingly, clinical morbidity caused by two types of PEDV strains were quite different, strains in G2-2 caused relatively minor mild clinical manifestations, presenting a smaller mortality and shorter onset period , while strains in G1-2 and G1-3 is caused by an outbreak of acute infectious diseases, showed a larger mortality and longer period especially PEDV strains in G1-3.

DISCUSSION

Since its emergence in late 2010, PEDV has continued to cause huge damage related to the economic and management in the swine industry in China (Sun *et al*., 2012; Wang *et al*., 2013; Sun *et al*., 2015). Although several control methods including vaccination and strict biosecurity have been implemented, several herds continue to experience repeated outbreaks (Lee *et al*., 2015; Opriessnig, 2015). The disease has developed to an endemic stage that has led to the necessity for further investigation into the genetic diversity of PEDV which may facilitate the development of a more successful control program and vaccines.

From five yearlong monitoring, it showed that the number of piglets died were enormous and the economic losses were significant while the impact of the health of other pig groups were not as severe as the impact on piglets to some degrees and the economic losses were less profound on other pig groups during this epidemic of PEDV.The result was consistent with national reports in 2011-2013 (Pan *et al*., 2012; Sun *et al*., 2012; Yang *et al*., 2013).

It was noteworthy that the pregnant sows assumed the highest morbidity (for example, the diarrhea epidemic caused the most significant impact on the pregnant sows with nearly one thousand sick pigs at No.7 farm in Aril 2012), followed by nursing sow while the nursed piglets and the growing pigs had lower morbidity. It was indicated that the effect way to prevent PEDV should focus on reduce the morbidity of pregnant sows. Jung (Jung *et al*., 2015) also indicated that prevention and control measures to reduce the impact of PEDV during epidemics should focus on pregnant sows mainly to decrease the mortality in suckling pigs. The active immunization in growing pigs was crucial to prevention the endemic infection of PEDV.

By generating the phylogenetic tree, some characters of prevalent PEDV strains in this diarrhea epidemic were found. The current epidemic strains are divided into two groups, Group 1 and Group 2. The two types of the PEDVs had unique genetic features from the genetic analysis of the S genes. Group 2 had 4.18% mutation rate while Group 1 owned 6.92%-7.21% mutation rate when compared with CV777. Group 2 felled into the same branch with the previous Chinese strains while Group 1 had a close relationship with the United States strain. Between the two types of the PEDV strains identified in China 2012-2015, Group 1 has a closely relationship to US strains reported in 2013, whereas the precise propagation path related to the introduction of PEDV into America remains undetermined (Jung *et al*., 2015 ; Scott *et al*., 2015; Sun *et al*., 2015) . Interestingly, clinical morbidity caused by two types of PEDV strains were quite different, strains in G2-2 (the farm number was No.6, No.4 and No.9)caused relatively minor mild clinical manifestations, presenting a smaller mortality and shorter onset period, while strains in G1-2 and G1-3 is caused by an outbreak of acute infectious diseases, showed a larger mortality and longer period especially PEDV strains in G1-3.Could we conclude, Group 1 and Group 2 (the farm number was No.1, No.3, No.7, *et al*.) on behalf of the highly pathogenic strains and the low pathogenic strains during the PEDV epidemic during 2011- 2015 in south China respectively?

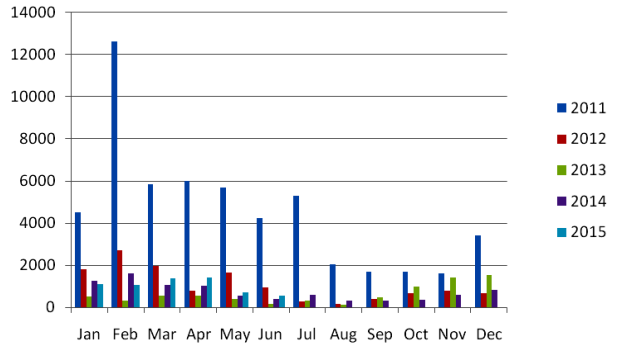

Fig 1: The mortality of piglets in nine farms during 2011 to 2015

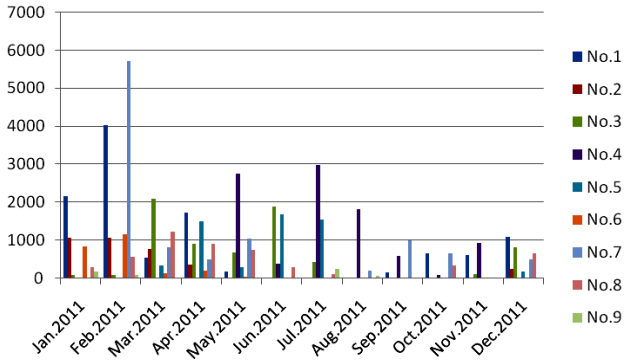

Fig 2: The mortality of piglets in nine farms in 2011

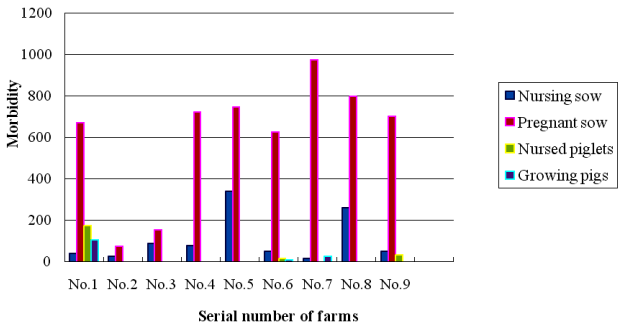

Fig. 3: The morbidity of pregnant sows, nursing sows, nursed piglets and the growing pigs in 2011.

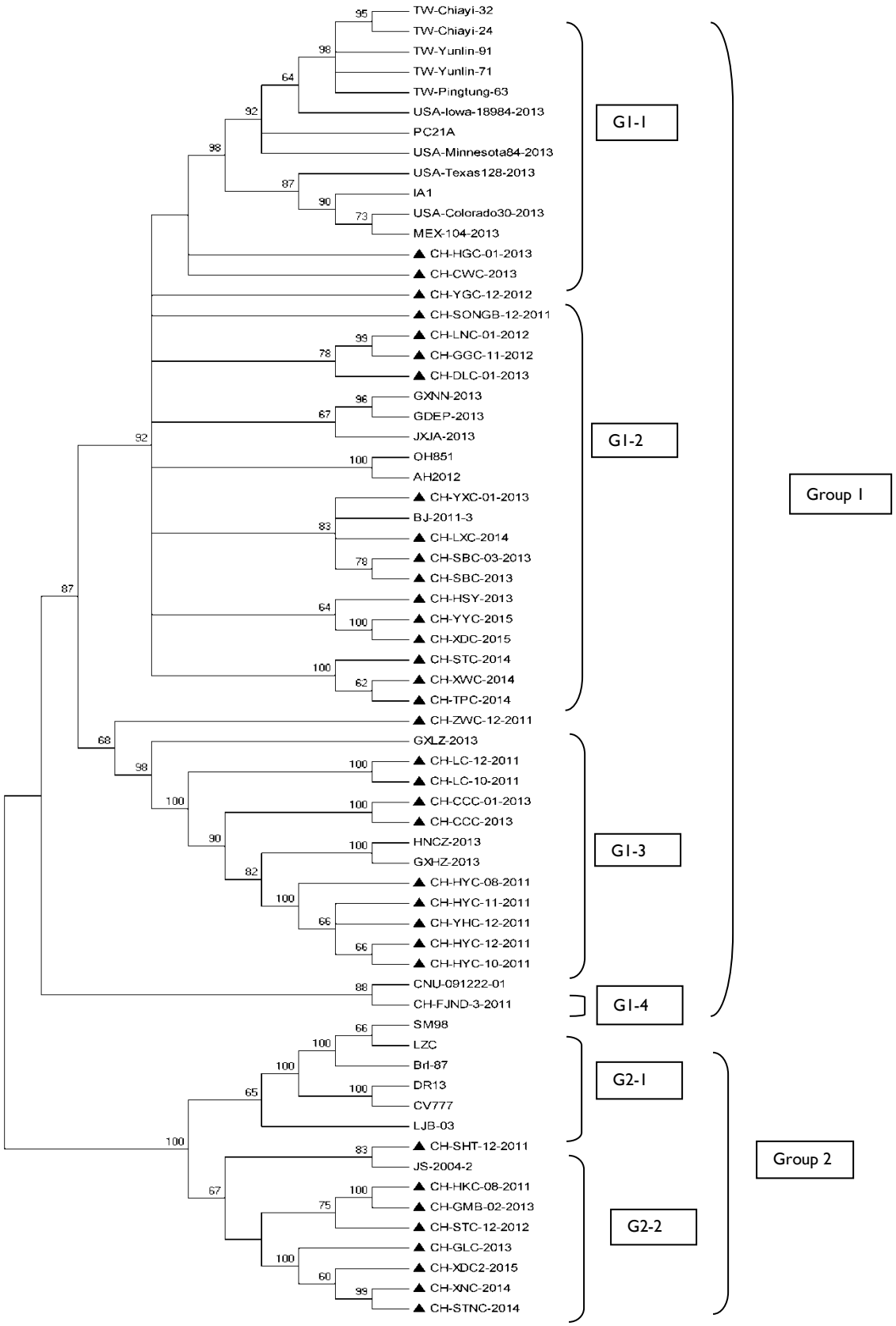

Fig. 4: Phylogenetic analysis by neighbor-joining method based on nucleotide sequences of full-length S genes of 35 field isolates of porcine epidemic diarrhea virus (PEDV) in south China and other PEDV reference strains. The 35 field isolates in this study are marked by solid triangle symbols.

It is notably that most of the strains isolated in 2012-2015 distributed in the subgroup G1-2 while most of the strains isolated in 2011 distributed in subgroup G1-3. This was consistent with the reports in United States (Jung *et al*., 2014; Sun *et al*., 2015).The two subgroups G1-2 and G1-3 have 97.9% -98.7% homologies with each other. It is showed that the PEDV strains had some minor variations from 2011 to 2015. Chen JF confirmed that PEDV had a high detection rate which indicated the S genes were heterogeneous in China (Chen *et al*., 2013).

Pan also reported that PEDV strains prevalent in China was the new variant (Pan *et al*., 2012), which brought a certain degree of difficulty to choice the vaccine strain to prevent PEDV epidemic.

Although vaccination has been encouraged to use to induce specific immunity to PEDV in all pigs and to prevent the virus spread and epidemic, it seemed that vaccination against PEDV did not induce mucosal immune responses and effective protection, at least did not provide lifelong protection from PEDV as pigs in the same farm suffered re-infection within one year. The epidemic lasted for a long time and relapsed several times (Lee *et al*., 2015; Sun *et al*., 2015). Massive feedback to the pregnant sows using piglet faeces or minced piglet gut was performed in most of swine farms during our study with the aims to pass the sow's protective immunity to the piglets. However, the effect was varied from farm to farm. The continuing monitoring of PEDV isolates in diarrhea farms has helped us to gain new insight of the biology of these viruses and will contribute to the development of effective vaccine to control the spread of PED.

Conclusions: In the study, the molecular epidemiological investigation and five yearlong monitoring of nine swine herds with the outbreak of diarrhea located in geographically separate regions of south China was executed. All S genes in the study could be divided into two types, the ones had 96 amino acids different when compared with CV777 and the mutation rate was 6.92% while the others' mutation rate was 4.18%. The amino acids variations of the former were identical to the United States strains and Taiwan strains. The two types on behalf of the highly pathogenic strains with acute virulent, caused significant economic loss and the low pathogenic strains caused a relative moderate diarrhea in clinical with less economic losses in this PEDV epidemic during 2011-2015 in south China. It was also indicated that the effect way to prevent PEDV should focus on reduce the morbidity of pregnant sows.

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Author's contribution: ZL, ZL and LC conceived and designed the study. YC, XZ, BS, QX, XL and HG executed the experiment. JYM and YB analyzed the data. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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