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Attenuation of an original US porcine epidemic diarrhea virus strain PC22A *via* serial cell culture passage

Chun-Ming Lin[a], Yixuan Hou[a], Douglas G. Marthaler[b], Xiang Gao[a], Xinsheng Liu[a,c], Lanlan Zheng[a,d], Linda J. Saif[a,*], Qiuhong Wang[a,*]

[a] Food Animal Health Research Program, Ohio Agricultural Research and Development Center, College of Food, Agricultural and Environmental Sciences, Department of Veterinary Preventive Medicine, The Ohio State University, Wooster, OH, USA
[b] Department of Veterinary Population Medicine and Veterinary Diagnostic Laboratory, University of Minnesota,1333 Gortner Avenue, St. Paul, MN 55108, United States
[c] State Key Laboratory of Veterinary Etiological Biology, OIE/National Foot-and-Mouth Disease Reference Laboratory of China, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou, China
[d] College of Animal Science and Veterinary Medicine, Henan Agricultural University, Zhengzhou, China

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ABSTRACT

Although porcine epidemic diarrhea (PED) has caused huge economic losses in the pork industry worldwide, an effective live, attenuated vaccine is lacking. In this study, an original US, highly virulent PED virus (PEDV) strain PC22A was serially passaged in Vero CCL81 and Vero BI cells. The virus growth kinetics in cell culture, virulence in neonatal pigs and the whole genomic sequences of selected passages were examined. Increased virus titers and sizes of syncytia were observed at the 65th passage level (P65) and P120, respectively. Based on the severity of clinical signs, histopathological lesions and the distribution of PEDV antigens in the gut, the virulence of P100 and above, but not P95C13 (CCL81), was markedly reduced in 4-day-old, caesarian-derived, colostrum-deprived piglets. Subsequently, the attenuation of P120 and P160 was confirmed in 4-day-old, conventional suckling piglets. Compared with P120, P160 replicated less efficiently in the intestine of pigs and induced a lower rate of protection after challenge. Sequence analysis revealed that the virulent viruses [P3 and P95C13 (CCL81)] had one, one, sixteen (including an early termination of nine amino acids) and two amino acid differences in non-structure protein 1 (nsp1), nsp4, spike and membrane proteins, respectively, from the fully attenuated P160. However, the overall pattern of attenuation-related genetic changes in PC22A differed from those of the other four pairs of PEDV wild type strains and their attenuated derivatives. These results suggest that PEDV attenuation can occur through multiple molecular mechanisms. The knowledge provides insights into potential molecular mechanisms of PEDV attenuation.

1. Introduction

Porcine epidemic diarrhea virus (PEDV) is an enveloped, positive-sense, single-stranded RNA virus belonging to the genus *Alphacoronavirus*, family *Coronaviridae,* and order *Nidovirales*. The genome of a PEDV is approximately 28 kb in length and is composed of seven open reading frames (ORFs) (Duarte et al., 1993; Zuñiga et al., 2016). The 5′ two thirds of the genome contains two overlapping open reading frames (ORFs), ORF1a and ORF1b, which encode nonstructural proteins (NSP) that direct genome replication, transcription, translation and viral polyprotein processing. The remaining one third contains 5 ORFs encoding four structural and one accessory proteins in the following order: spike (S), ORF3, envelope (E), membrane (M) and nucleocapsid (N) proteins (Duarte et al., 1993). The S protein on the viral surface has the highest sequence diversity among PEDV structural proteins. It is a glycoprotein that binds to the host cell receptors and induces the subsequent virus-cell membrane fusion (Wicht et al., 2014). Several neutralizing epitopes, including the CO–26 K equivalent epitope (COE) (aa position 499–638), SS2 (aa 748–755), SS6 (aa 746–771), and 2C10 (aa 1368–1374) have been identified (Oh et al., 2014; Sun et al., 2007). Sequence comparison of virulent and attenuated PEDV strains revealed that most attenuation-related mutations were located in the NSP3, S, and ORF3 genes (Beall et al., 2016; Chen et al., 2015; Park et al., 2012; Sato et al., 2011; Zuñiga

* Corresponding authors.
E-mail addresses: saif.2@osu.edu (L.J. Saif), wang.655@osu.edu (Q. Wang).

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et al., 2016). However, the detailed molecular mechanisms of PEDV attenuation remain unclear.

Currently, at least two types of genetically distinct PEDV strains are co-circulating on US swine farms (Lin et al., 2016; Vlasova et al., 2014). Sequence analysis of the original, highly virulent US PEDV strains, such as USA/Colorado/2013 and PC22A, revealed that these strains are very similar to the Chinese PEDV strains that have emerged since 2010 (Huang et al., 2013). Without adequate protection *via* lactogenic immunity, 100% morbidity and 50–100% mortality resulting from severe diarrhea were typically observed in neonatal piglets (Stevenson et al., 2013). Subsequently, PEDV variants, including OH851 and Iowa106 strains, sharing the same insertions and deletions in the S1 region of S protein as the "classical" PEDV strains, such as PEDV prototype CV777 strain, were reported and designated as "S INDEL" PEDV strains (Vlasova et al., 2014; Wang et al., 2014). Compared with the original US PEDV (non-S INDEL) strains, some US S INDEL strains showed lower pathogenicity but could still cause severe diarrhea and death in some pig litters (Lin et al., 2015a) similar to the CV777 strain (Pensaert and Martelli, 2016).

Traditional live, attenuated PEDV vaccines, such as attenuated CV777 (Sun et al., 2015), DR13 (Park et al., 2012) and 83P-5 (Sato et al., 2011), have been licensed in Asian countries and were established *via* serial passaging of the original virulent classical PEDV strains in cell cultures. However, the efficacy of these traditional vaccines against the emerging PEDV strains are questionable because epidemic PED outbreaks have been reported on routinely vaccinated swine farms in China (Sun et al., 2015). The high virulence of the emerging non-S INDEL PEDV strains (Liu et al., 2015; Stevenson et al., 2013) and/or the antigenic variations between the classical and emerging PEDV strains (Lin et al., 2015b, 2016) contribute to vaccine failure. Currently, a single inactivated PEDV vaccine (Zoetis, Florham Park, NJ) and an alphavirus-based PEDV subunit vaccine based on the emerging non-S INDEL PEDV strains (Merck, Ames, IA) are conditionally licensed in the US to vaccinate sows to produce lactogenic immunity and provide suckling piglets with passive immunity against PEDV. These vaccines are safe, but lack protective efficacy (Crawford et al., 2016). The protection of nursing piglets against PEDV relies mainly on lactogenic immunity induced by natural infection and/or live, attenuated PEDV vaccination of sows, as was observed for another swine enteric coronavirus, transmissible gastroenteritis virus (TGEV) (Langel et al., 2016). After antigenic sensitization in the gut of sows, pathogen-specific IgA plasmablasts migrate to the mammary glands, where they localize and produce secretory IgA (sIgA) antibodies into colostrum/milk (gut-mammary-sIgA axis) (Langel et al., 2016). As reported in previous PEDV and TGEV studies, inactivated vaccines usually elicit less robust protective immune responses than live, attenuated vaccines when used in native sows (Chattha et al., 2015; Song et al., 2007).

In the present study, we aimed to: 1) generate attenuated US PEDV vaccine candidates *via* serial cell culture passaging of the PC22A strain; 2) evaluate the pathogenicity of PC22A at high passage levels in 4-day-old caesarian-derived, colostrum-deprived

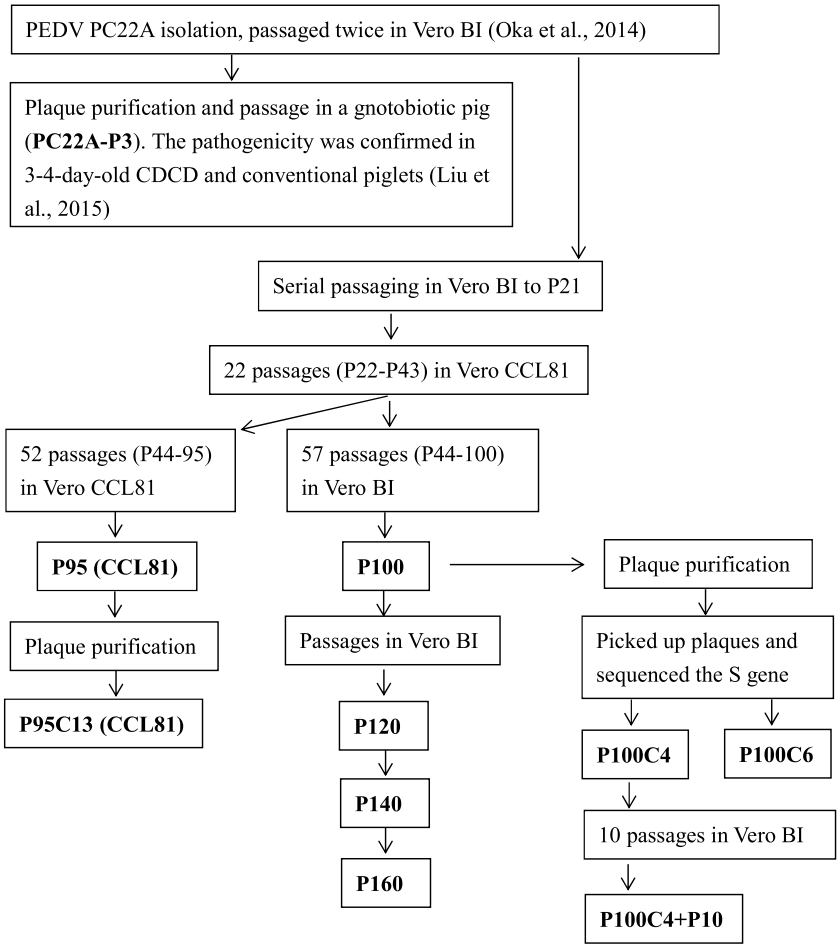

Fig. 1. The history of the isolation and passaging of PEDV PC22A strain in two Vero cells.

(CDCD) pigs; 3) examine the attenuation and immunogenicity of the two most promising virus candidates in conventional suckling piglets; and 4) identify genetic changes related to attenuation by performing comparative, complete genomic sequence analysis of virulent and attenuated PC22A variants.

2. Materials and methods

2.1. Viruses and cells

The isolation and identification of PEDV PC21A and PC22A strains were reported previously by our laboratory (Oka et al., 2014). They were isolated from the same farm outbreak with three SNPs and belong to the original US highly virulent PEDV strains. PC22A had been isolated and passaged three times in Vero cell culture before passaging once in a gnotobiotic pig, and is designated as PC22A P3 virus pool. Its virulence was confirmed in neonatal pigs (Liu et al., 2015; Jung et al., 2014). However, its challenge dose for weaned pigs has not been determined. In the present study, PC22A was passaged regularly to a total of P160 in Vero cells every 4 to 6 days (Fig. 1) as described previously (Oka et al., 2014). On the other hand, PC21A virus pool was prepared by passaging the original PC21A intestinal contents in gnotobiotic piglets before the virus was isolated in Vero cell culture (Jung et al., 2014). The challenge dose (12 $\log_{10}$ genomic equivalent (GE) per pig) of PC21A in PEDV-exposed weaned pigs has been examined and optimized in our earlier study evaluating the pathogenicity and cross-protection activity between the original US and S INDEL PEDV strains (Lin et al., 2015a). Therefore, we used PC21A to challenge weaned pigs in this study. Two Vero cell lines, Vero CCL81 (ATCC No.CCL-81) and Vero BI (a gift from Boehringer-Ingelheim in 1990), were used. At P95 (passaged in CCL81) and P100 (passaged in BI), virus was plaque-purified as described previously (Oka et al., 2014). The S1 was amplified by conventional RT-PCR and sequenced from 8 plaques of P95 (CCL81) and P100. All the clones of P95 (CCL81) had identical S1 sequences and clone#13 was selected for animal studies. Two clones of P100 (P100C4 and P100C6) had 2 nucleotide differences in S1 region and were selected for further characterization. P100C4 was subjected to additional 10 passages (P100C4 + P10) to examine genetic stability.

2.2. In vitro *virus growth kinetics in Vero cells*

Briefly, Vero BI cell monolayer was inoculated with PC22A at selected passages at a MOI = 0.001. The virus inoculum was removed at 2 h post inoculation (hpi), washed with PBS twice and replaced by maintenance medium containing 10 μg/mL of trypsin (Life Technologies) (Oka et al., 2014). Culture supernatant and cell lysates were collected at 12, 24, 36, 48, 72 and 96 hpi, respectively. After freezing and thawing once, the mixtures of culture supernatants and cell lysates were subjected to titration for 50% tissue culture infectious dose ($TCID_{50}$) in 96-well plates and calculated using Reed-Muench method (Reed and Muench, 1938).

2.3. Animal experiment design

PC22A variants at seven passage levels [P95C13 (CCL81), P100C4, P100C6, P100C4 + P10, P120, P140 and P160] were selected for pathogenesis studies in CDCD pigs. Fifty-seven CDCD piglets were derived and raised as described previously (Liu et al., 2015). They were housed in individual cages and randomly allocated to nine experimental groups (Table 1). Each group was maintained in a separate room (biosafety level 2). (Liu et al., 2015). At four days of age, piglets of each group were orally inoculated with the seven different passages of PC22A, highly virulent P3 (positive control), and mock (negative control). The dose of viral inoculum was 100 plaque forming unit (PFU) per piglet. After inoculation, clinical signs were observed 3 to 4 times daily. Rectal swabs were collected daily. Fecal consistency (FC) was scored as follows: 0, solid; 1, pasty; 2, semi-liquid; 3, liquid, respectively. One piglet of each group was euthanized at 1, 3, 6, and 9 days post inoculation (dpi), respectively, for histopathological examination. The remaining piglets were euthanized at the end of the study (10 or 15 dpi).

To confirm the virulence attenuation and assess the *in vivo* cross-protection, an inoculation-challenge study was performed in conventional piglets as described previously (Lin et al., 2015a). Briefly, four sows at 93 to 100-day of gestation were sourced from a specific, pathogen free swine herd of The Ohio State University (OSU). The sows tested negative for PEDV antibody and nucleic acid by immunofluorescence assay (IFA) and reverse transcription-PCR (RT-qPCR), respectively. Each sow was housed in a biosafety level 2 animal room and farrowed naturally. At 4 days of age, each litter of piglets received orally 100 PFU/pig of P3 (positive control), 1000 PFU/pig of P120, 1000 PFU/pig of P160, or mock (negative control), respectively. Throughout the study, piglets were nursed by their sows.

All piglets were challenged with 3 mL of the intestinal contents of gnotobiotic pigs containing 12 $\log_{10}$ GE of highly virulent PEDV PC21A strain on 19–20 dpi to assess cross-protection as described previously (Lin et al., 2015a). Given PC21A has been propagated in Gn piglets, but not in vitro cell culture, the possibility of virulence decrease *via* cell culture adaption can be eliminated. Observation

Table 1
Clinical signs and fecal virus shedding of 4-day-old CDCD piglets inoculated with selected passages of PEDV PC22A.

Passage level (Cell line)	No of Pigs	Diarrhea rate [A,B]	Mortality rate [A]	Onset of diarrhea (dpi), if present	Highest FC score [B]	Duration of diarrhea (days) [B]	Onset of viral shedding (dpi)	Highest viral shedding titer (log GE/mL)
P3	5	100 (5/5)	100 (5/5) [a]	<1	3.00 ± 0.00 [a]	> 3 [D]	< 1	12.70 ± 0.99 [a]
P95C13 (CCL81)	5	100 (4/4)	100 (3/3) [a]	2.75 ± 0.95 [b]	3.00 ± 0.00 [a]	> 4 [D]	1.75 ± 0.50 [c]	12.50 ± 0.76 [a]
P100C4	6	75 (3/4)	0 (0/4) [b]	5.66 ± 1.15 [a]	2.74 ± 0.48 [a]	0, 1 or > 2 [D]	2.33 ± 1.51 [b,c]	12.03 ± 0.58 [a]
P100C6	6	100 (4/4)	0 (0/4) [b]	5.60 ± 2.51 [a]	3.00 ± 0.00 [a]	> 4 [D]	2.75 ± 1.50 [b,c]	11.46 ± 1.25 [a]
P100C4 + P10	6	100 (4/4)	0 (0/4) [b]	3.50 ± 1.73 [a,b]	3.00 ± 0.00 [a]	> 4 [D]	2.80 ± 1.22 [b,c]	11.24 ± 0.68 [a]
P120	6	75 (3/4)	0 (0/4) [b]	6.67 ± 2.08 [a]	2.00 ± 1.41 [a, b]	2.00 ± 1.82	6.00 ± 2.65 [a]	10.19 ± 2.47 [a]
P140	6	50 (2/4) [C]	0 (0/4) [b]	6, 8 [C]	1.50 ± 0.58 [b]	1.00 ± 1.41	4.24 ± 1.07 [a,b]	7.65 ± 0.44 [b]
P160	7	40 (2/5) [C]	0 (0/4) [b]	2, 5 [C]	0.80 ± 0.96 [b]	1.00 ± 0.00	3.75 ± 2.06 [b]	5.00 ± 0.30 [c]
Mock	10	43 (3/7) [C]	0 (0/4) [b]	1, 1, 8 [C]	0.57 ± 0.98 [b]	0.29 ± 0.49	NA	NA

NA: not applicable; dpi: days post-inoculation; Fecal consistency, FC; GE: genomic equivalent.
[a,b,c] Different letters in each column indicate different significance levels among groups ($P < 0.05$).
[A] Piglets euthanized at 1 and 3 dpi for pathology examination were excluded.
[B] FC score: 0 = normal, 1 = pasty, 2 = semi-liquid, 3 = liquid feces. A FC score ≥ 2 was considered as diarrhea.
[C] Mild diarrhea (FC score = 2) when there was no or lower (< 8.30 $\log_{10}$ GE/ml) PEDV RNA shedding.
[D] Piglet(s) still had diarrhea (RS ≥ 2) on the day when it died or was euthanized.

of clinical signs, collection of rectal swabs, blood, and colostrum/milk samples were conducted as described (Lin et al., 2015a; Liu et al., 2015). The animal use protocols (# 2014A00000004) employed in this study were reviewed and approved by the OSU Institutional Animal Care and Use Committee.

2.4. Gross pathology, histopathology and immunohistochemistry examination

The procedure of necropsy, tissue sampling, processing and IHC staining were conducted as described previously (Lin et al., 2015a; Liu et al., 2015). Antigen retrieval and unmasking were performed by treating with 0.05% pronase E (Sigma-Aldrich1, St. Louis, MO) for 20 min. Mouse monoclonal antibody SD6-29 was a gift from Drs. Nelson E. and Lawson S., South Dakota State University used for the detection of PEDV N proteins.

2.5. PEDV RT-qPCR

The PEDV RNA fecal shedding titers were determined by TaqMan real-time RT-qPCR with the primers and probe targeting PEDV N gene (Oka et al., 2014). The detection limit was 10 GE per 20 μL of reaction, corresponding to 4.8 $\log_{10}$ GE per mL of original fecal samples.

2.6. Immunology assays

Serum/milk IgA and IgG and viral neutralization (VN) antibody titers were determined by immunofluorescence assays (IFA) and $TCID_{50}$ reduction assay (Lin et al., 2015b).

2.7. Genomic sequencing

Genomic sequences of PEDV were determined by next-generation sequencing (NGS) technology at the UM Genomic Center (St. Paul, MN) as previously described (Vlasova et al., 2014). GenBank accession numbers for the PEDV PC22A P3, P95C13 (CCL81), P100C4, P100C6, P120, P140 and P160 are KU893861, 893869-72, KX580958 and KU893873, respectively. The genomic sequence of P100C4 + P10 was the same as P100C4.

2.8. Sequence analysis

The genome and/or S gene sequences of various PEDV strains were obtained from GenBank. Multiple-sequence alignments were constructed using Cluster W method in the Lasergene MegAlign (DNASTAR Co., Ltd., Madison, WI). The location of nucleotide/aa changes between virulent/attenuated strains on consensus sequence were then identified manually. N-glycosylation sites were predicted by services available online at http://www.cbs.dtu.dk/services/NetNGlyc.

2.9. Statistical analysis

Statistical analyses were done using SAS (Statistical Analysis System; SAS for windows 9.12; SAS Institute Inc., Cary, NC, USA). Fisher exact test was applied to compare the proportions of presence or absence of diarrhea or death between groups. The serial variables among groups were compared using one way analysis of variance (ANOVA) followed by Duncan's multiple range test. The correlation between FC scores and fecal PEDV RNA shedding titers was determined by Spearman's rank correlation. A P value of less than 0.05 was considered statistically significant.

3. Results

3.1. PEDV PC22A strain at high passage levels replicated to high infectious titers in cell culture

The history of the isolation, characterization and passaging of PEDV PC22A strain is shown in Fig. 1. The infectious titers of PC22A were titrated every 4–5 passages, which were generally lower than 6 $\log_{10}$ PFU/mL before ~P65. In subsequent passages, the virus replicated to 7 $\log_{10}$ PFU/mL. Interestingly, we observed that the sizes of PEDV-induced syncytia increased in P120 and above passages. The diameter of P120-syncytia (600.98 ± 113.39 μm) was significantly larger than those of P9 (207.18 ± 60.13 μm) and P100C6 (212.82 ± 69.69 μm), respectively ($n = 50$; $P < 0.01$).

The multi-step growth kinetics of selected passages of PC22A in Vero BI cells indicated the lag phase of P120, P140 and P160 (12 hpi) was shorter than those of P9, P95C13 (CCL81), P100C4 and P100C6 (24 hpi) (Fig. 2). The highest titers of P9 (6.00 ± 0.67 $TCID_{50}$/mL) and P95C13 (CCL81) (5.50 ± 0.84 $TCID_{50}$/mL) were similar, but

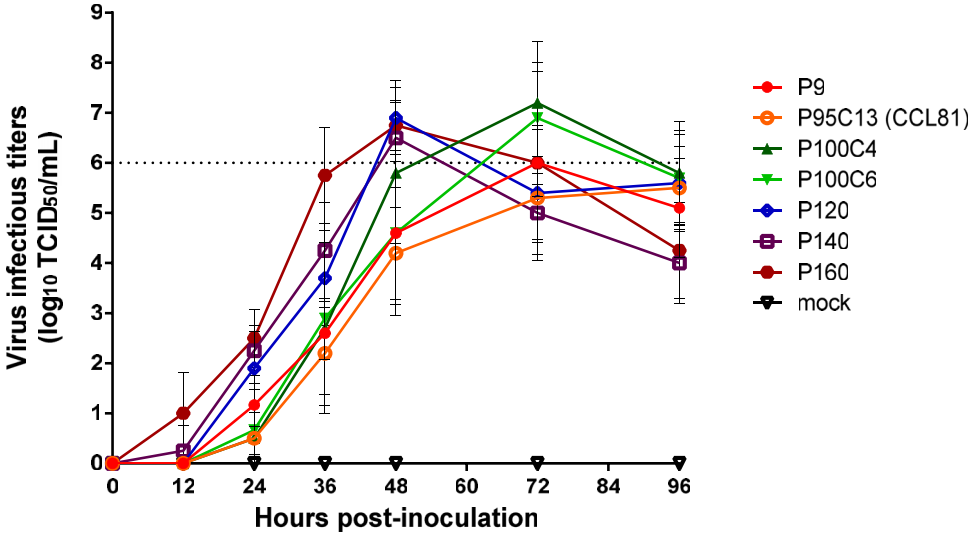

Fig. 2. The growth kinetics of PC22A at selected passages in Vero BI cells. Vero BI cells were inoculated with PC22A at selected passages at MOI = 0.001. Cell lysates were sampled at designated time points and titrated using $TCID_{50}$ infectivity assay.

significantly lower ($P < 0.05$) than those of P100C4 (7.22 ± 1.23 $TCID_{50}$/mL), P100C6 (6.90 ± 1.10 $TCID_{50}$/mL), P120 (6.9 ± 0.74 $TCID_{50}$/mL), P140 (6.5 ± 1.00 $TCID_{50}$/mL) and P160 (6.75 ± 0.50 $TCID_{50}$/mL) (Fig. 2).

3.2. As the cell culture passage level of PC22A increased, viral pathogenicity decreased gradually in 4-day-old CDCD piglets

3.2.1. PEDV PC22A strain at P100 and above demonstrated reduced clinical signs and fecal virus shedding in piglets

The clinical signs and fecal virus shedding of 4-day-old CDCD piglets orally inoculated with 100 PFU/pig of PEDV PC22A at different passage levels are summarized in Table 1. Acute onset of watery diarrhea and transient vomiting were observed in all PEDV PC22A-P3 inoculated piglets within 1 day post-inoculation (dpi). Thereafter, all piglets showed severe anorexia and dehydration and became moribund (1–3 dpi) (Table 1). In the mock control group, two and one piglets had semi-liquid feces (FC score: 2) transiently at 5 and 12 days of age, respectively. However, no fecal PEDV RNA shedding was detected by RT-qPCR in the mock control group throughout the experiment.

In the P95C13 (CCL81) group (n = 5), none of the piglets had clinical signs at 1 dpi. However, all of them had watery diarrhea and the peak of fecal viral shedding (12.50 ± 0.76 log_{10} GE/mL) within 3 dpi (Table 1). The piglets gradually became moribund and died or euthanized at 2, 3, 4 and 6 dpi, respectively.

Compared to the P95C13 (CCL81), no mortality was observed in piglets inoculated with P100 and higher passage levels of PC22A. In the P100C4 group (n = 6), the onset of viral shedding and diarrhea was delayed to 2.33 ± 1.51 and 5.66 ± 1.15 dpi, respectively (Table 1). Similar results were observed in the P100C6 group, except that two of the piglets in the P100C6 group had diarrhea longer than 4 days. There was no statistical difference between the P100C4 and P100C4 + P10 groups.

In the P120 group (n = 6), two piglets exhibited semi-liquid feces at 5, 6 or 9 dpi. Fecal viral shedding was not detectable until 6.00 ± 2.65 dpi. The highest viral shedding titer was 10.19 ± 2.47 log_{10} GE/mL, which was significantly lower ($P < 0.05$) than all the groups, expect for the P140, P160 and the mock control (Table 1). In the P140 group, only two piglets exhibited a transient change from normal to pasty/semi-liquid fecal consistency along with moderate fecal virus shedding (7.50–8.30 log_{10} GE/mL) at 6 and 8 dpi, respectively. In the P160 group (n = 7), there were also two piglets with transient semi-liquid feces at 2 and 5 dpi, respectively. However, the titers of fecal viral shedding were usually below or around the detection limit (4.80 log_{10} GE/mL) (Table 1).

3.2.2. PEDV PC22A strain at high passage levels caused reduced histopathological lesions and had limited tissue distribution

The highly virulent P3 caused severe histopathological changes throughout the entire small intestine of 4-day-old CDCD piglets. PEDV N proteins were observed in the cytoplasm of most villous enterocytes in jejunum (Fig. 3A) and ileum, in some cells of duodenum and cecum, and a few crypt cells of jejunum (Table 2).

In the P95C13 (CCL81) group, piglets showed moderate to severe villous atrophy with villous height: crypt depth (VH:CD) ratios ranging from 1.19 ± 0.33 (6 dpi) to 3.73 ± 0.54 (2 dpi) in jejunum (Table 2). Approximately 40–80% of villous enterocytes were positive for PEDV N proteins (Fig. 3B). In addition to jejunum and ileum, PEDV N proteins were also detected in duodenum and colon (Table 2). However, no PEDV-positive cells were found in the intestinal crypt cells.

In the P100C4 group, one and three piglets euthanized at 6 and 10 dpi, respectively, showed moderate villous atrophy with VH:CD ratios ranging from 2.34 ± 0.67 to 3.91 ± 1.01 in jejunum. PEDV N proteins were detected in the jejunum, ileum, and/or duodenum, but not colon (Table 2). The highest percentage (35%) of PEDV-positive enterocytes was detected in the jejunum of a piglet euthanized at 6 dpi. Otherwise, the percentage of PEDV-positive enterocytes was lower than 10%. Similar findings were observed in the P100C6 (Fig. 3C) and P100C4 + P10 groups, except for one P100C6-inoculated piglet that was euthanized at 10 dpi and showed severe villous atrophy (VH:CD ration = 0.74 ± 0.55) in jejunum (Table 2).

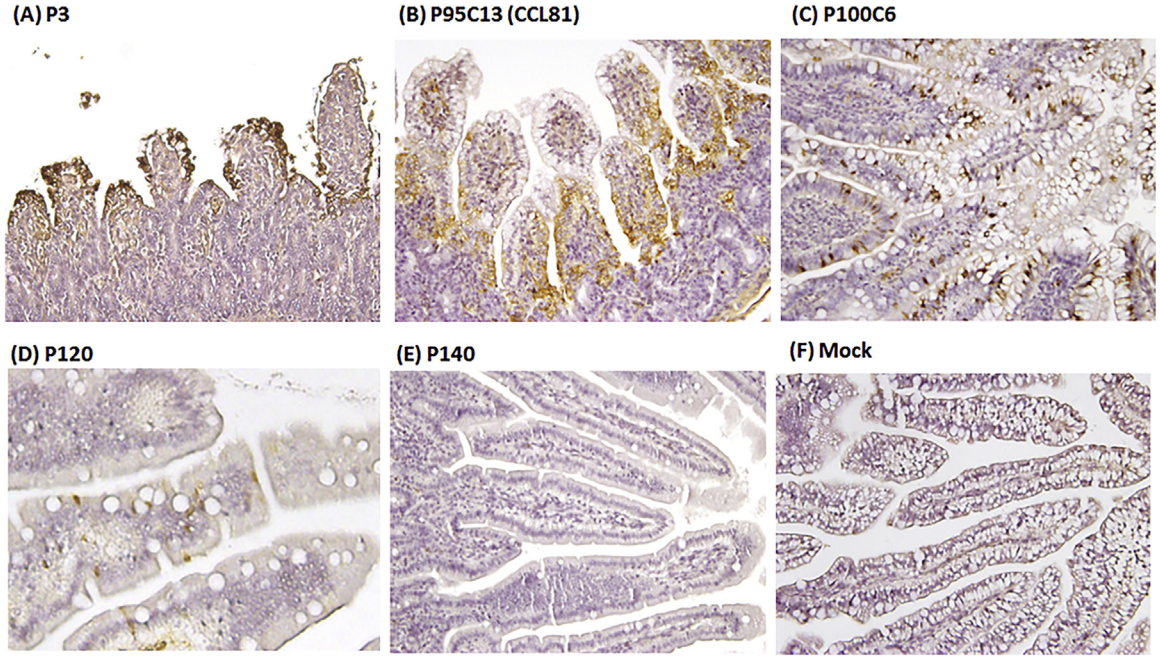

Fig. 3. Immunohistochemical staining of PEDV antigens in the jejunum of pigs. Piglets were inoculated with PC22A P3 (A), P95C13 (CCL81) (B), P100C6 (C), P120 (D), P140 (E) and mock (F), respectively. PEDV N proteins (brown) were detected using monoclonal antibody SD6-29 as the primary antibody. Images were taken at a 200- (A, B, C, E and F) or 400- (D) fold magnification.

Table 2
Histopathological changes in the gastro-intestinal tract of 4-day-old CDCD piglets inoculated with different passages of PEDV PC22A.

PC22A passage	Villous atrophy [A,B,C]	VH:CD ratios (Sampling time, dpi) [A,B]	Infection Sites in intestines				Intra-cellular PEDV N protein staining pattern
			Vertical (location) [B]		Longitudinal (extent)		
			Villous [D]	Crypt	D, J, I	C	
P3	Severe	0.99–2.80 (1–3)	+++ (entire)	+	D (patchy), J, I (cont.)	+	diffuse
P95C13 (CCL81)	Moderate to severe	3.73 (2), 2.17 (3), 1.19 (6)	+++ (entire)	–	D (patchy), J, I (cont.)	+	diffuse
P100C4	Moderate	2.34 (6), 2.54–3.91 (10)	+/++ (entire)	–	D (patchy), J, I (cont.)	–	diffuse
P100C6	Moderate to severe	6.32 (6), 5.02–6.33 (10), 0.74 (10)	+/++ (entire)	–	J, I (patchy)	–	diffuse
P100C4 + P10	Moderate	7.71 (6), 4.76 (9)	+/++ (entire)	–	J, I (patchy)	–	diffuse
P120	Moderate	3.59 (9)	+ (entire/top)	–	J, I (patchy)	–	stippled [E]
P140	Mild	4.99 (9)	–	–	–	–	–
P160	No to mild	5.21 (9)	–	–	–	–	–
Mock	No	9.01 (3), 7.31 (6), 6.80 (9)					

dpi: days post-inoculation, VH:CD: villous height: crypt depth, D: duodenum, J: jejunum, I: ileum, C: colon; cont.: continuous.
[A] Only piglets dead/euthanized near onset of clinical signs and/or peak of fecal PEDV RNA shedding, if presented, are reported.
[B] In jejunum.
[C] Severe, moderate or mild villous atrophy was interpreted when VH:CD ratios <1/3, 1/3 to 2/3 and >2/3 of their age-matched controls.
[D] –, +, ++, and +++ denotes none, less than 30%, 30% to 60% and more than 60% of villous enterocytes showing PEDV N protein positive cells, respectively.
[E] Diffuse: positive signal presented in whole cytoplasm except nucleus. Stippled: pinpoint signal scattered in the cytoplasm.

P120-inoculated piglets euthanized at 9 dpi showed moderate villous atrophy and lower VH:CD ratios compared with the age-matched mock pigs (3.59 ± 0.97 *vs.* 6.80 ± 0.62) (Table 2). PEDV N proteins were stained as tiny stipples within the cytoplasm of the P120-infected enterocytes (Fig. 3D). These positive signals were sporadically detected in less than 10% of enterocytes in jejunum and, occasionally, in the ileum of piglets euthanized at 6 dpi and 15 dpi (Table 2). In the P140 (Fig. 3E), P160 and mock (Fig. 3F) groups, no significant microscopic lesions and PEDV-positive enterocytes were detected, although the VH:CD ratios of the P140 (4.99 ± 0.32) and P160 (5.21 ± 0.31) groups were slightly lower than those (6.80 ± 0.62) of the mock group (Table 2). No PEDV-positive cells were detected in the respiratory system of any pigs in this experiment (lung and trachea epithelia) (data not shown).

3.3. P120 and P160 were attenuated in neonatal, conventional piglets and P120 infection induced partial protection against challenge with the highly virulent PC21A strain

Four litters of 4-day-old conventional, suckling piglets were inoculated orally with P120 (1000 PFU/piglet), P160 (1000 PFU/piglet), P3 (100 PFU/piglet) and virus-free culture medium (mock), respectively. The P3-inoculated litter served as a positive control, where 66.67% (8/12) of piglets had died by 9 dpi, and the four remaining piglets were moribund and euthanized at 9 dpi based on the early removal criteria in our IACUC protocols. Neither piglets (n = 7) nor sow in the mock-inoculated litter (negative control, one litter) showed clinical signs and viral shedding. Serum samples collected from mock-inoculated piglets at 25 days of age and their sow had low viral neutralization (VN) titer (≤8). However, no PEDV-specific IgG and IgA antibody was detected by IFA.

In the P120-inoculated litter, all piglets had semi-liquid to watery diarrhea starting from 2 dpi and lasting for 2–5 days (from 2 to 6 dpi). Both FC scores and viral shedding titers peaked at 5 dpi. Moderate villous atrophy and scattered cells staining for PEDV N proteins in jejunum and ileum were detected in two piglets euthanized at 2 and 3 days after onset of diarrhea. On the other hand, 71% (5/7) of P160-inoculated piglets had moderate diarrhea (semi-liquid feces) for 1 or 2 days observed at 6 (n = 2), 10 (n = 2) or 11 (n = 2) dpi. Compared with the P120 group, P160-inoculated piglets had significantly lower FC scores and viral shedding titers during 2 to 9 dpi ($P < 0.01$). Piglets inoculated with P160 gained

Table 3
Clinical signs, fecal PEDV RNA shedding and serum PEDV antibody titers in conventional suckling pigs inoculated with P120, P160 or mock and challenged with highly virulent homologous US PC21A strain at 19–29 dpi.[A]

	Before PC21A challenge						After PC21A challenge					
	P120-inoculated litter (n = 1)		P160-inoculated litter (n = 1)		Mock-inoculated litter (n = 1)		P120-inoculated litter (n = 1)		P160-inoculated litter (n = 1)		Mock-inoculated litter (n = 1)	
	Piglets (n = 8)	Sow	Piglets (n = 7)	Sow	Piglets (n = 7)	Sow	Piglets (n = 6)	Sow	Piglets (n = 7)	Sow	Piglets (n = 5)	Sow
Morbidity (%)	100	no	71	no	0	no	67	no	100	yes	100	yes
Mortality (%)	0	no	0	no	0	no	0	no	0	no	0	no
Onset of diarrhea (dpi/dpc)	2.75 ± 1.04 *	ND	10.4 ± 5.27	ND	ND	ND	2.00 ± 0.00	ND	2.71 ± 0.76	3	1.80 ± 0.45	3
Highest FC score	3.00 ± 0.00 *	0	1.71 ± 0.49	0	0.00 ± 0.00	0	2.17 ± 0.98 *	0	3.00 ± 0.00	3	3.00 ± 0.00	3
Duration of diarrhea (day)	3.33 ± 1.21 *	ND	0.86 ± 0.69	0	0.00 ± 0.00	0	0.67 ± 0.52 *	0	2.71 ± 0.49	3	3.80 ± 0.84	4
Onset of viral shedding (dpi/dpc)	1.88 ± 0.35 *	2	4.86 ± 0.69	6	ND	ND	1.00 ± 0.00	2	1.43 ± 0.53	1	1.60 ± 0.55	1
Highest viral shedding titer (log_{10} GE/ml)	11.24 ± 0.77 *	7.1	8.90 ± 0.54	8.9	ND	ND	8.92 ± 1.67 *	7.5	10.94 ± 1.05	10.2	10.57 ± 0.81	10.44
Weekly body weight gain (kg)	1.45 ± 0.52 *	NT	2.10 ± 0.28	NT	NT	NT	1.63 ± 1.30 *	NT	0.17 ± 0.80	NT	NT	NT
Serum IgG Ab titer (log_2) [B]	5.67 ± 2.34 *	1	1.58 ± 1.11	1	<2	<2	8.50 ± 1.64	9	8.67 ± 0.98	10	<2	<2
Serum IgA Ab titer (log_2) [B]	6.42 ± 1.59 *	5	<2	<2	<2	<2	8.25 ± 0.27 *	7	4.08 ± 1.04	4	<2	<2
Serum VN Ab titer (log_2) [B]	3.33 ± 1.03	2	2.67 ± 1.40	3	2.57 ± 0.53	3	3.50 ± 1.52	4	4.00 ± 0.69	3	3.00 ± 0.00	3

ND, not detectable; NT, not tested; FC, fecal consistency, Ab, antibody; dpi, days post-inoculation; dpc, days post-challenge; GE, genomic equivalents; VN: viral neutralization.
* Significant difference between P120- and P160-inoculated litters tested by *t*-test ($P < 0.05$).
[A] One P3-inoculated litter serviced as positive control and had a mortality rate of >67% (8/12) and all piglets were euthanized by 9 dpi based on the early removal criteria in our animal experiment protocols.
[B] Serum sample collected at 0 and 7 dpc, respectively.

2.10 ± 0.28 kg of body weight by 7 dpi, which was significantly higher ($P < 0.05$) than that (1.45 ± 0.51 kg) of P120-inoculated pigs (Table 3). Serum IgG and IgA PEDV antibody titers of P160-inoculated piglets were significantly lower than those of P120-inoculated piglets at 19/21 dpi. However, both groups of pigs developed similar low VN antibody titers (≤16) (Table 3). Vertical transmission of PEDV occurred in both sows in the P120 or P160 group by direct contact with their piglets, and viral shedding occurred for 8 and 2 days, respectively. However, clinical signs were not observed in either sow. The serum IgA PEDV antibody titers of P120- and P160-infected sows were 32 and <2, respectively. Similarly, the milk PEDV antibody titers (IgG, 16; IgA, 32) of the P120-infected sow were higher than those (IgG, 2; IgA, < 2) of the P160 PEDV contact-exposed sow at 23 days post-farrowing.

To examine whether P120 and P160 induced cross-protection against the highly virulent PEDV, the surviving conventional pigs were challenged with 12 $\log_{10}$ GE of highly virulent PEDV PC21A strain at 19 (P120 group), 21 (P160 group), and 29 (mock group) dpi, respectively. All pigs in the mock-inoculation group developed severe diarrhea after challenge. In both P120 and P160 groups, onset of diarrhea and viral shedding were observed within 3 days post-challenge (dpc). However, several parameters, including the piglet morbidity, magnitude and duration of diarrhea and viral shedding and weekly body weight gain, differed significantly ($P < 0.05$) between the P120 and P160 groups (Table 3). In addition, the serum IgA PEDV antibody titers of piglets in the P120 group were significantly ($P < 0.05$) higher than those in the P160 group at 7 dpc (Table 3). Viral shedding of contact-exposed sows in both P120 and P160 groups peaked within 4 dpc. However, only the sow in the P160 group developed diarrhea along with high fecal PEDV RNA shedding titers for 4 days. At 7 dpc, the serum IgA PEDV antibody titers of P120- and P160-infected sows were 128 and 16, respectively. Furthermore, the milk collected from the sow in the P120 group had higher PEDV antibody titers (IgG, 128; IgA, 128) than those (IgG, 32; IgA, 8) of the sow in the P160 group. However, the VN PEDV antibody titers in milk remained low for both sows (< 16).

3.4. Accumulation of mutations through serial passaging of PEDV in Vero cells

Amino acid changes of PEDV PC22A at different passage levels are listed in Table 4. Compared with P3, P95C13 (CCL81) had deletions and insertions: 1) three nucleotide deletions (CTA) at positions 4691–4693 of NSP3 gene, resulting in SI1564-1565F in the NSP3 protein; and 2) three nucleotide insertions (GGC) at position 1397 of the S gene, resulting in a change of one amino acid (D) to two amino acids (GH). In addition, several nucleotide point mutations resulted in aa substitutions in ORF1ab (D1622G in NSP3, A3627V in NSP7, and L4622F in NSP12), S (V695I, V881F, Q893K, and G1009V), and ORF3 (I98T) proteins.

Except for V695I in the S protein, all the aa changes in P95C13 (CCL81) also occurred in P100 and above passage levels. On the other hand, the mutations listed as below were consistently detected in P100 and above, but not in P95C13 (CCL81): 1) six nt deletion (164-TTGGTG-169) in S gene, resulting in one aa (I55 K) substitution and two aa (G56E57) deletions in the S protein; 2) three nt point mutations (496A > G, 1360C > A, 2912C > T) in S gene, resulting in three aa substitutions (I166 V, Q454 K and A971 V); and 3) one nt point mutation (673T > A) in M gene, resulting in L225I substitution.

Eight clones from P100 were picked up by plaque assay. Sequencing results showed that clones no. 4 and no. 6 [P100C4 and P100C6] had one single nucleotide polymorphisms (SNP) in the S gene and were selected for further analysis. Whole genomic sequence analysis revealed three aa differences (at aa 173 of NSP1,

Table 4
Changes of nucleotides and amino acids among PEDV PC22A strain at different passage levels.

Gene	Nucleotide position	Amino acid position	PC22A passage						
			P3	P95C13 CCL81	P100C4	P100C6	P120	P140	P160
ORF1ab									
NSP1	518	173	A**C**A (T)	–	A**T**A (I)	–	–	–	–
	661	221	**T**CT (S)	–	–	–	–	–	**A**CT (T)
NSP2	2462	821	G**C**A (A)	–	–	G**T**A (V)	–	–	–
NSP3	4691–4693	1564–1565	T**CTA**TT (SI)	TTT (F)	TTT (F)	TTT (F)	TTT (F)	TTT (F)	TTT (F)
	4865	1622	G**A**T (D)	G**G**T (G)	G**G**T (G)	G**G**T (G)	G**G**T (G)	G**G**T (G)	G**G**T (G)
NSP4	8578	2860	**A**TT (I)	–	–	–	–	**C**TT (L)	**C**TT (L)
NSP7	10880	3627	G**C**G (A)	G**T**G (V)	G**T**G (V)	G**T**G (V)	G**T**G (V)	G**T**G (V)	G**T**G (V)
NSP12	13863	4622	**C**TT (L)	**T**TT (F)	**T**TT (F)	**T**TT (F)	**T**TT (F)	**T**TT (F)	**T**TT (F)
NSP14	17811	5938	**C**AC (H)	–	–	**T**AC (Y)	–	–	–
S	164–169	55–57	A**TTGGTG**AA (IGE)	–	AAA (K)	AAA (K)	AAA (K)	AAA (K)	AAA (K)
	496	166	**A**TA (I)	–	G**T**A (V)	G**T**A (V)	G**T**A (V)	G**T**A (V)	G**T**A (V)
	1360	454	**C**AA (Q)	–	**A**AA (K)	**A**AA (K)	**A**AA (K)	**A**AA (K)	**A**AA (K)
	1397–1399	466	GAT (D)	G**GGC**AT (GH)	G**GGC**AT (GH)	G**GGC**AT (GH)	G**GGC**AT (GH)	G**GGC**AT (GH)	G**GGC**AT (GH)
	2038	680	**GAT**(D)	–	–	–	–	–	**A**AT (N)
	2083	695	**G**TT(V)	**A**TT(I)	–	–	–	–	–
	2641	881	**G**TT (V)	**T**TT (F)	**T**TT(F)	**T**TT(F)	**T**TT(F)	**T**TT(F)	**T**TT(F)
	2677	893	**C**AA (Q)	**A**AA (K)	**A**AA (K)	**A**AA (K)	**A**AA (K)	**A**AA (K)	**A**AA (K)
	2901	967	**T**TT (F)	–	–	–	–	–	TT**A** (L)
	2912	971	G**C**G (A)	–	G**T**G (V)	G**T**G (V)	G**T**G (V)	G**T**G (V)	G**T**G (V)
	3025	1009	G**G**T (G)	G**T**T (V)	G**T**T (V)	G**T**T (V)	G**T**T (V)	G**T**T (V)	G**T**T (V)
	3045	1015	TT**T** (F)	–	–	–	TT**G** (L)	TT**G** (L)	TT**G** (L)
	4135	1379	**G**AA (E)	–	–	–	**T**AA (STOP)	**T**AA (STOP)	**T**AA (STOP)
ORF3	293	98	A**T**T **(I)**	A**C**T (T)	A**C**T (T)	A**C**T (T)	A**C**T (T)	A**C**T (T)	A**C**T (T)
M	448	150	**C**TT (L)	–	–	–	–	–	**T**TT (F)
	673	225	**T**TA (L)	–	**A**TA (I)	**A**TA (I)	**A**TA (I)	**A**TA (I)	**A**TA (I)

NSP: nonstructural protein; STOP: stop codon.
The bold letters indicate mutated/deleted/inserted nucleotides based on P3 virus.

aa 821 of NSP2 and aa 5938 at NSP14, respectively) between P100C4 and P100C6. However, these mutations were not carried over to P120. In addition, the genomes of P100C4 and P100C4 + P10 were identical.

In P120, two additional nt changes appeared in the S gene. One (3045T > G) resulted in F1015L substitution and the other (4135G > T) resulted in a stop codon, shortening the S protein by 9 aa. In P140, an additional one nt change (8578A > C) was found in ORF1ab gene, resulting in an I2860L aa substitution in the NSP4 protein. Compared with P140, P160 had an additional one (S221T), two (D680N and F967L) and one (L150F) aa substitutions in NSP1, S and M proteins, respectively (Table 4).

Interestingly, the reported neutralizing epitopes (COE, SS2, SS6, and 2C10), signal peptide, transmembrane domain and putative S2 fusion peptide of S protein were all conserved in different passage levels of PC22A. Except for one of the 21 Asn-Xaa-Ser/Thr sequons (1294-NTTE) in P160, the other predicted N-linked glycosylation sites were conserved in all passages of PC22A.

3.5. Comparison of the amino acid changes among pairs of virulent and attenuated PEDV strains

To study whether different attenuated PEDV strains shared common mutation patterns, the amino acid changes of the attenuated strains compared with individual parental/low passage level virus were summarized for four strain pairs (PC22A P3/PC22A P160, YN1/YN144, virulent DR13/attenuated DR13 and wild type CV777/vaccine CV777) at the genomic level, and for one pair (parental 83P-5/83P-5-100th) at the S protein level (Akimkin et al., 2016). S protein-based phylogenetic analysis illustrated that PC22A and YN strains belonged to emerging non-S INDEL clade while CV777, DR13, and 83P5 strains clustered within classical clade (Lin et al., 2016). Compared with their parental viruses, the completely attenuated viruses PC22A P160, YN144, attenuated DR13 and CV777 vaccine had 34, 112, 292 and 351 aa changes, respectively. In general, the pattern of amino acid changes in the PC22A P3/P160 pair differed from the other three pairs, although there was high S protein sequence identity (98.82%) between YN1 and PC22A P3. On the other hand, 216/292 (73.97%) aa changes in the attenuated DR13 were also found in the CV777 vaccine strain. In addition, 78.57% (11/14) and 85.71% (12/14) aa changes found in the S protein of the attenuated 83P-5-100th were also found in the S proteins of the attenuated DR13 and CV777 vaccine, respectively.

We also compared the S protein sequences of PC22A at high passage levels and those of other field PEDV variants to exam if they shared similar molecular features. Partial/complete attenuated P100C4, P100C6, P120, P140 and P160 of PC22A, but not the virulent P95C13 (CCL81), had four amino acid substitutions (I55K, I166V, Q454K, and A971V) and a 2 aa-deletion (G56E57) in the S protein. The same aa substitution (I55K) and deletion (G56E57) were also found in the naturally occurring, highly virulent US PEDV Minnesota188/2014 strain (Fig. 4A). In addition, classical PEDV strains and S INDEL strains had a 4 aa-deletion (ΔGENQ56-59) in this region (Fig. 4A). Similar to the 9 aa-deletion at the end of the S proteins of P120 and higher passage levels of PC22A, a 7 aa-deletion was found in the clinically mild Chinese non-S INDEL PEDV strain FL2013 and the classical Korean PEDV vaccine strain SM98 (Fig. 4B).

4. Discussion

In Asian countries, several live, attenuated PEDV vaccine strains were generated by serial passaging of classical PEDV strains in Vero cells (Park et al., 2012; Sato et al., 2011; Song et al., 2007; Sun et al., 2015). In the present study, the original US PEDV strain PC22A was passaged in two lineages (CCL81 and BI) of Vero cells (Fig. 1). Compared with PC22A at low passage level (P3), P100C4, P100C6, P100C4 + P10, P120, P140 and P160, but not P95C13 (CCL81), showed virulence attenuation *in vivo* (Tables 1 and 2; Fig. 3). In addition, P160 replicated less efficiently in pigs than P120 and induced lower serum and milk IgG and IgA antibody titers, corresponding to lower cross-protection after homologous challenge with highly virulent PEDV PC21A strain (Table 3). Furthermore, the location of aa changes in attenuated PC22A P160 differed from other cell culture-attenuated PEDV strains, including CV777 (Sun et al., 2015), DR13 (Park et al., 2012), 83P-5-100th (Sato et al., 2011) and YN144 (Chen et al., 2015), but shared some common

A

PEDV strain	50----------60----------	Pathogenicity	GenBank No.
PC22A-P3	GGYLPIGENQGVNSTWYC	Virulent	KU893861
PC22A-P95C13 (CCL81)	GGYLPIGENQGVNSTWYC	Virulent	KU893869
PC22A-P100C4	GGYLPK- -NQGVNSTWYC	Partially attenuated	KU893870
PC22A-P160	GGYLPK- -NQGVNSTWYC	Attenuated	KU893873
Minnesota188	GGYLPK- -NQGVNSTWYC	Virulent	KM077139
Iowa106	GGYLPS - - - - MNSSSWYC	Mild	KJ645695
83P-5-100th	GGYLPS - - - - MNSSSWYC	Attenuated	AB548621
attenuated DR13	GGYLPS - - - - MNSSSWYC	Attenuated	JQ023162
CV777	GGYLPS - - - - MNSSSWYC	Attenuated	KT323979

B

PEDV strain	-------1380----------1390--	Pathogenicity	GenBank No.
PC22A-P3	CRGPRLQPYEVFEKVHVQ	Virulent	KU893861
PC22A-P95C13 (CCL81)	CRGPRLQPYEVFEKVHVQ	Virulent	KU893869
PC22A-P100C4	CRGPRLQPYEVFEKVHVQ	Partially attenuated	KU893870
PC22A-P120	CRGPRLQPY	Attenuated	KU893872
FL2013	CRGPRLQPYEA	Mild	KP765609
SM-98	CRGPRLQPYEA	Attenuated	GU937797
83P-5-100th	CRGPRLQPYEAFEKVHVQ	Attenuated	AB548621
attenuated DR13	CRGPRLQPYEAFEKVHVQ	Attenuated	JQ023162
CV777	CRGPRLQPYEAFEKVHVQ	Attenuated	KT323979

Fig. 4. Sequence alignments of PEDV PC22A at different passage levels and other PEDV strains. Sequence alignments of the amino acid 50–67 of S1 (A) and the C-terminal region of S protein (B) were performed using Cluster W method.

mutations with PEDV variants naturally occurred in the field (Fig. 4).

In our previous study, approximately 0.1 (10^{-1}) and one PFU of PC22A P3 caused acute onset of watery diarrhea in 40% and 100% of 4-day-old CDCD piglets within 24 h post-inoculation, respectively (Liu et al., 2015). To evaluate the reduction of virulence among different passages of PC22A, 100 and 1000 PFU were used in CDCD and conventional suckling piglet studies, respectively. Compared with piglets inoculated with P3, the onset of diarrhea and viral shedding was slightly delayed in piglets inoculated with P95C13 (CCL81). However, the morbidity and mortality rates of P95C13 (CCL81)-inoculated piglets were still 100%. On the other hand, lower morbidity, no mortality, milder clinical signs and histological lesions were found in P100C4-, P100C6- and P100C4 + P10-inoculated piglets, indicating higher degree of attenuation *in vivo* (Table 1). However, one piglet inoculated with P100C6 still had severe villous atrophy at 10 dpi (Table 2), suggesting the incomplete attenuation of this virus. In the P120 group, the onset of clinical signs and viral shedding were postponed until 5 dpi. Longer incubation time, lower viral shedding titers (Table 1) along with less and limited viral antigen distribution in the intestines (Table 2; Fig. 3) suggested that P120 replicated less efficiently *in vivo* than P100C4, P100C6 and P100C4 + P10. In the P140 and P160 groups, viral shedding titers and duration were reduced further, suggesting P160 full attenuation *in vivo* (Table 1). We hypothesized that adaptation of PC22A to non-porcine Vero cells decreased its replication efficiency in its natural host (Sato et al., 2011). Piglets are known to develop age-dependent resistance to PEDV disease (Lin et al., 2015a; Shibata et al., 2000). Thus, a prolonged incubation time reduces the severity of PEDV infection and disease, leading to low mortality rates.

One strategy for PEDV vaccination is to immunize the sows to induce adequate lactogenic immunity to passively protect neonatal piglets from PEDV infection and disease *via* antibodies in colostrum and milk (Chattha et al., 2015; Langel et al., 2016; Saif et al., 1972). This objective may be accomplished by the selection of an optimal vaccine candidate, whose antigenicity is close to that of the epidemic strain and virulence is minimal or absent in young piglets. In the present study, the attenuation of PC22A P120 and P160 in CDCD piglets was further confirmed in conventional piglets, although short-term (3.33 ± 1.21 days) diarrhea was still observed in some piglets inoculated with the high dose (1000 PFU) of P120. In general, the level of virus replication in piglets and sows correlated with the titers of serum/milk IgG and IgA PEDV antibodies and the level of *in vivo* cross-protection against challenge with the highly virulent PEDV PC21A strain (Table 3). Although mucosa immunity was not measured in the present study, it was reported that PEDV-specific IgA and IgG antibody-secreting cell responses in blood correlated well with the responses found in gut-associated lymphoid tissues (de Arriba et al., 2002) and a strong positive correlation between the anti-PEDV IgA levels in fecal and serum samples has been identified (Gerber and Opriessnig, 2015). Compared with P160, P120 induced higher titers of serum IgA and IgG PEDV antibodies in piglets, corresponding to a higher protection rate. A similar phenomenon was observed in previous studies on TGEV and PEDV: compared with virulent strains, several attenuated TGEV/PEDV vaccines administered orally replicated poorly in the gut, induced low milk antibody titers in sows and resulted in low protection efficacy in suckling pigs (Chattha et al., 2015; de Arriba et al., 2002).

The molecular basis for the observed PEDV attenuation is not yet completely elucidated. The virulence of P100C4 and P100C6, but not P95C13 (CCL81), was significantly reduced in 4-day-old CDCD piglets. A six nucleotide (TTGGTG) deletion at nt positions 164–169 of S protein, which corresponded to the change of IGE55–57 K, occurred in P100C4 and P100C6, but not in P95C13 (CCL81). Interestingly, the same mutation was also observed in an US S 2aa-DEL PEDV variant (USA/Minnesota188/2014) which still caused severe disease at a conventional pig farm (Marthaler et al., 2014) (Fig. 4A). The full genome and S gene sequence identity between Minnesota/188 and prototype of US PEDV Colorado/2013 were 99.9% and 99.4%, respectively. Thus, it is likely that the 2 aa-deletion alone is insufficient to cause viral attenuation. The S1 protein sequences of S INDEL PEDV and other historical PEDV strains have longer (4 aa) deletions at the corresponding position (aa 56–59) (Fig. 4A). Recent studies by us and others showed that the US S INDEL PEDV strains have lower pathogenicity than non-S INDEL PEDV strains (Chen et al., 2016; Lin et al., 2015a). A large deletion in S1 protein also occurred in a field PEDV strain JPN/Tottori2/2014 (194 aa-deletion, residues 23–216 of the S protein) (Masuda et al., 2015) and US PEDV strain PC177 (197 aa-deletion, residues 34–230 of the S protein) (Oka et al., 2014). Both PEDV Tottori2 and PC177 strains showed reduced virulence in pigs (Masuda et al., 2015) (Hou et al., presented at the 35th Annual Meeting of American Society of Virology, Blacksburg, VA, June 18–22, 2016).

While the S1 domain of PEDV S protein is responsible for binding to cell receptors, the S2 domain is responsible for cell membrane fusion activity (Shirato et al., 2011; Zuñiga et al., 2016). In the murine-adapted PEDV MK-p10 strain, loss of the ER retrieval signal KxHxx motif in the cytoplasmic tail of S allowed the S proteins to be efficiently transported from endoplasmic reticulum-Golgi intermediate compartment (ERGIC) onto the cell surface and subsequently increased fusion activity *in vitro* (Shirato et al., 2011). P120 had an early termination of S protein by 9 aa (EVFE**KVHVQ**), including the ER retrieval motif. Similarly, P120 showed increased cell fusion activity *in vitro* and decreased virulence *in vivo* when compared with P9 and P100. In CDCD piglets, much lower fecal virus shedding titers (Table 1), more restricted intestinal virus infection (Table 2) and much less intracellular PEDV N proteins (Fig. 3) were observed in P120-inoculated pigs than in pigs inoculated with the lower passages (P3, P100C4, P100C6 and P100C4 + P10) of PC22A. These results support that the infection of P120 was less extensive than the lower passages of PC22A *in vivo*. Similarly, a 7 aa (FE**KVHVQ**) deletion at the end of S protein was reported for a clinically mild Chinese non-S INDEL PEDV strain FL2013 (Zhang et al., 2015) and a Korean vaccine strain SM-98 (Fig. 4B). These data suggest that the early termination of the S protein and the lack of ER retention signal of PEDV may be a marker for PEDV attenuation, which can be tested using reverse genetics technology.

It is likely that several regions of the S protein (S1 region and/or C-terminal of S2, and others) can affect the virulence of PEDV. In addition, aa changes were also found in ORF1ab, M, ORF3 and E proteins in high passages of PC22A. However, except for one aa (L225I) in M protein, the sequences of ORF1ab, ORF3, E and N proteins of attenuated P120 and virulent P95C13 (CCL81) were the same. Therefore, it is more likely that the attenuation of P120 may not be the result of mutations in ORF1ab, ORF3, E and N genes. However, additional mutations in P160 in ORF1ab (S221T in NSP2), S (D680N, and F967L), and M (L150F) further attenuated P160 compared with P120. Also, comparative analysis of paired virulent/attenuated strains (PC22A, 83P5, DR13, YN CV777) showed different patterns of attenuation-related mutations. Collectively, these results suggest that the complete attenuation of a PEDV strain is the result of a combination of multiple mutations along the genome, and can occur *via* multiple molecular mechanisms. Whether individual or a combination of genetic changes (point mutations, deletions and insertions) of PEDV strains alter viral infectivity, pathogenicity and replication efficiency can be further examined by reverse genetics technology.

5. Conclusion

In summary, PC22A at high passage levels fits the criteria for a good vaccine candidate: 1) Cell culture adaption of PC22A strain facilitated the generation of high titers of virus stocks, which potentially can be used to develop cost-effective vaccines; 2) Based on clinical signs and histopathological examinations, P120 and P160 were attenuated in neonatal CDCD piglets and in conventional suckling piglets. In addition, no alteration of tissue tropism was observed; 3) While P160 was highly attenuated compared to P120, P120 induced active immunity in piglets and lactogenic immunity in the sow, and provided better cross-protection against the challenge with highly virulent homologous PEDV PC21A strain than P160; and 4) The mutations found in P120 were consistently identified throughout the next 40 passages in cell cultures, suggesting genetic stability *in vitro*. The *in vivo* genetic stability will be examined in the future if P120 and/or P160 induce efficacious lactogenic immunity in sows and can protect suckling pigs from PEDV challenge.

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