Molecular characteristics and pathogenicity of porcine epidemic diarrhea virus in some areas of China from 2015 to 2018

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Research

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Abstract

Background

Since 2010, porcine epidemic diarrhea virus (PEDV) has caused severe diarrhea disease in piglets, which has led to large economic losses in China. To understand the genetic characteristics of PEDV strains that have been circulating in some provinces of China from 2015 to 2018, 362 feces and small intestine samples in pigs were collected and tested.

Methods

The S gene of PEDV in collected samples were amplified by RT-PCR. A phylogenetic tree was constructed using MEGA6.0 software with the neighbor-joining method to analyze the evolutionary relationship. Nucleotide and deduced amino acid (AA) sequences of S were aligned using the MegAlign program of DNASTAR7.1 software to determine sequence homology. PEDV GDgh16 strain isolation, IFA identification and titer detection were performed in Vero cells. Six 4-day-old healthy colostrum deprived suckling piglets were used for challenging experiment of PEDV GDgh16 strain. Virus copies from the small intestine were detected by RT-qPCR. The other section was stained with the anti-N protein McAb at 1:1000 dilution for immunohistochemical (IHC) examinations.

Results

The results showed that 160 samples tested positive and the PEDV-positive prevalence was 44.20%. Phylogenetic tree analysis of entire S genes showed that these strains were clustered into four subgroups, G1-b, G1-c, G2-a and G2-b, and that the G2-b strains had become dominant in recent years. Compared with previous strains, these strains had multiple variations in the SP and S1-NTD domains and in the neutralizing epitope of the S protein. Furthermore, we successfully isolated and identified a new virulent G2-b strain, GDgh16, which was well adapted to Vero cells and had a high mortality rate in piglets through challenge experiments.

Conclusions

Our study provides full insights into the genetic characteristics of prevalent PEDV strains in parts of China, suggesting that the development of novel effective vaccines is necessary and urgent.

Background

Porcine epidemic diarrhea virus (PEDV) is the pathogen of porcine epidemic diarrhea (PED), which is a severe diarrhea disease in piglets that has the characteristics of severe watery diarrhea, vomiting, dehydration, weight loss and nearly high mortality up to 100% [1]. PED was sporadic around the world from 1990 to 2009. However, since 2010, an acute and severe outbreak of PED in piglets occurred in China and spread to other Asian countries, causing large economic loss [2–8]. In April 2013, PED suddenly outbreak in the United States which causing many piglets to die, and the mortality rate of Suckling piglets reached 100%[9, 10]. It was demonstrated

that the disease was caused by a highly pathogenic PEDV variant. Compared with the PEDV variant strains, the S genes of classical CV777 and new OH851 had the same insertions and deletions (S-INDEL strains) [11, 12].

The genome of PEDV is approximately 28 kb in length and consists of seven open reading frames (ORFs), which encode four structural proteins and three non-structural proteins [13]. S is the largest structural protein, which contains neutralizing antibody epitopes and a specific receptor binding site for virus entry [14]. At present, four antigenic epitopes has been characterized in the S protein, including the CO equivalent (COE) domain (aa positions 499–638), the epitopes SS2 (aa positions 748–755) and SS6 (aa positions 764–771), and the epitope 2C10 (1368 GPRLQPY 1374) [15, 16]. Because of the vital role of the S protein and extensive mutation of the S gene, it is often used as a target gene for the analysis of virus genetic variation. Based on the S gene,PEDV strains can be classified into genogroup 2 (G2) and genogroup 1 (G1). At present, most isolated recovered in China belong to G2 [17]. Recently, a new mutation in the S gene of PEDV has been reported[18]. Different recombinant PEDV strains have also been reported in different areas of China[19, 20]. Studies have shown that one province in particular has the co-existence of different genotyped PEDV strains. These results indicate that PEDV has been continuing to spread widely to most areas of China and has caused serious economic loss to the pig industry, thereby manifesting the complex evolution of the virus. Therefore, extensive research of the evolutionary pathogenic mechanism is essential in China.

To control the PEDV spread, the classical CV777-derived vaccine has been widely used in many areas of China; however, it cannot provide adequate protection against PEDV invasion. In contrast, the wide-scale use of the vaccines has increased the environment stress and led to PEDV variation to escape immune protest. To further and fully understand the prevalence and evolution of PEDV in South China, in this study, the diarrhea samples of piglets were collected, and the variation of the S genes of PEDV positive samples were analyzed by sequence alignment and a phylogenetic tree.

Materials And Methods

Sample collection

A total of 362 diarrheic samples from small intestine tissues or diarrhea feces of suckling piglets in pig farms in five provinces of China (Guangdong, Guangxi, Jiangxi, Hu’nan and Hainan) were collected from June 2015 to October 2018. The piglets had severe watery-diarrhea and dehydration. The diarrhea feces were re-suspended in 1 mL phosphate buffer saline (PBS) solution in 1.5 mL Eppendorf tubes. After centrifugation at 10,000 × g for 5 min, 200 μL supernatants were transferred into new tubes for RNA extraction and virus isolation.

RNA extraction and sequencing

The total RNA of collected supernatants was extracted using TRIzol reagent (TaKaRa) according to the manufacturer’s instructions. To extract RNA, reverse transcription PCR (RT-PCR) was performed using three pairs of newly designed primers for PEDV S gene amplification and detection (Table 1). The overlapping three PCR products were identified by 1.5% agarose gel electrophoresis. The positive PCR products were sequenced by Sangon Biological Engineering Co. Ltd., and the entire sequence of the S gene was obtained by using DNAStar

7.1 software. The complete S gene sequence was submitted to GenBank, and the accession no. is listed in Table 2.

Table 1
Primers used for PEDV complete S gene amplification

Primer name	Nucleotide sequence, 5'-3'	Size(bp)
PEDV S1-F	GGTAAGTTGCTAGTGCGTA	1630
PEDV S1-R	CACAGAAAGAACTAAACCC	
PEDV S2-F	CTGCCATTCAGCGTATTCTTT	1768
PEDV S2-R	CTGCGAGTTAACAACCTCTTGA	
PEDV S3-F	GTGCGCAGTATTACTCTGGT	1559
PEDV S3-R	AAGAAGACGCTTTAAACAGTG	

Table 2
Information of S genes of 62 PEDV isolates

No.	Designation	Area	Region	Year	S (bp)	Accession no
1	FJly15	Longyan	Fujian	2015	4161	MN368663
2	FJqz15	Quanzhou	Fujian	2015	4161	MN368664
3	FJzz15	Zhangzhou	Fujian	2015	4161	MN368665
4	GDgz15-1	Guangzhou	Guangdong	2015	4161	MN368666
5	GDgz15-2	Guangzhou	Guangdong	2015	4161	MN368667
6	GDhy15	Heyuan	Guangdong	2015	4161	MN368668
7	GDhz15	Huizhou	Guangdong	2015	4161	MN368669
8	GDjm15	Jiangmen	Guangdong	2015	4161	MN368670
9	GDmm15	Maoming	Guangdong	2015	4161	MN368671
10	GDsg15-1	Shaoguan	Guangdong	2015	4161	MN368672
11	GDsg15-2	Shaoguan	Guangdong	2015	4161	MN368673
12	GDsg15-3	Shaoguan	Guangdong	2015	4161	MN368674
13	GDzq15-1	Zhaoqing	Guangdong	2015	4161	MN368675
14	GDzq15-2	Zhaoqing	Guangdong	2015	4161	MN368676
15	GXnn15	Nanning	Guangxi	2015	4161	MN368678
16	GZgy15	Guiyang	Guizhou	2015	4161	MN368679
17	HBhg15	Huanggang	Hubei	2015	4161	MN368680
18	JXgz15	Ganzhou	Jiangxi	2015	4161	MN368681
19	JXyc15	Yichun	Jiangxi	2015	4161	MN368662
20	FJqz16	Quanzhou	Fujian	2016	4158	MN368683
21	GDfs16	Foshan	Guangdong	2016	4158	MN368684
22	GDhy16	Heyuan	Guangdong	2016	4161	MN368685
23	GDhz16	Huizhou	Guangdong	2016	4161	MN368686
24	GDjm16-1	Jiangmen	Guangdong	2016	4158	MN368687
25	GDjm16-2	Jiangmen	Guangdong	2016	4161	MN368688
26	GDjx16	Jiexi	Guangdong	2016	4158	MN368689
27	GDsg16-1	Shaoguan	Guangdong	2016	4158	MN368690
28	GDsg16-2	Shaoguan	Guangdong	2016	4158	MN368691
29	GDyj16	Ynagjiang	Guangdong	2016	4161	MN368692

No.	Designation	Area	Region	Year	S (bp)	Accession no
30	GDgh16	Guanghui	Guangdong	2016	4158	MG983755
31	GDdg17	Dongguan	Guangdong	2016	4158	MN368693
32	FJfz17-1	Fuzhou	Fujian	2017	4161	MN368695
33	FJfz17-2	Fuzhou	Fujian	2017	4161	MN368696
34	FJqz17-1	Quanzhou	Fujian	2017	4161	MN368697
35	FJqz17-2	Quanzhou	Fujian	2017	4158	MN368698
36	GDhy17	Heyuan	Guangdong	2017	4158	MN368699
37	GDhz17	Huizhou	Guangdong	2017	4158	MN368700
38	GDjm17-1	Jiangmen	Guangdong	2017	4152	MN368701
39	GDjm17-2	Jiangmen	Guangdong	2017	4158	MN368702
40	GDjm17-3	Jiangmen	Guangdong	2017	4161	MN368703
41	GDmm17-1	Maoming	Guangdong	2017	4158	MN368704
42	GDmm17-2	Maoming	Guangdong	2017	4158	MN368705
43	GDsg17	Shaoguan	Guangdong	2017	4161	MN368706
44	HNcz17	Chenzhou	Hunan	2017	4161	MN368707
45	JXnc17	Nanchang	Jiangxi	2017	4158	MN368708
46	FJfz18-1	Fuzhou	Fujian	2018	4161	MN368710
47	FJfz18-2	Fuzhou	Fujian	2018	4158	MN368711
48	FJqz18	Quanzhou	Fujian	2018	4158	MN368712
49	GDhy18-1	Heyuan	Guangdong	2018	4158	MN368713
50	GDhy18-2	Heyuan	Guangdong	2018	4158	MN368714
51	GDhy18-3	Heyuan	Guangdong	2018	4158	MN368715
52	GDhz18	Huizhou	Guangdong	2018	4158	MN368716
53	GDjm18-1	Jiangmen	Guangdong	2018	4158	MN368717
54	GDjm18-2	Jiangmen	Guangdong	2018	4149	MN368718
55	GDmm18-1	Maoming	Guangdong	2018	4158	MN368719
56	GDmm18-2	Maoming	Guangdong	2018	4158	MN368720
57	GDsg18-1	Shaoguan	Guangdong	2018	4158	MN368721
58	GDsg18-2	Shaoguan	Guangdong	2018	4158	MN368722
59	GDst18	Shantou	Guangdong	2018	4158	MN368723

No.	Designation	Area	Region	Year	S (bp)	Accession no
60	GDzj18-1	Zhanjiang	Guangdong	2018	4155	MN368724
61	GDzj18-2	Zhanjiang	Guangdong	2018	4161	MN368725
62	SDbz18	Binzhou	Shandong	2018	4158	MN368709

S gene sequence analyses

The representative strains of complete genome sequences that were available in GenBank were collected and used for phylogenetic analyses (Table 3). The phylogenetic tree of all the S genes of the representative strains and isolates was constructed by the neighbor joining method with 1000 bootstrap replicates using Molecular Evolutionary Genetics Analysis (MEGA) software (version 6.0) (http://www.megasoftware.net/).

Table 3
Information of the representative strains

Virus Strain	Countries	Year	accession no.	Virus Strain	Countries	Year	accession no.
CV777	Belgium	2001	AF353511	83P-5	Japan	2013	AB548618
JS-2004-2	China	2004	AY653204	OKN-1-JPN-2013	Japan	2013	LC063836
DX-S	China	2007	EU031893	CH-LXC-2014	China	2014	KT388418
LZC	China	2007	EF185992	PEDV-14	China	2014	KM609207
DR13/virulent	Korea	2007	DQ862099	CH-HNQX-3-14	China	2014	KR095279
JS2008	China	2008	KC109141	CH-HNYF-14	China	2014	KP890336
BJ-2011-1	China	2011	JN825712	CH-GD-22-2014	China	2014	KP870132
CH-JLCC-2011	China	2011	JQ638920	USA-Minnesota271-2014	USA	2014	KR265813
CH-S	China	2011	JN547228	MEX-124-2014	USA	2014	KJ645700
CH-FJND-1-2011	China	2011	JN543367	OH851	USA	2014	KJ399978
SM98	Korea	2011	GU937797	USA-Ohio126-2014	USA	2014	KJ645702
CH-GXNN-2012	China	2012	JX018179	AOM-2-JPN-2014	Japan	2014	LC063837
GD-A	China	2012	JX112709	AOM-3-JPN-2014	Japan	2014	LC063833
GD-B	China	2012	JX088695	KCH-2-JPN-2014	Japan	2014	LC063845
CH-SDDZ-2012	China	2012	KU133240	KPEDV-9	Korea	2014	KF898124
AH2012	China	2012	KC210145	KNU-1310	Korea	2014	KJ451045
JS-HZ2012	China	2012	KC210147	KNU-1401	Korea	2014	KJ451047
CH-ZJCX-1-2012	China	2012	KF840537	KNU-1406-1	Korea	2014	KM403155
CH9-FJ	China	2012	JQ979287	L00721-GER-2014	Germany	2014	LM645057
CV777/attenuated	China	2012	JN599150	FR-001-2014	France	2014	KR011756
CH7	China	2012	JQ239435	PEDV-WS	China	2015	KM609213
CH-HBXX2-11	China	2013	JX501319	CH-XBC-01-2015	China	2015	KR296677
CH-ZMDZY-11	China	2013	KC196276	CH-YGC-01-2015	China	2015	KR296678

Virus Strain	Countries	Year	accession no.	Virus Strain	Countries	Year	accession no.
CH-SBC-03-2013	China	2013	KC787542	CH-ZWBZa-01-2015	China	2015	KR296680
CH-YNKM-8-2013	China	2013	KF761675	CH-HNAY-2015	China	2015	KR809885
CH-JX-1-2013	China	2013	KF760557	CH-JPYC-02-2015	China	2015	JN547228
CH-HBQX-10	China	2013	JX501318	TW-Pingtung-63	China	2015	KP276250
USA-Indiana-17846-2013	USA	2013	KF452323	CBR2	Thailand	2015	KR610994
USA-Iowa-16465-2013	USA	2013	KF452322	HUA-PED47	Korea	2015	KP455314
USA-Minnesota90-2013	USA	2013	KJ645682	HUA-PED45	Korea	2015	KP455313
MN	USA	2013	KF468752	HUA-PED67	Korea	2015	KP455319
IA1	USA	2013	KF468753	15V010-BEL-2015	Belgium	2015	KR003452
IA2	USA	2013	KF468754	CH-HNCD-2016	China	2016	MF152600
NPL-PEDV-2013	USA	2013	KJ778615	HUA-14PED96	Korea	2016	KT941120
USA-Colorado-2013	USA	2013	KF272920	14JM-226	Japan	2018	KY619763
NK	Japan	2013	AB548623	14JM-126	Japan	2018	KY619740
MK	Japan	2013	AB548624	13JM-291	Japan	2018	KY619768

Virus isolation

Vero cells which grown in a 24-well cell culture plate were infected with the previous supernatants and were maintained in Dulbecco's modified Eagle's medium (DMEM) (Thermo Scientific) contains 7ug/ml trypsin without EDTA (Thermo Scientific). The cells were monitored daily for cytopathic effects (CPE). When the CPE appeared in 70% of the cells, the cells were fixed with anhydrous ethanol and immunofluorescence assay (IFA) was performed using the anti-N protein McAb (Alpha Diagnostic International Inc, Cat#PEDV12-F, USA) at 1:1000 dilution.

Titer detection for the virus growth curve

Vero cells cultured in 24-well cell culture plates were infected with PEDV at a MOI 0.01. The cells and supernatants were collected at 12, 24, 36, 48, 60, 72 and 96 hours postinfection (hpi). Then, the cells were frozen and thawed three times. After centrifugation at 10,000 × g for 5 min at 4 ℃, the supernatants were collected and $TCID_{50}$ was determined using a microtitration infection assay.

Piglet challenge experiment

To determine the virulence of isolated strain GDgh16, six 4-day-old healthy colostrum deprived suckling piglets were obtained and were artificially fed with bovine milk from birth. All the piglets without colostrum were randomly divided into two groups with three piglets in each group. One group was challenged orally with 0.5 mL PEDV at $10^{5.0}$ $TCID_{50}$/mL. Then, the other group received a cell culture medium. The small intestine samples of the euthanized 48 hours post challenge piglets were collected in duplicate. One was crushed in a grinder with 2 mL PBS. Then, the crushed intestine was centrifuged at 10,000 × g for 10 min at 4 ℃. Next, the supernatants were collected and the RNA was extracted. Finally, virus copies from the small intestine were detected by real-time quantitative PCR (RT-qPCR). The other section was stained with the anti-N protein McAb (Alpha Diagnostic International Inc, Cat#PEDV12-F, USA) at 1:1000 dilution for immunohistochemical (IHC) examinations.

Statistical analysis

The numerical data were expressed as the mean ± SD, and the data were analyzed using GraphPad Prism software (version 5.02 for Windows; GraphPad Software Inc.). Differences between groups were assessed using ANOVA. Differences were considered statistically significant at a P value of < 0.05 and were extremely significant at a value of $P < 0.01$ or $P < 0.001$.

Results

PEDV detection and phylogenetic analysis of the S gene

Of all the 362 feces and small intestine samples being tested, 160 samples were PEDV positive (44.20%) from 2015 to 2018, and the positive prevalence was 44.83%(26 positive samples and 58 test samples), 66.67%(46 positive samples and 69 test samples), 42.86%(27 positive samples and 63 test samples),35.47%(61 positive samples and 172 test samples) in 2015, 2016, 2017 and 2018, respectively. The positive prevalence of 2016 was the highest, and that of 2018 was the lowest. Sequence alignment showed that all these strains shared 92.9–100% homology for nucleotides and 91–100% identity for amino acids. Compared to the reference strain CV777, these strains shared 93.1–96.8% nucleotide homology and 91.5–96.8% amino acid identity. These strains shared 93.8–99% nucleotide homology and 92.5–98.9% amino acid identity with Chinese PEDV strains.

Sixty-two S genes of the tested strains and the representative strains that were published in GenBank were analyzed by a phylogenetic tree. As shown in Fig. 1, the phylogenetic analysis showed that these strains could be divided into two groups, namely, G1 and G2. G1 included the classical strains (CV777 and SM98) and some isolates from China, USA and Japan after 2010. Thus, G1 was further divided into three subgroups: G1-a, G1-b and G1-c. G1-a and G1-b were classical S-INDEL strains, and G1-c was a new S-INDEL strain. G2 was a non-S-INDEL strain and was also divided into two subgroups, G2-a and G2-b, which consisted of a number of severely

virulent strains from all over the world since 2010. The strains in our study belonged to G1-b, G1-c, G2-a and G2-b. One strain, GDjm18-2, was categorized as subtype G1-b, which included classical vaccine strains CV777/attenuated and JS2008. One strain GDjm17-1 belonged to the G1-c cluster. The other strains that were identified in our study formed eight clusters. In these strains, 25 isolates from Guangdong, 3 isolates from Fujian and 1 isolate from Jiangxi formed three clusters and belonged to G2-b, having high similarity with GD-A and CH-GXNN-2012. The other 34 isolates formed five clusters and belonged to G2-a. In the 34 strains, JXyc15 has a close relationship with the C4 cluster (North American strains). The other strains had a closer identity with CH-ZMDZ-11, CH-HNAY-2015 and CH-HNCDE-2016L. As shown in Table 4, all the isolated strains from 2015 belonged to G2-a (100%). In 2016 and 2017, there were 46.15% and 43.75% isolated strains belonging to G2-a, respectively. Compared with G2-a, the rate slightly increased, and there were 53.84% and 50% isolated strains belonging to G2-b, respectively. However, in 2018, there were 72.22% isolated strains that belonged to G2-b, which was much higher than that of G2-a in 2017 (22.22%).

Table 4
The PEDV positive prevalence of different groups of tested strains in our study.

Group	2015	2016	2017	2018
G1-b	0	0	0	5.56%
G1-c	0	0	6.25%	0
G2-a	100%	46.15%	43.75%	22.22%
G2-b	0	53.84%	50%	72.22%

Amino acid sequence analysis of the neutralizing epitope in the S protein

To analyze the genetic characteristics of the South China PEDV strains, the deduced amino acids of the S protein isolated in our study were aligned and compared with that of the representative PEDV strains, including strains from G1-a (CV777 and DR13 virulent), G1-b (CV777-attenuated), G1-c (OH851 and CH-ZWZBa-01-2015), G2-a (CH-HNQX-3-14, CH-HNAY-2015, CH-ZMDZY-11) and G2-b (CH-GXNN-2012, CD-A). As shown in Fig. 2, compared to the CV777 strain, the G1-b strain GDjm18-2 had three amino acid (AA) substitutions in the COE domain, and 1 AA substitution in the epitope SS6. The G2-a strains had AA substitutions at thirty-five positions in the COE domain, at two positions in the epitope SS2 and at five positions in the epitope SS6. In these positions, many new AA substitutions have been found in the COE regions of the G2-a strains, including 502 (S/P), 507 (P/M), 510 (N/S), 516 (N/D), 522 (S/A), 527 (S/G), 533 (A/V), 535 (D/E), 547 (D/E), 559 (V/I or A), 562 (S/D), 567 (S/A), 568 (K/T or N), 570 (Q/H), 571 (D/N or Y), 575 (P/L), 580 (S/A), 588 (S/G), 594 (T/R or C), 608 (Y/H), 613 (S/I or G), 614 (G/V), 626 (K/E or S), and 637 (L/ F or S). 2C10 was conserved in all G2-a strains. In the G2-a strains, GDhz16 had four continuous AA mutations in the epitope SS6, which was different from that of the other strains and the reference strains. Compared to the CV777 strain, except for having the AA substitution at one position in three epitopes (SS2, SS6 and 2C10), the G2-b strains had AA substitutions at seventeen positions in the COE domain. In addition to the common AA mutations that were similar to the reference strains in G2-b, there were novel AA substitutions at eight positions in the COE regions, including 504 (V/L), 510 (N/D), 535 (D/H), 542 (S/H), 567 (S/Y), 614 (G/V), 626 (K/T), and 637 (L/V).

Mutated amino acid number analysis of different domains in the S protein

To further analyze the AA mutations in different domains of the S protein in the isolates, the different domains of the S protein were aligned with CV777, and the average number of AA mutations each year were computed. The S protein could be divided into an S1 protein and an S2 protein; the S1 protein contained four domains: SP (1–18), S1-NTD (19–233), COE and RBD (501–629). The S2 protein included five domains: SS6 (764–771), HR1 (978–1117), HR2 (1274–1313), TM (1324–1346) and 2C10 (1368–1374). The previous data indicated that 2C10 was conserved, so we did not analyze the 2C10 domain. As shown in Fig. 3, compared with the S2 protein sequence in these strains, S1 had more AA mutations. From 2015 to 2018, the mutated AA numbers of S1 maintained a high level, but that of S2 decreased. Furthermore, the mutated AA numbers of SP (1–18) and S1-NTD (19–233) slightly increased. However, the mutated AA numbers of the COE and RBD domain decreased. SS6, HR1, HR2 and TM in the S2 protein did not obviously change from 2015 to 2018.

Discussion

At present, PEDV has become a vital viral causing diarrhea and has caused much damage to pig farms worldwide. Because there is no effective vaccine against the emerging prevalent strain in China, the variant PEDV strain has been prevalent in many farms of different areas[21]. Considering the viral variant and limited protection of commercial vaccines, it is necessary to fully understand the genetic variation and epidemiology of PEDV for next-generation vaccine development.

In our study, the genetic variation of PEDV in parts of China was analyzed from 2015 to 2018.

The S gene encodes the largest structural proteins and could stimulate the body to produce neutralizing antibodies. Because of its extensive variant, the S gene has been commonly used as a target gene in studies on the genome characteristics of PEDV[22]. Phylogenetic analysis showed that the strains of four subgroups existed from 2015 to 2018, and G2-a and G2-b are the two most prevalent subgroups in China. From 2015 to 2018, eight strains that belonged to four subgroups (G1-b, G1-c, G1-a and G1-b) were epidemic in Jiangmen of Guangdong, which suggested that PEDV had mutated widely and the epidemic of PEDV was becoming more complex. These results agree with Wen et al.'s report[23]. In 2015, all the isolated strains belonged to G2-a, but in 2018, there were 72.22% strains belonging to G2-b, and only 22.22% strains belonging to G2-a. Interestingly, different from G2-a, which included the strains of other countries, such as America, South Korea, and Japan, the G2-b subgroup only contained Chinese-isolated strains. Combined with previous studies, these results suggest that G2-b strains might be the dominant strains in the future in China [24, 25].

The S protein is much more variable, and many studies showed that the AA changes in the S protein might affect the virulence and pathogenicity. Our study showed that the number of AA mutations in the SP1 and S1-NTD domains increased in 2017 and 2018. It had been reported that S1-NTD might be a vital domain related to viral virulence [26, 27]. The conformation change of S1-NTD might be related to the high pathogenicity of the PEDV strain FJzz1 [24, 28]. Recently, increasingly more virulent PEDV strains emerged [24, 28, 29]. Whether these mutations changed the major conformations and altered the pathogenicity of these strains will be further explored in the future. Our data showed that the positive rate increased from 2015 to 2016, but it decreased from 2016 to 2018, which might be due to the improvement of disease prevention and control strategies. Many pig farms used the mode of "feed-back" to give sows immunity for protecting piglets against PEDV invasion. This was an effective measure to prevent PED, but a risk of virus dispersal also existed, which was why many recombinant PEDV strains were reported [30–34].

At present, four neutralizing epitopes of PEDV S protein have been determined, which were the COE domain (499–638), epitope SS2 (748–755), epitope SS6 (764–771) and epitope 2C10 (1368–1374) [15, 16]. In our study, there were AA changes in as many as 35 positions in the COE domain. Moreover, one strain, GDhz16, had four continuous AA mutations in epitope SS6. Epitopes SS2 and 2C10 also had AA substitutions. Because of the mutation, especially some insertions and deletions in the S protein, antigenicity, pathogenicity and neutralization properties of isolated strains have changed[35, 36]. That was why the prototype strain CV777-derived vaccine could protect against the disease induced by classical strains but not prevent the disease induced by variant strains [37, 38]. Whether these AA changes affect the antigenicity and neutralization properties of the four neutralizing epitopes needs be explored in the future.

Based on previous epidemiological and clinical observations of field strains since 2010, the emerging G2 strains were highly pathogenic[39]. To investigate the pathogenicity of isolated variant strains, three piglets were infected orally with GDgh16. The results showed that the piglets in the infected group began to have clinical signs of diarrhea at 12 h, and the piglets developed a typical symptom of PED at 16 h. The morbidity reached 100%. The piglets began to die at 24 hpi, and all had died by 48 hpi. Moreover, the small intestine had high viral copies and many viral antigens, which indicated that GDgh16 was a highly pathogenic strain. Other researchers demonstrated that different types of pigs infected with variant PEDV strains shared consistent outcomes[40–43]. These results indicated that the variant strains were a large threat to the pig industry, and how to control the PED spread has become a critical issue.

Conclusion

The PEDV strains circulating in parts of China from 2015 to 2018 were clustered into four subgroups: G1-b, G1-c, G2-a and G2-b. The G2-b strains became dominant in 2018. Compared with previous strains, these strains had multiple variations in the SP and S1-NTD domain and in the neutralizing epitope of the S protein. Furthermore, we successfully isolated and identified a new virulent G2-b strain, GDgh16, which was well adapted to Vero cells and had a high mortality rate in piglets. Our study provides full insight into the genetic characteristics of prevalent PEDV strains in parts of China, which suggests that the development of novel effective vaccines is necessary and pressing.

Abbreviations

PEDV: Porcine epidemic diarrhea virus; PED: Porcine epidemic diarrhea; ORFs: Open reading frames; S: Spike; AA: Amino acid; PBS: Phosphate buffer saline; RT-PCR: Reverse transcription polymerase chain reaction; MEGA: Molecular Evolutionary Genetics Analysis; DMEM: Dulbecco's modified Eagle's medium; CPE: Cytopathic effects; IFA: Immunofluorescence assay; IHC: Immunohistochemical; McAb: Monoclonal antibody; hpi: Hour post infection; S-INDEL: Same insertions and deletions; COE: CO equivalent; SS2: Amino acid positions 748–755; SS6: Amino acid positions 764–771; 2C10: 1368 GPRLQPY 1374; G1: Genogroup 1; G2: Genogroup 2.

Declarations

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Availability of data and materials

Genetic data presented in this paper are publicly available via GenBank.

Authors' contributions

Conceived and designed the experiments: Linyang Yu, Jianguo Dong, Li Huang, Changxu Song. Performed the experiments: Linyang Yu, Jianguo Dong, Shuangyun Wang, Yanling Liu , Leyi Zhang, Pengshuai Liang, Lei Wang, Bin Chen. Analyzed the data and wrote the paper: Linyang Yu, Jianguo Dong, Li Huang, Changxu Song. All authors read and approved the final manuscript.

Ethical Statement

All of the samples were collected according to the animal ethics regulations of the National Engineering Center for Swine Breeding Industry (NECSBI 2015-16).

Consent for Publication

Not applicable.

Competing interests

The authors declare no conflicts of interest.

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Figures

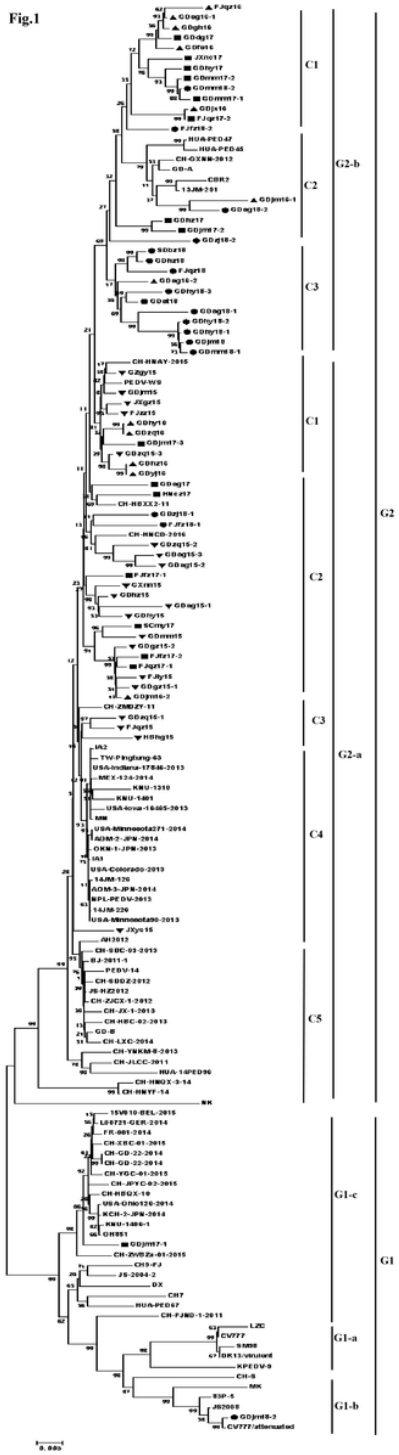

Figure 1

Phylogenetic tree based on the complete S genes of 62 Chinese PEDV strains identified in this study and in other global reference strains. The tree was constructed by the neighbor-joining method in the MEGA V.6.0 program. The Chinese PEDV strains in this study are marked by black triangle, black square and solid circle symbols.

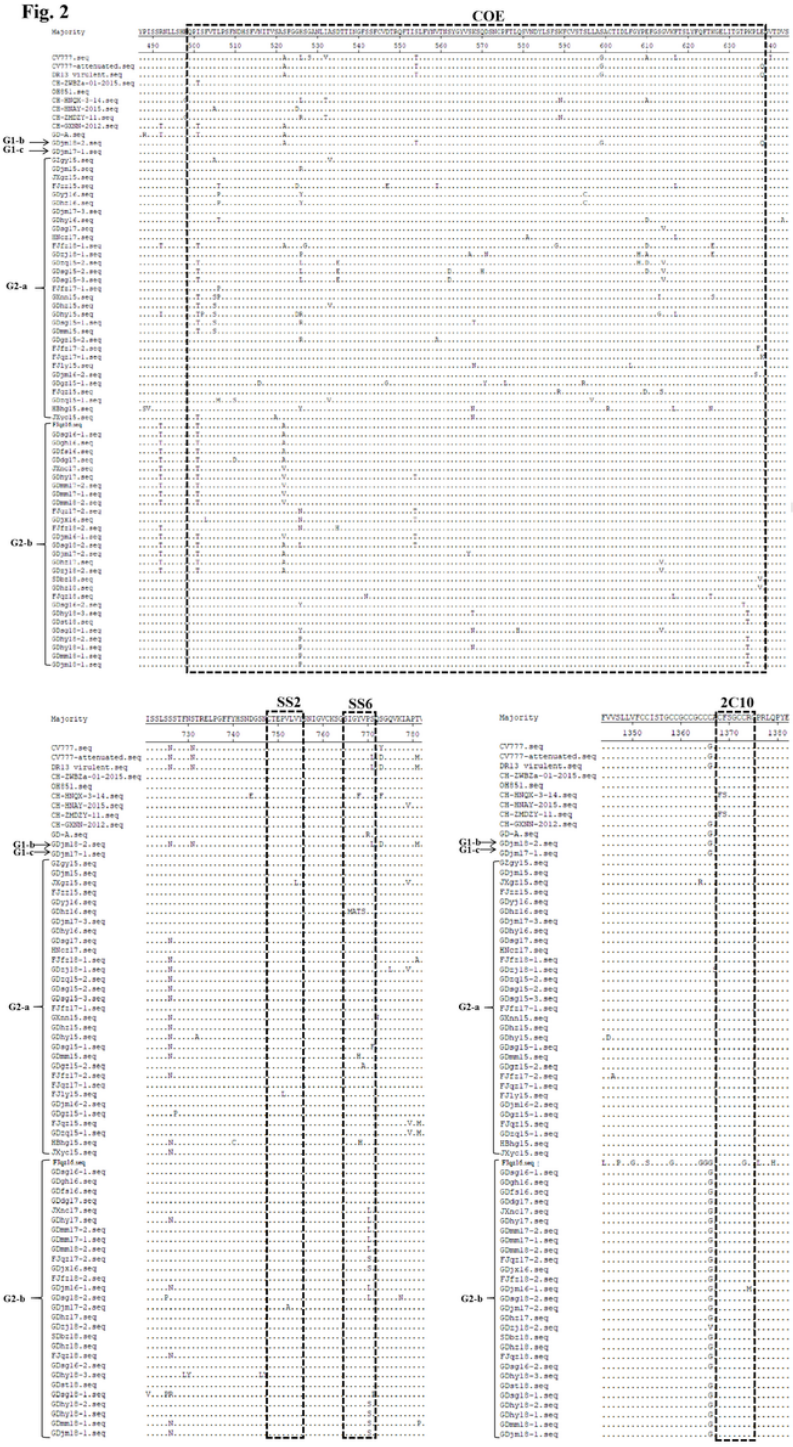

Figure 2

Amino acid sequence analysis of the neutralizing epitope in the S protein The amino acid sequence alignment of S protein neutralizing epitopes of isolated strains and reference strains using the Clustal W method.

Fig.3

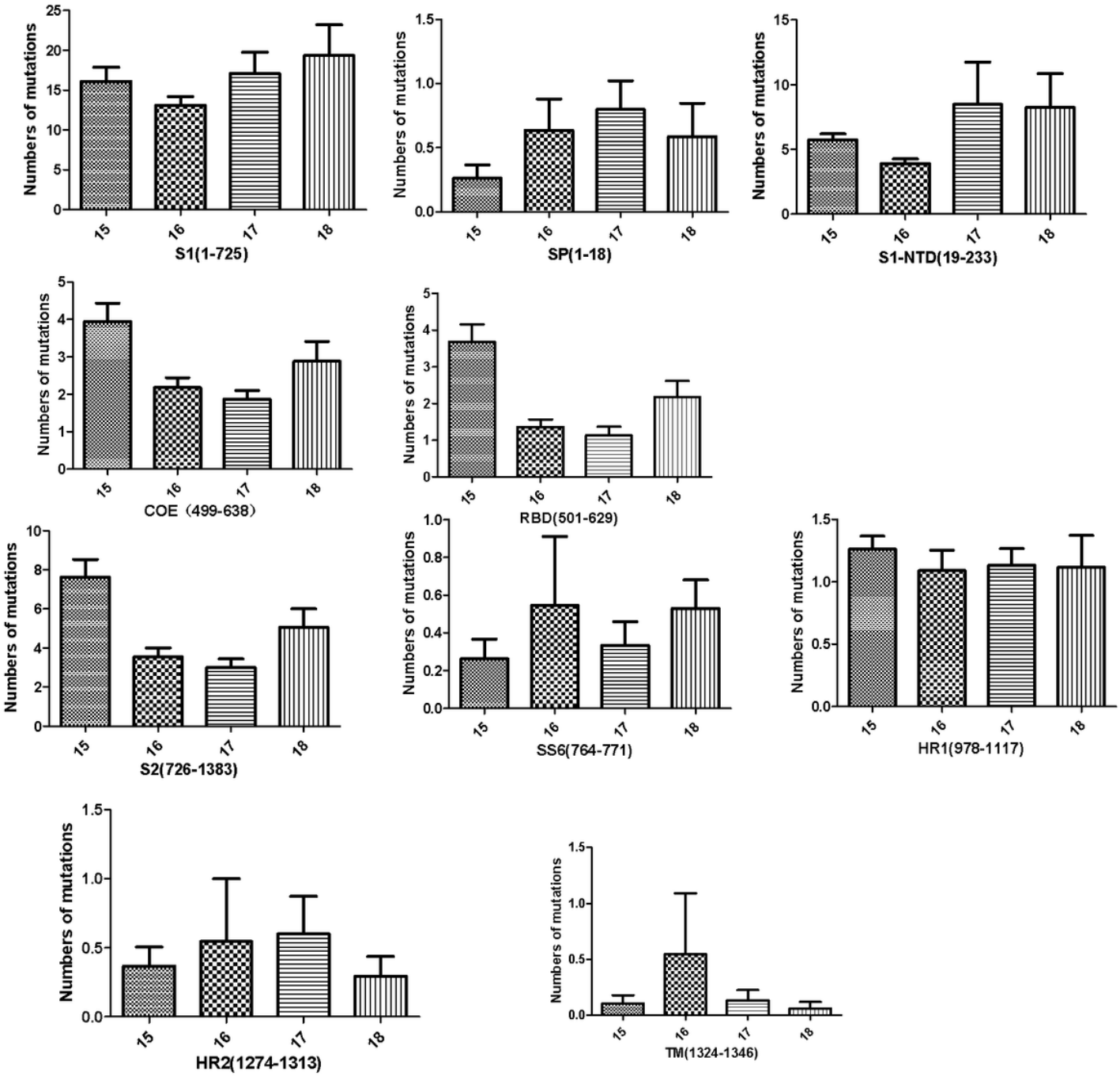

Figure 3

Number of amino acid mutations in different domains in the S protein The number of amino acid mutations in different domains in the S protein, including the S1 subunit (residue 1–725), the S2 subunit (residue 726–1383), the signal peptide (SP, residues 1–18), the N-terminal domain of S1 (S1-NTD, residue 19–233), neutralizing epitopes (COE, residues 499–638; SS2, residues 748-755; SS6, residues 764–771; 2C10, residues 1368–1374), two heptad repeat regions (HR1, residues 978–1117 and HR2, residues 1274–1313), and the transmembrane domain (TM, residues 1324–1346)

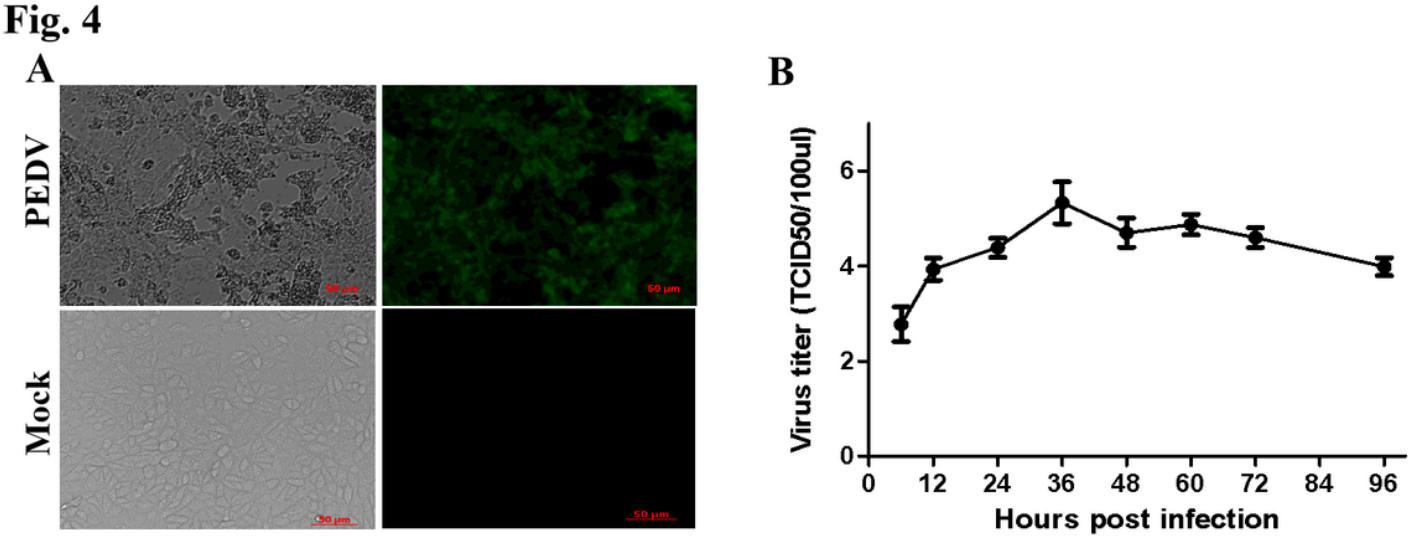

Figure 4

Detection and proliferation kinetic curve of PEDV strain GDgh16 (A) Identification of GDgh16 in Vero cells. The CPE of GDgh16 at 24 hpi was observed by white light and was tested by IFA using mAb against the PEDV N protein. (B) Proliferation kinetic curve of the PEDV strain GDgh16. Vero cells were infected with GDgh16 at MOI of 0.01. The cells and culture solution were collected at 6 hpi, 12 hpi, 24 hpi, 36 hpi, 48 hpi, 60 hpi and 72 hpi, frozen, thawed and centrifuged. Then, the supernatant was collected, and the TCID50 was detected.

Fig. 5

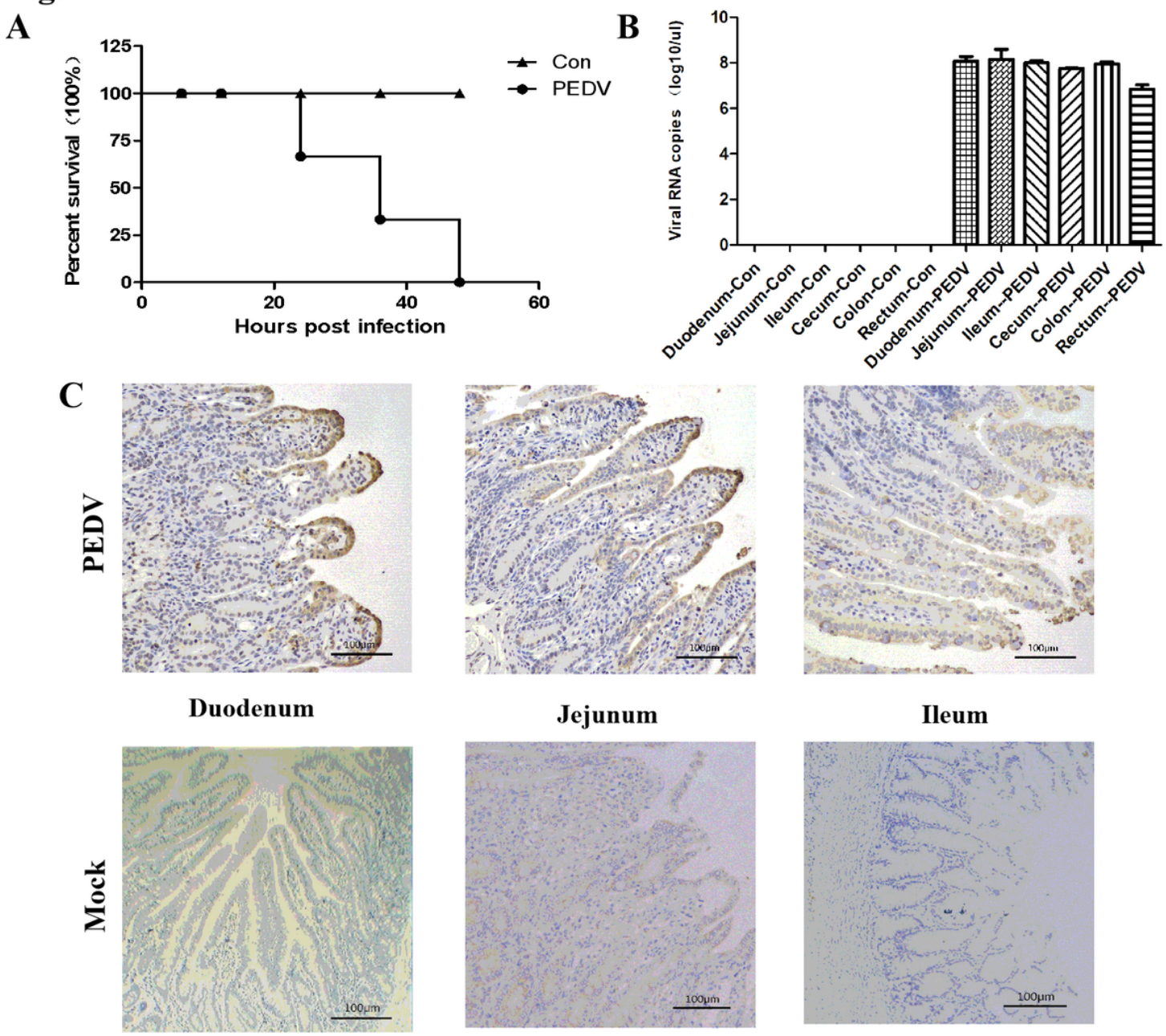

Figure 5

Pathogenicity analysis of GDgh16. (A) The survival rate of piglets in each group. (B) Quantification of the viral load in different parts of the intestine. The different parts of the intestine were collected, and the viral load was quantified by the TaqMan real-time RT-PCR targeting the PEDV N gene. (C) Immunohistochemical detection of intestines. The duodenum, jejunum and ileum of each group were stained with the PEDV monoclonal antibody against the N protein (1:1000 dilution).