Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
29 (4): 373-380, 2023
DOI: 10.9775/kvfd.2023.29358

RESEARCH ARTICLE

Molecular Characterization and Phylogenetic Analysis of a Novel *Porcine Epidemic Diarrhea Virus* Circulating in Large-scale Pig Farms in Xinjiang, China

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How to cite this article?

Chen J, Tian L, Li Z, Wang L, Guo Y, Shang Y, Sun Y, Huang X, Meng Q, Cai X, Xia X, Qiao J: Molecular characterization and phylogenetic analysis of a novel Porcine Epidemic Diarrhea Virus circulating in large-scale pig farms in Xinjiang, China. *Kafkas Univ Vet Fak Derg,* 29 (4): 373-380, 2023.
DOI: 10.9775/kvfd.2023.29358

Article ID: KVFD-2023-29358
Received: 14.03.2023
Accepted: 11.07.2023
Published Online: 17.07.2023

ABSTRACT

Porcine epidemic diarrhea (PED) is a highly contagious disease caused by *porcine epidemic diarrhea virus* (PEDV), which is characterized by severe diarrhea and vomiting in lactating piglets, resulting in serious economic losses to the pig industry worldwide. Here, a novel variant strain of PEDV (named PEDV/CH/XC/2020) was isolated from the feces of infected piglets, and subjected to genetic variation and recombination analysis based on whole genome sequencing. The results showed that PEDV/CH/XC/2020 belonged to a GIIa subtype variant strain of PEDV, with the genome of 28128 nt in length, which shared only 94% identities with the vaccine strain CV777. Furthermore, multiple amino acid mutations were occurred in the neutralizing antigenic epitopes of COE (499-638 aa) and S1D (636-789 aa) regions of spike protein. It was worth noting that genetic recombination was occurred in the 24010-26546 nt and 27000-27663 nt regions, suggesting that this isolate may arise from genetic recombination with parental strains such as GD-1, CH/ZMDZY and PEDV-CHZ and continuous mutation in epidemic process. In contrast to other mutant strains, the mutation sites $_{767}$F, $_{838}$L and $_{1060}$C of S protein are unique, which might result in the alterations of virulence and immunogenicity of PEDV/CH/XC/2020. These findings of the molecular characteristic of this novel variant strain provided new insights into the genetic variation and diversity and enriched molecular epidemiological date of PED.

Keywords: Porcine epidemic diarrhea virus, Molecular characterization, Phylogenetic analysis, Genetic recombination

INTRODUCTION

Porcine epidemic diarrhea virus (PEDV) is a member of the genus Coronavirus in the *Coronaviridae* family, which can cause severe diarrhea, vomiting and dehydration in lactating piglets, often with a mortality rate of 85% or more [1-3]. Since this infectious disease was first reported in UK in 1971, it has been prevalent in many countries, including Japan, Canada, France and Bulgaria [4,5]. As an emerging and re-emerging epizootic swine virus, a variant strain of PEDV was first reported in Korea in 2009 [6]. Subsequently, PEDV variant strains have become widespread in many regions in Asia, America and Europe, causing great economic losses to the global pig industry [7,8].

The genome of PEDV is a linear positive-stranded single-stranded RNA with an entire genome length of 27.000-33.000 nucleotides, which harbors seven open reading frames, with the gene arrangements of 5'UTR-ORF1a-1b-S-ORF3-E-N-S-3'UTR [9,10]. Among them, S gene encodes the spike protein, a protective antigen that induces neutralizing antibodies in the body [11-13]. Due to being located on the surface of the viral particle, the spike (S) protein is most susceptible to mutation under the immune pressure of vaccine, leading to changes in the virulence

and immunogenicity of PEDV, thereby evading the body's immune response [14-16].

Xinjiang province is one of the major bases of the pig industry in China, with a current pig stock of over 4 million head. In recent years, the incidence of PEDV infection in piglets has been increasing despite the increasing immunization with PEDV inactivated vaccine, which has brought huge economic losses to the local pig industry. However, the genetic characterization of prevalent strains of PEDV remains unknown. The main objective of this study was to characterize the molecular characterization of PEDV epidemic strain from large-scale pig farms in Xinjiang, China, and clarify the reasons for the decreasing immunization effect of inactivated PEDV vaccine. Here, a novel variant strain of PEDV was isolated from fecal samples of piglets with diarrhea, and its whole genome was sequenced to analyze the genetic variation characteristics of this PEDV isolate. This study not only provides valuable molecular epidemiological data for the study of PEDV variation, but also provides a scientific basis for the rational use of PED vaccine in large-scale pig farms in Xinjiang.

Material and Methods

Collection of Clinical Samples

The feces of piglets with diarrhea were collected in September 2020 from a large-scale pig farm in a region of Xinjiang, China, where there was prevalent of PEDV infection. All sows in this farm were immunized with Transmissible gastroenteritis (TGE) and porcine epidemic diarrhea (PED) inactivated vaccine 40 days before farrowing. Clinical feces were collected from 8-day-old piglets with diarrhoea and kept in sterilized centrifuge tubes at 4°C for PEDV detection and isolation.

RT-PCR Detection

Primers were designed according to the conserved sequences of PEDV (GenBank accession number: AF353511.1). Then, RT-PCR was performed to detect PEDV in piglet feces using FP1 (5'-GCAGGACACATTCTTGGT-3') and RP1 (5'-AGATGAAGCATTGACTGAAC-3') primers. Briefly, total RNA was isolated from the diarrhea feces according to the instructions of RNA extraction kit (Qiagen, Germany), followed by reverse transcription using SuperScript® II Reverse Transcriptase (Invitrogen, USA) following the manufacturer's protocol. The PCR reaction mixture contains 20 µL of water, 1 µL (0.2 µmol/L) of each FP1-RP1 primer, 25 µL of 2× Premix Ex Taq (TaKaRa, Japan), and 3 µL of DNA template. After that, PCR products were detected by 1.5% agarose gel electrophoresis.

Virus Isolation

The positive feces were homogenized and mixed with saline at a volume ratio of 1:6, centrifuged at 12000 rpm for 10 min at 4°C, and then filtered through a 0.45 µm membrane. Vero-E6 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (Gibco, USA) and antibiotics (100 IU/mL penicillin and 100 IU/mL streptomycin). The virus filtrate (2%) and trypsin solution were added into Vero-E6 cells, respectively. After 1 h of adsorption at 37°C, the DMEM medium was discarded and cells were cultured in DMEM medium containing trypsin (a final concentration of 10 µg/mL) for 3-4 days. The inoculated Vero-E6 cells were passaged blindly until the stable cytopathic effects (CPE) were observed.

Virus Identification

The infected cells were repeatedly freeze-thawed three times and centrifuged at 4°C, 12000 r/min for 10 min. Then, the cells were negatively stained with 2% phosphotungstic acid staining solution (pH 6.8), followed by the observation of the morphological features of viral particle under transmission electron microscopy (TEM) (Hitachi, Japan). To further identify the isolate, indirect immunofluorescence assay (IFA) was performed in infected Vero-E6 cells. Briefly, Vero-E6 cells was inoculated with the viral solution at 2% v/v, and cultured for 24 h and then fixed in 4% paraformaldehyde for 20 min. These cells were treated with Triton X-100 for 10 min, washed with PBS and then closed with 0.5% BSA at room temperature for 2 h. The anti-N protein monoclonal antibody was incubated at 37°C for 1 h, and then added to Alexa fluor 488-labeled goat anti-mouse IgG antibody (1:1000) (Sigma, USA), incubated for 1 h at 37°C, washed with PBS and the results observed under a fluorescent microscope. In parallel, normal Vero-E6 cells were used as a negative control.

Full-length Genome Sequencing

Viral particle was purified by cesium chloride (CsCl) gradient centrifugation. After that, the total viral RNA was extracted using RNA simple Total RNA Kit (TaKaRa, Japan). Then, total RNA was reverse transcribed into cDNA using PrimeScript™ RT Master MixcDNA (TaKaRa, Japan) and sent to Beijing Bio Sequencing Co Ltd. for sequencing. Then, these sequences were assembled to obtain the whole genome sequence of the isolate.

Genetic Variation and Phylogenetic Analysis

To clarify the evolutionary relationship between this isolate and other virulent strains, the sequence was compared with the reference gene sequences registered in GenBank to analyze the homology. Phylogenetic trees based on the whole genome and S gene were constructed using MEGA10.0 software *(https://www.megasoftware.net),* respectively. Meanwhile, the variant sites in spike protein encoded by S gene were analyzed using DNAMAN software.

Table 1. *Reference sequences used in genetic recombination analysis of porcine epidemic diarrhoea coronavirus strains*

Strain / Isolate	GenBank Accession No.	Country	Strain / Isolate	GenBank Accession No.	Country
CV777	AF353511.1	Belgium	CH/JX-1/2013	KF760557.2	China
SM98	GU937797.1	South Korea	JS-HZ2012	KC210147.1	China
CHYJ130330	KJ020932.1	China	JS2008	KC210146.1	China
EF185992.1 LZC	EF185992.1	China	AH2012	KC210145.1	China
CH/JX-2/2013	KJ526096.1	China	CH/ZMDZY	KC196276.1	China
CHZ	KM609209.1	China	CH/FJZZ-9/2012	KC140102.1	China
CH/S	JN547228.1	China	GD-1	JX647847.1	China
JS	KC109141.1	China	ZJCZ4	JX524137.1	China
CH/ZJCX-1/2012	KF840537.1	China	LC	JX489155.1	China
AH-M	KJ158152.1	China	CHGD-01	JX261936.1	China
CH/HNQX-3-14	KR095279.1	China	AJ1102	JX188454.1	China
JSHA2013	KR818833.1	China	GD-A	JX112709.1	China
CH/GDZHDM/1401	KR153326.1	China	GD-B	JX088695.1	China
CH/HNYF/14	KP890336.1	China	CH/FJND-3/2011	JQ282909.1	China
HLJBY	KP403802.1	China	DR13	JQ023162.1	South Korea
SC1402	KP162057.1	China	BJ-2011-1	JN825712.1	China

Recombination Analysis

The whole genome sequences of 32 strains registered in GenBank were compared with those of different regions in China *(Table 1)*, and the genetic recombination were analyzed using RDP4 software to speculate on the recombination events that occurred during its evolution.

Results

Porcine epidemic diarrhea virus was successfully detected by RT-PCR in feces of piglet with diarrhoea *(Fig. 1)*. Compared to Vero-E6 control cells, a significant CPE

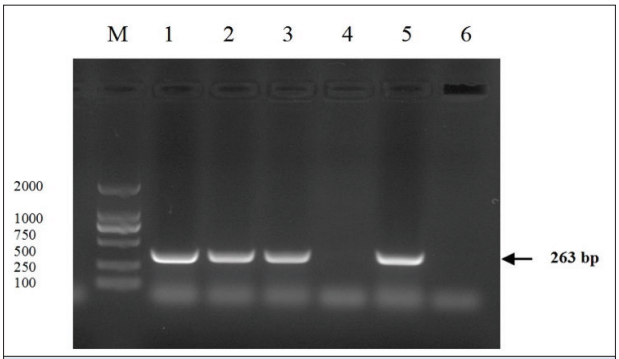

Fig 1. Molecular detection of *porcine epidemic diarrhea virus* in feces of piglet. M: Standard DNA marker DL-2000 (2000, 1000, 750, 500, 250, 100 bp);1: Positive control; 2, 3, 5: Positive feces detected by RT-PCR; 4: Negative feces; 6: Negative control

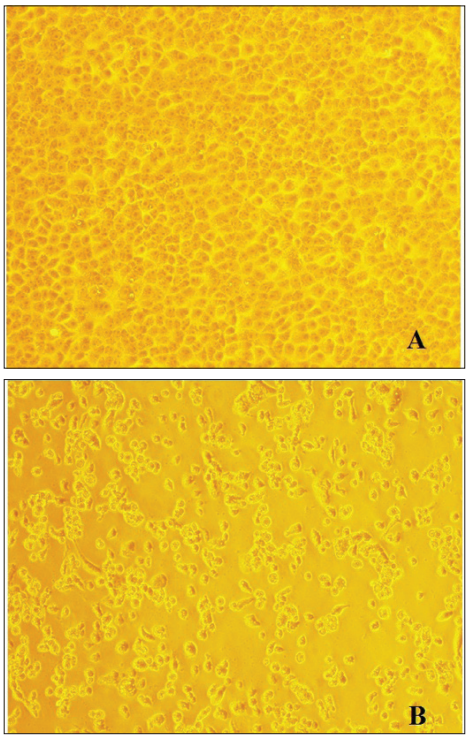

Fig 2. Cytopathic effects of PEDV CH/XC/2020 isolate infected Vero E6 cells. **A**- Mock-inoculated Vero cells culture showing normal cells; **B**- PEDV infected Vero E6 cells. PEDV CH/XC/2020 induced an obvious cytopathic effects at 36 h, the Vero E6 cells that infected by PEDV were changed to round shaped and shedding

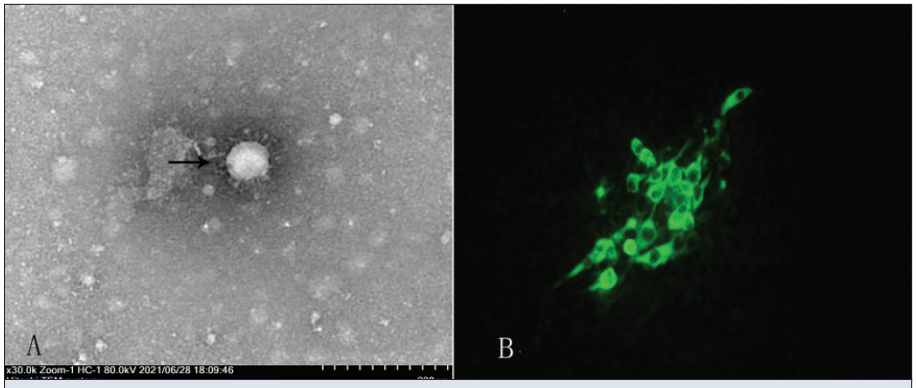

Fig 3. Identification of the isolated PEDV CH/XC/2020 strain. **A-** Transmission electron microscopic (TEM) of the supernatants of the PEDV CH/XC/2020-inoculated Vero E6 cells by using negative staining with phosphotungstic acid, typical Coronavirus particles with a corolla shape and approximately 100 nm in diameter were visible as arrow indicates; **B-** IFA detection of Vero-E6 cells infected by PEDV CH/XC/2020 isolate at 36 h, PEDV CH/XC/2020 strain in Vero E6 cells was stained using monoclonal antibody against anti-N protein and Alexa fluor 488-labelled goat anti-mouse IgG antibody, green fluorescence can be observed in infected cells

began to appear in the 6th generation. The characteristic CPE were mainly manifested by fusion of cells with each other, rounding and shedding *(Fig. 2)*. After the isolated virus was purified by etching, virus titer was measured, with $10^{5.5}$ $TCID_{50}$/mL after 20 generations of successive passages.

After negative staining, the viral particle was observed under transmission electron microscopy, which owned the typical morphological features of PEDV, with a corolla shape and approximately 100 nm in diameter *(Fig. 3-A)*. Furthermore, green fluorescence can be observed in infected cells, whereas no fluorescence was detected in control cells, indicating that a PEDV strain (namely PEDV/CH/XC/2020) was successfully isolated using Vero-E6 cells *(Fig. 3-B)*.

The whole genome of PEDV/CH/XC/2020 isolate was 28128 nt in length (GenBank accession number: OM393722), which shared 99.11% and 98.74% identities with CH/HBTS/2017 and AJ1102 strains, respectively, but only 94% identities with CV777 vaccine strain. The sequence comparisons based on S gene revealed that PEDV/CH/XC/2020 shared 99.2% identities with CH/GZZY/12/2020 (MZ161063.1) and CH/SXXY/11/2020 (MZ161017.1), but only 94.1% identities with vaccine strain CV777.

Phylogenetic analysis based on the complete genome showed that the 33 PEDV strains were mainly divided into 2 genogroups, GI (classical strain) and GII (variant strain), and each genogroup was further divided into two subgroups, GIIa and GIIb *(Fig. 4)*, among which PEDV/CH/XC/2020 isolate was located in GIIa branch. Similarly, the Phylogenetic tree based on S gene also showed that this isolate belonged to the GIIa branch of GII. However, PEDV CH/XC/2020 was genetically distant from other domestic and foreign GIIa strains *(Fig. 5)*.

The length of S gene of PEDV/CH/XC/2020 is 4158 nt, encoding 1386 amino acids. Compared with vaccine strain CV777, the isolate had multiple mutations in the neutralizing antigen epitopes of COE (499-638 aa) and S1D (636-789 aa) regions of S protein, which were mainly

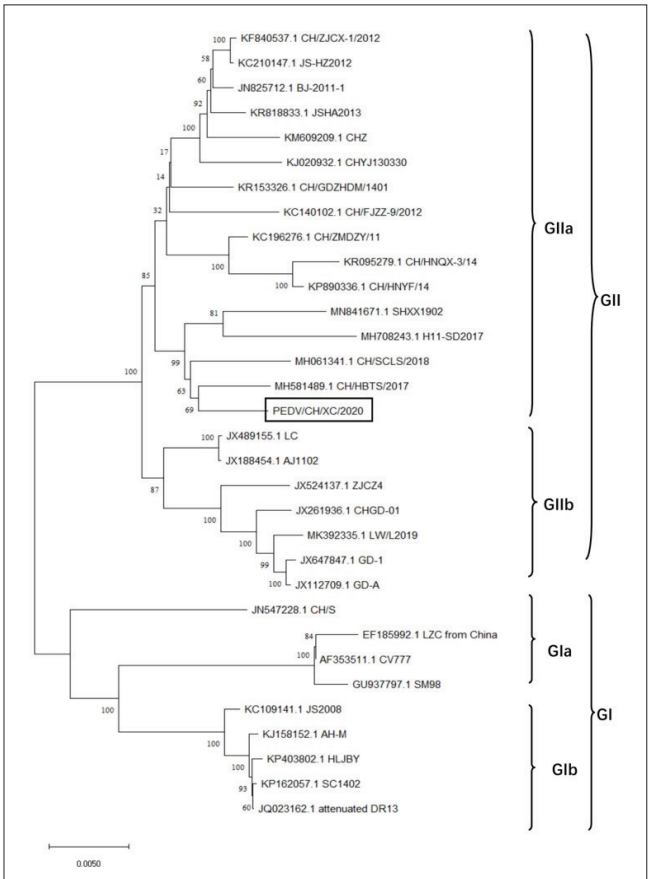

Fig 4. Phylogenetic analysis of the different epidemic strains based on the complete genome of PEDV. The phylogenetic tree was constructed by the neighbor-joining method using the MEGA 10.0 software (1000 bootstrap replicates). Reference virus genomes from different serogroups were analysed. The PEDV CH/XC/2020 isolate was marked with black box

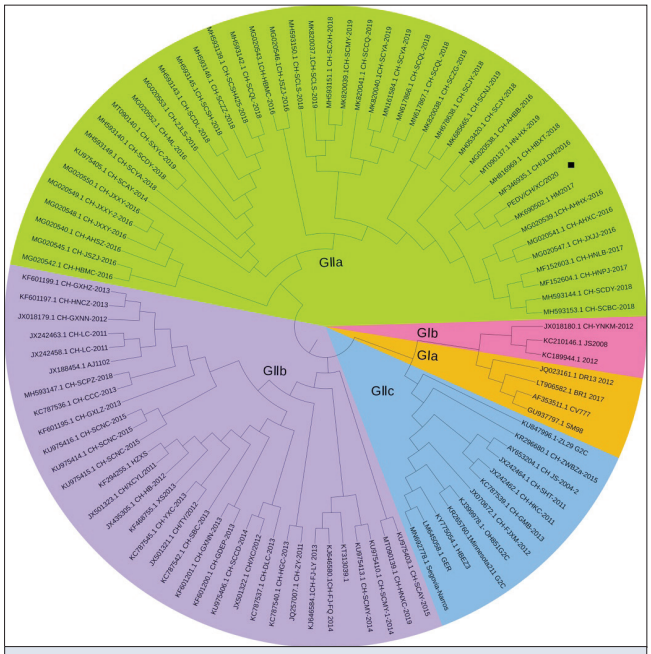

Fig 5. Phylogenetic analysis of prevalent strains in different geographical areas based on spike protein gene of PEDV. The phylogenetic tree was constructed by the neighbor-joining method using the MEGA X program; MEGA 10.0 software (1000 bootstrap replicates). The PEDV CH/XC/2020 isolate was marked with black square

manifested in COE region as $_{526}$A→S, $_{530}$L→H, $_{532}$S→G, $_{536}$V→I, $_{558}$T→S, $_{603}$G→S, $_{614}$A→E, $_{621}$L→F, $_{640}$P→S, $_{644}$I→V, and in S1D region as $_{640}$P→S, $_{676}$I→F, $_{716}$N→D, $_{728}$N→S, $_{733}$N→S, $_{773}$S→F, and $_{775}$Y→S. There were also multiple amino acid mutations in other regions, such as $_{2}$R→K, $_{5}$I→T, $_{15}$P→S, $_{27}$QST$_{29}$→SAN, $_{55}$SMN$_{57}$→IGE, $_{58}$NQGV$_{61}$ and the insertion of $_{139}$D. These mutation sites were also present compared to strain AJ1102 and other reference strains *(Table 2)*.

Recombination analysis revealed that PEDV/CH/XC/2020 might undergo recombination events in the regions 24010-26546 nt and 27000-27663 nt *(Table 3)*, with the related parental strains GD-1, CH/ZMDZY and PEDV-CHZ *(Fig. 6)*.

Discussion

As the most mutation-prone gene in PEDV genome, spike (S) gene encodes spike protein that is primarily involved in binding to viral receptor and invading host cell *via* membrane fusion. Therefore, spike protein plays an important role in the process of infection and the induction of neutralizing antibody [17,18]. So far, only one serotype of PEDV has been identified; however, PEDV strains can

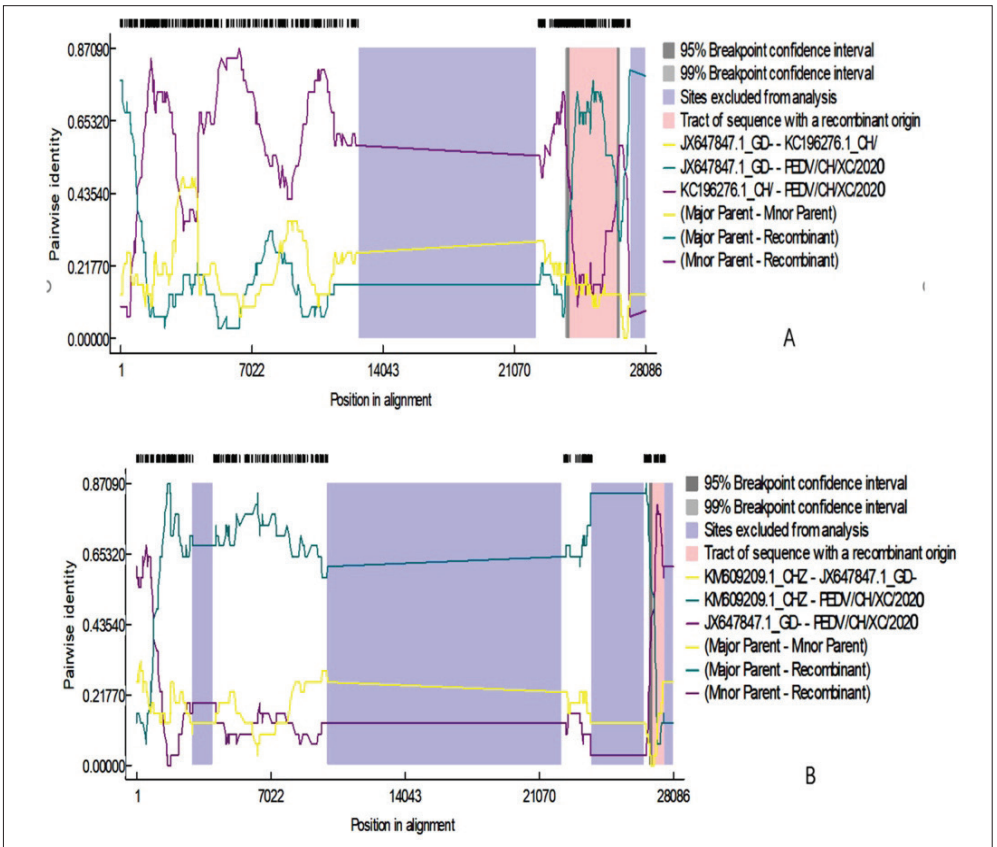

Fig 6. Analysis of potential recombination breakpoints in the genome of PEDV CH/XC/2020 isolate by RDP4 software. A- Event 1 occurred in the regions of 24010-26546 nt, the potential parent strains were GD-1 (JX647847.1) and CH/ZMDZY (KC196276.1), respectively, with the Av.P-Val of 4.035×10^{-10}; B- Event 2 occurred in the regions of 27000-27663 nt, the potential parent strains were GD-1 (JX647847.1) and PEDV-CHZ (KM609209.1), respectively, with the Av.P-Val of 2.330×10^{-6}

Table 2. *Amino acid mutations in COE (499-638 aa) and S1D (636-789 aa) regions of 25 reference strains of porcine epidemic diarrhoea coronavirus (PEDV)*

Accession no.	Variant Sites in Amino Acid Sequences																												
	505	526	529	530	532	536	545	551	558	572	603	610	614	621	639	640	641	642	643	644	676	680	716	728	733	772	773	775	783
AF353511.1	I	A	G	L	S	V	F	D	T	K	G	F	A	L	K	P	L	E	G	I	I	V	N	N	N	P	S	Y	T
CH/XC/2020	.	S	.	H	G	I	.	.	S	.	S	.	E	F	.	S	.	.	.	V	F	.	D	S	S	.	F	S	.
JQ023161	.	.	.	H	G	I	.	.	.	.	.	.	E	F	.	.	.	.	.	V	F	.	D	.	.	.	.	.	.
JX188454.1	T	.	.	H	G	I	.	.	S	.	S	.	E	F	.	.	.	.	.	V	F	F	D	S	S	.	.	S	.
KC210146.1	.	.	.	H	G	I	.	.	.	.	.	.	E	F	.	.	.	Q	.	V	F	.	D	.	.	.	.	S	.
MF152605.1	.	S	.	H	G	I	.	.	S	.	S	.	E	F	.	.	.	.	.	V	F	.	D	.	S	.	.	S	.
MF346935.1	.	S	.	H	G	I	.	.	S	.	S	.	E	F	.	S	.	.	.	V	F	.	D	S	S	.	.	S	.
MK685665.1	.	S	.	H	G	I	.	.	S	T	S	.	D	F	T	.	.	.	.	V	F	.	D	.	S	S	.	S	.
MK690502.1	.	S	.	H	G	I	.	.	S	.	S	.	E	F	.	S	.	.	.	V	F	.	D	.	S	.	.	S	.
MK820039.1	.	S	.	H	G	I	L	.	S	.	S	.	E	F	.	.	.	.	.	V	.	.	D	S	S	.	.	S	.
MN841671.1	.	S	.	H	G	I	.	E	S	N	S	.	E	F	.	.	.	.	.	V	F	.	D	.	S	.	.	S	.
MT090139.1	.	S	D	H	G	I	.	.	S	N	S	L	D	F	.	.	.	Q	.	V	F	.	D	S	S	.	.	S	M
MZ161017.1	.	S	.	H	G	I	.	.	S	.	S	.	E	F	.	S	.	.	.	V	F	.	D	S	S	.	.	S	.
MZ161063.1	.	S	.	H	G	I	.	.	S	.	S	.	E	F	.	S	.	.	.	V	F	.	D	S	S	.	.	S	.
MT263014.1	T	S	.	.	G	I	.	.	S	.	S	.	E	F	.	.	.	.	.	V	F	.	D	S	S	.	.	S	.
MT090140.1	T	S	.	R	G	I	.	.	S	.	S	.	D	F	.	.	.	G	.	V	F	.	D	.	S	.	.	S	.
MN759311.1	T	.	.	H	G	I	.	.	S	.	S	.	E	F	.	.	.	.	.	V	F	.	D	S	S	R	.	S	.
MN213154.1	T	.	.	H	G	I	.	.	.	.	S	.	E	F	.	.	.	.	.	V	F	F	D	S	.	S	.	S	.
MN114121.1	T	S	.	H	G	I	.	.	S	.	S	.	E	F	.	.	F	.	.	V	F	.	D	.	S	.	.	S	.
MK841495.1	.	S	.	H	G	I	.	E	S	.	S	.	E	F	.	.	.	V	.	V	F	I	D	.	S	L	.	S	.
MK841494.1	.	S	.	H	G	I	.	E	S	.	S	.	E	F	.	.	.	V	.	V	F	I	D	.	S	L	.	S	.
MK539948.1	T	S	.	H	G	I	.	.	S	.	S	.	E	F	.	.	F	.	.	V	F	.	D	.	S	.	.	S	.
KR296677.1	.	S	.	H	G	I	.	.	S	.	S	.	E	F	.	.	.	.	.	V	F	.	D	S	S	.	.	S	.
KR296673.1	T	S	.	H	G	I	.	.	S	.	S	.	E	F	.	.	.	.	.	V	F	.	D	S	S	.	.	S	.
KR296663.1	.	S	.	H	G	I	.	.	S	.	S	.	E	F	.	.	.	.	.	V	F	.	D	S	S	.	.	S	.
GU937797.1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	P	.	.

Parsimony informative sites are indicated in bold; the remaining sites are singleton sites; a period indicates no base mutation

Table 3. *Analysis of potential gene recombination events by RDP4 software*

Recombination Event no.	Methods	Major Parent	Minor Parent	Position (nt)	Recombinant Score
Event 1	RDP/GENECONV/ MaxChi	GD-1 (JX647847.1)	CH/ZMDZY (KC196276.1)	24010~26546	0.643
Event 2	RDP/GENECONV/ MaxChi	PEDV-CHZ (KM609209.1)	GD-1 (JX647847.1)	27000~27663	0.686

The recombinant score more than 60% was considered as a potential recombinant strain

be divided into two genotypes, namely Gl and GII, based on phylogenetic analysis of S gene. Among them, each genotype can be further divided into two or three subtypes, GIa and GIb, GIIa, GIIb and GIIc respectively [19,20]. In recent years, the frequent outbreaks of diarrhea in piglets caused by the variant strains of PEDV in China have resulted in massive deaths in newborn piglets, suggesting that the emerging variant strains are more pathogenic than that of classical strains [8,21,22]. Considering that vaccine strain CV777 belongs to GIa subtype, which does not provide effective protection against GIIa subtype variant, so it is urgent to develop a new vaccine against GIIa subtype variant. Here, it was revealed that PEDV/CH/XC/2020 belongs to GIIa subgroup of variant strain. This strain has multiple mutations in S gene, especially in the S1 region (1-789 aa), which is highly variable, with deletions, insertions and multiple point mutations. Moreover, some point mutations also substantively occurred in E, M and N gene. Compared to reference strains, PEDV/CH/XC/2020 possesses the characteristic mutation sites of $_{767}$F, $_{838}$L and $_{1060}$C in S gene. Furthermore, PEDV CH/XC/2020 was genetically distant from other domestic and foreign GIIa strains, suggesting that PEDV CH/XC/2020 was a new variant that is emerging in large-scale pig farms in Xinjiang, China.

Currently, it had been revealed that genetic recombination was occurring continuously in different strains of PEDV during the course of the epidemic, and that the recombination occurred not only in S gene, but also in other regions of the genome [23-25]. Antas et al.[26] showed that the end (5-400 nt) of PEDV S gene in Poland may be generated through the recombination among SeCoV MU2 (MN692770) and 1556 (MN692763) strain in Spain or DR13 (JQ23161) strain in Korea. Nefedeva et al.[27] found that two recombination events had occurred at 20476 nt (ORF1b) and 24403 nt (S) in PEDV/Belgorod/dom/2008 strain, whereas LZC (EF185992) and SLO/JH-11/2015 (KU297956) were potential parental strains. Wang et al.[28] showed that PEDV strain prevalent in China may have been generated by the recombination of parental strains USAIllinois 972013, Korea K14JB01, CHYJ130330, and CHZMDZY1. Li et al.[25] showed that the PEDV CH/HNQX-3/14 strain was produced by natural recombination of the classic strains CV777, DR13 and CH/ZMDZY/11. The present study revealed that recombination was occurred not only in S gene but also in conserved N gene of PEDV/CH/XC/2020, which displayed novel molecular characterization as compared with other PEDV epidemic strains that had been identified.

Taken together, this study for the first time revealed that the genotype GIIa variant strain was present in piglets in Xinjiang, and confirmed that this variant strain has multi-locus amino acid mutations in antigenic epitopes of S protein, which might be generated by genetic recombination of various parental strains. These findings will provide new insights into the molecular characteristics of PEDV variant strain, and facilitate the development of effective vaccines for the prevention and control of PED in veterinary practice.

Availability of Data and Materials

All data supporting the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgments

We thank the field staff who provided the technical supports for this study.

Funding Support

This work was supported by the national key research and development program (No. 2016YFD0500900), Young and middle-aged leading science & technology innovation talents plan of Xinjiang Corps (No. 2016BC001) and the International Science & Technology Cooperation Program of China (No. 2014DFR31310).

Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Competing Interests

The authors declare no competing interests.

Author Contributions

JL Chen, LL Tian and ZY Li performed the experiment. LX Wang, Y Guo, YX Shang, YQ Sun and XT Huang contributed significantly to analysis and manuscript preparation. JL Chen and J Qiao performed the data analyses and wrote the manuscript. QL Meng, XP Cai and XZ Xia helped perform the analysis with constructive discussions.

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