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ORIGINAL ARTICLE

# Coinfection with porcine epidemic diarrhea virus and *Clostridium perfringens* type A enhances disease severity in weaned pigs

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## Abstract

*Clostridium perfringens* is a constituent of the normal gut microbiome in pigs; however, it can potentially cause pre- and post-weaning diarrhea. Nevertheless, the importance of this bacterium as a primary pathogen of diarrhea in piglets needs to be better understood, and the epidemiology of *C. perfringens* in Korean pig populations is unknown. To study the prevalence and typing of *C. perfringens*, 203 fecal samples were collected from diarrheal piglets on 61 swine farms during 2021–2022 and examined for the presence of *C. perfringens* and enteric viruses, including porcine epidemic diarrhea virus (PEDV). We determined that the most frequently identified type of *C. perfringens* was *C. perfringens* type A (CPA; 64/203, 31.5%). Among the CPA infections, single infections with CPA (30/64, 46.9%) and coinfections with CPA and PEDV (29/64, 45.3%) were the most common in diarrheal samples. Furthermore, we conducted animal experiments to investigate the clinical outcome of single infections and coinfections with highly pathogenic (HP)-PEDV and CPA in weaned piglets. The pigs infected with HP-PEDV or CPA alone showed mild or no diarrhea, and none of them died. However, animals that were co-inoculated with HP-PEDV and CPA showed more-severe diarrheal signs than those of the singly infected pigs. Additionally, CPA promoted PEDV replication in coinfected piglets, with high viral titers in the feces. A histopathological examination revealed more-severe villous atrophy in the small intestine of coinfected pigs than in singly infected pigs. This indicates a synergistic effect of PEDV and CPA coinfection on clinical disease in weaned piglets.

## Introduction

*Clostridium perfringens* is a ubiquitous Gram-positive spore-forming anaerobic bacterium that has been isolated from diverse environments, including swine ecosystems [1]. *C. perfringens* isolates from pigs are divided into five toxinotypes (A–E) based on their ability to produce a combination of six toxins: α- (*cpa*), β1- (*cpb*), β2- (*cpb2*), entero- (*cpe*), ε- (*etx*), and ι-toxins (*iap*) [2]. Although all types of *C. perfringens* can generally be found in the gastrointestinal tract of piglets, only *C. perfringens* types A (CPA) and C (CPC) are considered pathogenic in nursing pigs [3]. In particular, CPA is the most frequently detected bacterial pathogen in porcine neonatal diarrhea [3, 4].

Porcine epidemic diarrhea virus (PEDV) is a swine enteric coronavirus belonging to the subgenus *Pedacovirus* of the genus *Alphacoronavirus* within the family

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*Coronaviridae* of the order *Nidovirales* [5]. PEDV is highly contagious and can be fatal for neonatal piglets within their first week of life, with morbidity and mortality up to 100%. However, it causes milder clinical disease with lower mortality in older pigs [6, 7]. In 2013, a highly pathogenic (HP) PEDV strain emerged in the United States. Subsequently, it spread to other pig-raising countries in America, Asia, and Europe, posing a significant health threat to the global pig population [6, 8]. Since then, PEDV strains have been divided into two genotypes, each with two subgenotypes: the low-pathogenic genotype 1 (historical G1a and recombinant G1b) and the HP genotype 2 (local epidemic G2a and pandemic G2b) [6, 7].

Since the 2013–2014 pandemic, the HP-G2b strain of PEDV has evolved and become endemic to South Korea, resulting in year-round small- to large-scale outbreaks nationwide [6, 7]. In endemic circumstances, PEDV persists in weaning or growing-finishing barns, where the virus circulates, causing mild post-weaning diarrhea with low mortality rates [6]. PEDV infection in weaned pigs leads to decreased growth performance, including lower average daily gain (ADG) and average daily feed intake (ADFI) [9]. Furthermore, the severity of clinical disease in nursing and weaning piglets may be exacerbated by coinfections with other viral and bacterial enteropathogens [10]. Since the incidence and type of *C. perfringens* associated with pre- and post-weaning diarrhea in South Korea remain undetermined, the aim of the present study was to investigate the prevalence and typing of *C. perfringens* in diarrheic samples collected from domestic swine farms. We evaluated the pathogenic outcomes of single and sequential coinfections with PEDV (HP-G2b) and CPA in weaned pigs.

## Materials and methods

### Collection of clinical samples

In total, 61 commercial farrow-to-finish swine farms located in South Korea with a reported history of diarrheic disease were sampled to identify *C. perfringens*. Fecal samples ($n=203$) of affected piglets (2–4 weeks of age) showing pre- or post-weaning diarrhea collected from January 2021 through January 2022 were submitted for laboratory diagnosis. Fecal samples were diluted with PBS to make 10% (wt/vol) suspensions [11–13], which were vortexed and then centrifuged for 10 min at 4,500 × *g* in a Hanil FLETA5 centrifuge (Incheon, South Korea). The clarified supernatants were initially subjected to conventional PCR for the detection and typing of *C. perfringens* as described previously [2, 3]. In brief, *cpa*-gene-based PCR was performed to detect *C. perfringens* using diarrheic fecal samples, and *C. perfringens* isolates were further assigned to one of the five toxinotypes based on the combination of toxin genes detected by PCR (e.g., CPA was diagnosed by testing for the *cpa* and *cpb2* genes, while CPC was diagnosed by testing for the *cpa* and *cpb* genes). To determine if any other diarrhea-causing viral pathogens were present in the clinical samples, we performed virus-specific RT-PCR analysis for PEDV, transmissible gastroenteritis virus (TGEV), porcine deltacoronavirus (PDCoV), and porcine rotaviruses (PRV) as described previously [14–16]. PEDV-positive samples were further subjected to nucleotide sequence analysis for genotyping as described elsewhere [12, 15, 16].

### Pig infection experiments and clinical examination

The animal studies described here were approved by the Institutional Animal Care and Use Committee (IACUC) of the Choong Ang Vaccine Laboratory (CAVAC) and conducted at the CAVAC Animal Facility as described previously [11, 13, 17, 18]. A total of 16 weaned piglets (19 days old at the start of the experiment) were obtained from commercial crossbred sows (Great Yorkshire × Dutch Landrace) at a conventional breeding farm with a good health record and without a previous herd history of a PEDV outbreak or PEDV vaccination. All pigs were also tested to confirm that they were not infected with any porcine enteric viruses, including PEDV, TGEV, PDCoV, and PRV [14–16]. Pigs were assigned to four experimental groups and housed in four separate rooms: the PEDV (HP-G2b KNU-141112 strain)-inoculated group ($n=4$), the CPA (CAVAC strain carrying the *cpa* and *cpb2* genes encoding the α- and β2-toxins)-inoculated group ($n=4$), the PEDV/CPA co-inoculated group ($n=4$), and the control group ($n=4$). The animals were fed commercial milk replacer (3–4 times daily) and had *ad libitum* access to water for the duration of the study [11, 13, 17, 18]. At 21 days of age (after a 2-day acclimation period), the pigs were inoculated orally with KNU-141112 at a dose of $10^{4.0}$ 50% tissue culture infective doses ($TCID_{50}$) per pig [17] or CPA at a dose of $1.1 \times 10^{9.0}$ colony-forming units (CFU) per pig [19]. Pigs in the PEDV and CPA coinfection group were first inoculated orally with PEDV and then with CPA at the same dose as described above. The dose of PEDV was equivalent to 1,000 times the median pig diarrhea dose ($PDD_{50}$) of the KNU-141112 strain in neonatal piglets [11, 17, 20]. The sham-inoculated piglets were inoculated with cell culture medium as a control. All piglets were observed for 7 days post-inoculation (dpi), with daily recording of clinical signs (vomiting and diarrhea) and mortality [11, 13, 17, 18]. Stool samples from the pigs in all groups were collected prior to inoculation and thereafter daily using 16-inch cotton-tipped swabs and subjected to quantitative real-time RT-PCR (rRT-PCR) to

measure PEDV fecal shedding titers as described previously [13, 15, 17, 18]. The KNU-141112 strain with a known infectivity titer was serially diluted tenfold to generate a standard curve in each PCR plate. The virus concentrations (genome copies/ml) in the samples were calculated based on this standard curve. The mean cycle threshold (Ct) values were calculated for the PCR-positive samples, and the mean virus titers were calculated for all pigs within each group [13, 15, 17, 18]. In addition, the area under the curve (AUC) of viral shedding in each group was calculated using the log transformed values of the viral loads from 1 to 7 dpi [21]. The clinical significance score (CSS) was determined using the following scoring criteria for diarrheal severity based on the fecal consistency at 7 dpi as described previously [13, 17, 18]: 0, normal and no diarrhea (mean Ct values > 35); 1, mild and fluidic feces; 2, moderate mucous to watery diarrhea; 3, severe watery and projectile diarrhea (mean Ct values < 20); 4, death. The animals were weighed daily to measure their ADG. Any pigs that died during the study were necropsied, while the surviving pigs from the inoculation and control groups were euthanized at 7 dpi for post-mortem examination [11, 13, 17, 18].

### Histopathology and immunohistochemistry of the small intestine

At necropsy, intestinal tissues and other major organs were examined macroscopically. Small intestinal tissue (duodenum, proximal jejunum, and ileum) specimens (< 3 mm thick) were collected from each piglet, fixed in 10% formalin for 24 h at room temperature, and embedded in paraffin according to standard laboratory procedures as described previously [11, 13, 17, 18]. The formalin-fixed paraffin-embedded tissue samples were cut into 4- to 5-µm-thick sections on a microtome (Leica, Wetzlar, Germany), floated in a 40°C water bath containing distilled water, and transferred to glass slides. The tissue was then deparaffinized in xylene for 5 min and rehydrated in decreasing concentrations of ethanol (100%, 95%, 90%, 80%, and 70%, respectively) for 3 min each. The deparaffinized intestinal tissue sections were stained with hematoxylin and eosin (H&E; Sigma-Aldrich, St. Louis, MO) for histopathology or subjected to immunohistochemistry (IHC) for the detection of PEDV antigens using a PEDV nucleocapsid (N)-specific monoclonal antibody (MAb; CAVAC, Daejeon, South Korea) as described previously [11, 13, 17, 18]. The severity of villus atrophy was also quantified by measuring the ratio between villous height and crypt depth (VH:CD) throughout the H&E-stained small intestinal sections, and the mean ratio of VH:CD in each small intestine segment was calculated as described previously [18, 22].

### Statistical analysis

All values are expressed as the standard deviation of the mean (SDM). Statistical analysis was conducted using the GraphPad Prism 7 software package (GraphPad Software, San Diego, CA). *P*-values less than 0.05 were considered statistically significant.

## Results

### Prevalence of PEDV or/and CPA in the swine samples

A total of 203 fecal samples from 61 swine farms (3.3 samples on average) were examined bacteriologically for *C. perfringens* as well as for enteric viruses, including PEDV, TGEV, PDCoV, and PRV. On seven farms (11.5%), none of the pathogens investigated in this study were detected, but 129 fecal samples (63.5%) from 49 farms (80.3%) tested positive for PEDV. All of the PEDV isolates that were detected were further investigated by nucleotide sequencing and found to belong to the HP-G2b strain. These data indicated that most swine farms tested in this study were PEDV endemic. In 65 (32.0%) of the samples from 25 farms (41%), *C. perfringens* was identified. Subsequent typing of the *C. perfringens*-positive samples revealed that 64 samples (98.5%) from 25 farms (100%) contained CPA, while one sample (1.5%) from one farm (4%) was positive for CPC. Molecular toxin typing revealed that 82.8% of the CPA isolates (53/64) carried the coding genes for the α- (*cpa*) and β2- (*cpb2*) toxins, suggesting virulence potential. The remaining CPA-positive samples (11/64, 17.2%) contained only the *cpa* toxin gene. No sample or farm was positive for other toxinotypes. Among the CPA-positive samples, single CPA infections (30/64, 46.9%) and CPA/PEDV coinfections (29/64, 45.3%) were almost equally prevalent, and 29 out of 64 CPA-positive samples from 17 out of 25 CPA-positive farms were also positive for PEDV. CPA/PDCoV (2/58) and CPA/PRV (3/58) coinfections were detected on one and two of the CPA-positive farms, respectively. One CPC-positive fecal sample tested positive for PEDV (CPC/PEDV coinfection). An overview of the combination of pathogens that were detected in 203 diarrheal samples from 61 farms and toxin typing of 65 CPA isolates is shown in Fig. 1. The predominant combinations of pathogens detected in pre- and post-weaning diarrheic samples were PEDV (99/203, 48.8%), CPA (30/203, 14.8%), and CPA/PEDV (29/203, 14.3%), and the most relevant major toxin gene profile in the CPA isolates was the combination of *cpa* (α-toxin) and *cpb2* (β2-toxin) genes.

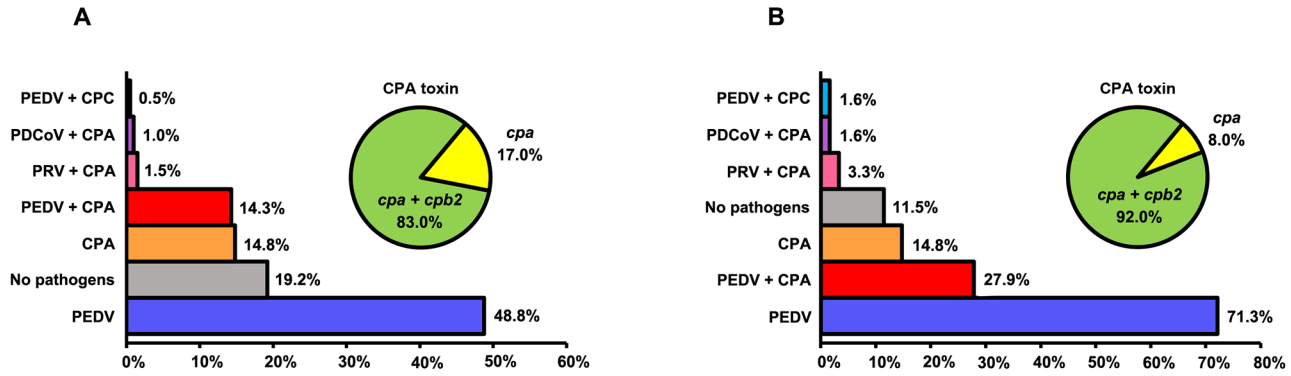


**Fig. 1** The combination of pathogens detected in pre- or post-weaning diarrheic samples (A, $n=204$) from commercial swine farms (B, $n=61$) in South Korea, 2020–2022. The circular graphs in each panel show the percentage of pigs (A) and farms (B) with at least one CPA isolate encoding *cpa* and/or *cpb2* toxin genes.

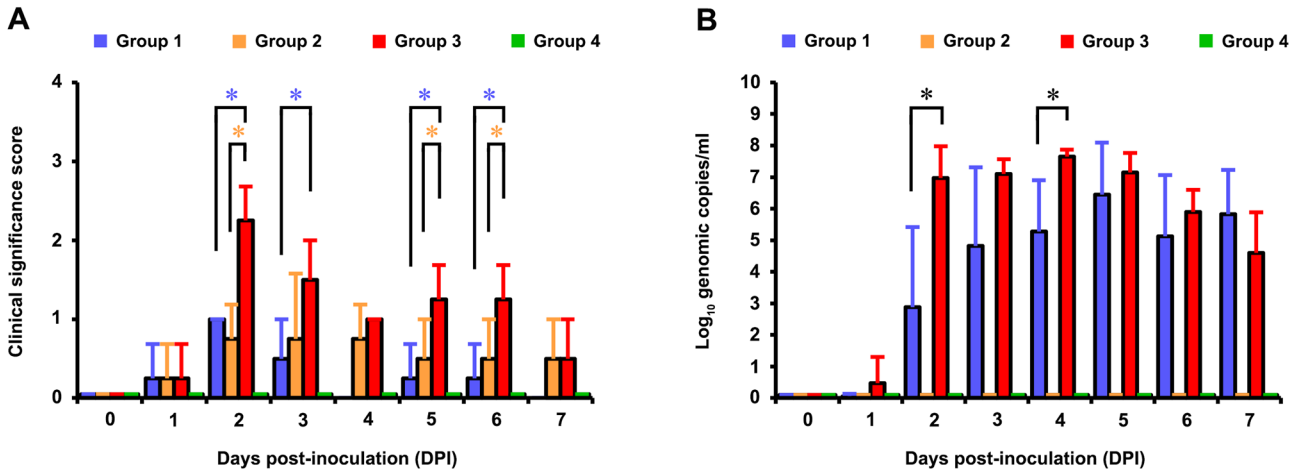


**Fig. 2** The clinical outcomes of single infections and coinfections with PEDV and CPA in weaned piglets. (A) The clinical significance scores (CSS) for each group. The CSS of individual pigs from each group was measured as described in Materials and methods. (B) The fecal viral RNA shedding profile of PEDV in each group. The PEDV RNA titers ($log_{10}$ genomic copies/ml) in rectal swaps at the indicated sampling time points were determined using rRT-PCR. The mean values for each group at each time point are presented, and error bars indicate the SDM. The *P*-values were calculated by comparing the data between the PEDV/CPA coinfection and single-infection groups. *, $P=0.001$–$0.05$; **, $P<0.001$

## Clinical outcomes of coinfection with PEDV and CPA in weaned pigs

Based on the CPA prevalence study, it was interesting to note that CPA/PEDV coinfection appeared to result in higher disease severity than a single infection with either pathogen. To determine the clinical consequences of the interaction between the co-circulating PEDV and CPA strains, we investigated the effects of coinfection with HP-PEDV and CPA in weaned piglets. Sixteen pigs were divided into four groups of four animals each and challenged orally with HP-PEDV (group 1), CPA (group 2), or HP-PEDV/CPA (group 3), and the remaining four animals in the control group were inoculated with cell culture medium. Clinical signs were recorded three times daily, and fecal swabs were collected before inoculation and daily after inoculation throughout the experiment. All animals were vigorous and clinically normal, showing normal stool consistency, and their fecal samples were negative during PEDV RNA for the 2-day acclimation period.

The negative-control piglets demonstrated normal clinical manifestations throughout the study. As shown in Fig. 2A, the piglets infected with PEDV KNU-141112 showed mild diarrhea at 2 dpi (mean CSS = 1.0) and then recovered at 3 or 4 dpi. PEDV-associated mortality was absent in the HP-PEDV group. Similarly, except one pig in group 2 that exhibited moderate watery diarrhea at 2 dpi, the animals inoculated with CPA alone displayed mild diarrhea at 1 or 2 dpi (mean CSS = 0.25 and 0.75, respectively) without mortality and recovered at 5 or 7 dpi. However, the piglets

from the HP-PEDV/CPA coinfection group showed moderate to severe watery diarrheal symptoms at 2 dpi (mean CSS = 2.25) but remained alive until the end of the study. The diarrhea of two piglets in group 3 lasted until 7 dpi, while the remaining animals recovered at 7 dpi (Fig. 2A).

Most inoculated piglets (3/4) in group 1 tested positive for PEDV by 2 dpi with a mean titer of $10^{2.9}$ genomic copies/ml. All of the animals shed large amounts of PEDV in their feces, with mean titers ranging from $10^{4.8}$ to $10^{6.4}$ genomic copies/ml (Fig. 2B). Although fecal shedding of PEDV was observed in all piglets of the HP-PEDV/CPA coinfection group by 2 dpi, these pigs shed much higher amounts of PEDV, with a mean titer of $10^{6.9}$ genomic copies/ml, than the piglets inoculated with PEDV alone. The titer then increased gradually, ranging from $10^{7.1}$ to $10^{7.7}$ genomic copies/ml at 5 dpi, and declined thereafter. Overall, a significantly larger amount of fecal shedding at 2 and 4 dpi was observed in the HP-PEDV/CPA coinfected animals (group 3) than in those in group 1, with a maximum of a 4-log elevation compared to group 1 upon coinfection (Fig. 2B). We also found that the mean viral AUC in the HP-PEDV/CPA coinfection group was greater than that in the HP-PEDV single-infection group (35.5 ± 0.8 vs. 27.5 ± 1.8 log10 copies/ml × days). The feces of the piglets in the CPA-inoculated and control groups were negative for PEDV RNA during the trial.

In addition, the starting and final body weights of individual piglets from each group were measured at 0 and 7 dpi during the experimental period (Fig. 3A). The pigs inoculated with CPA (group 2) weighed 2.3% less and those inoculated with HP-PEDV/CPA (group 3) weighed 7.3% less at the end of the study than the control pigs (group 4), which were not significantly different in weight from the HP-PEDV-inoculated animals (group 1). The ADG per group was calculated from 0 to 7 dpi, and the overall ADG in the challenged pigs from groups 1–3 was compared to that in the unchallenged animals (group 4) (Fig. 3B). Single infection and coinfection with CPA resulted in a significant decline in ADG compared with the control group. The HP-PEDV/CPA-coinfected pigs (group 3) had the greatest decrease in ADG (mean ADG of -0.525 kg) compared to the control pigs (mean ADG of + 0.65 kg). There was no significant variation in ADG between the HP-PEDV-infected and control groups.

All animals in the inoculated and control groups were euthanized for postmortem evaluation at 7 dpi (Fig. 4). No macroscopic or histologic intestinal alterations were recorded in the sham-inoculated piglets (Fig. 4D). Although all animals in the single-infection and coinfection groups displayed no gross macroscopic lesions at necropsy, they had microscopic intestinal changes demonstrating jejunal villous atrophy (Fig. 4A–C). IHC staining detected PEDV N proteins predominantly in the cytoplasm of epithelial cells in atrophied jejunal villi of the piglets infected with HP-PEDV alone or coinfected with HP-PEDV and CPA (groups 1 and 3; Fig. 4A and C). PEDV antigen was absent in the small intestines of all of the animals infected with CPA alone (group 2; Fig. 4B) or inoculated with cell culture medium (group 4; Fig. 4B). PEDV RNA titers were also determined in the intestinal tissue samples collected at necropsy. There were no statistical differences in the PEDV titers in the small intestinal segments between the HP-PEDV single-infection and HP-PEDV/CPA coinfection groups (Fig. 5A). However, the mean (± SDM) VH:CD ratios of the jejunum differed significantly among the groups (Fig. 5B). The lowest ratio was observed in the HP-PEDV/CPA coinfection group (3.12 ± 0.39), medium to low ratios were observed in the CPA (3.67 ± 0.92) and HP-PEDV single-infection

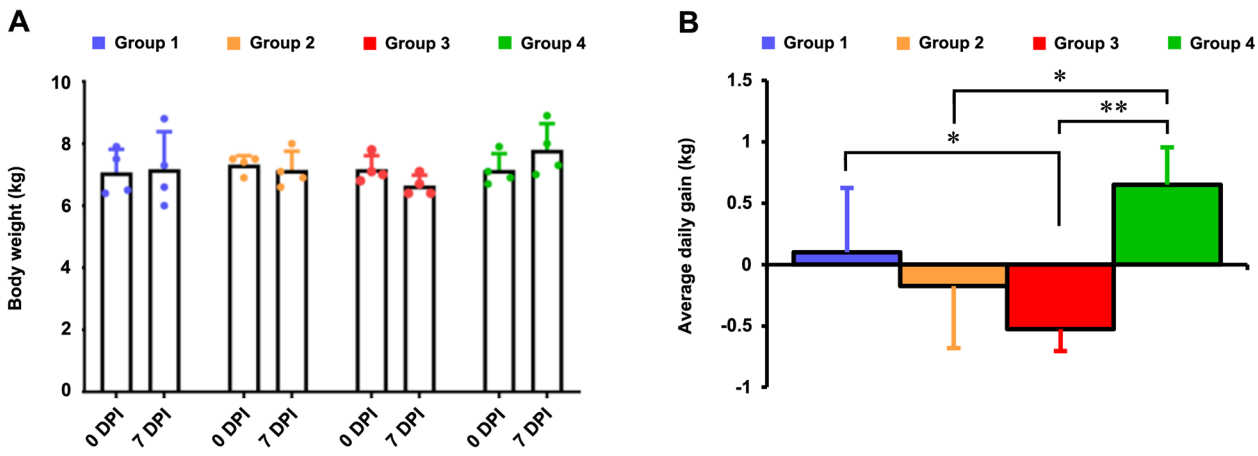


**Fig. 3** The mean body weight (A) and average daily weight gain (ADG) (B) in weaned piglets from each group after single infection and coinfection with PEDV and CPA through 7 dpi. The mean body weights and ADG between the PEDV/CPA coinfection and single-infection groups were compared. The error bars represent the mean ± SDM. *, $P = 0.001–0.05$; **, $P < 0.001$

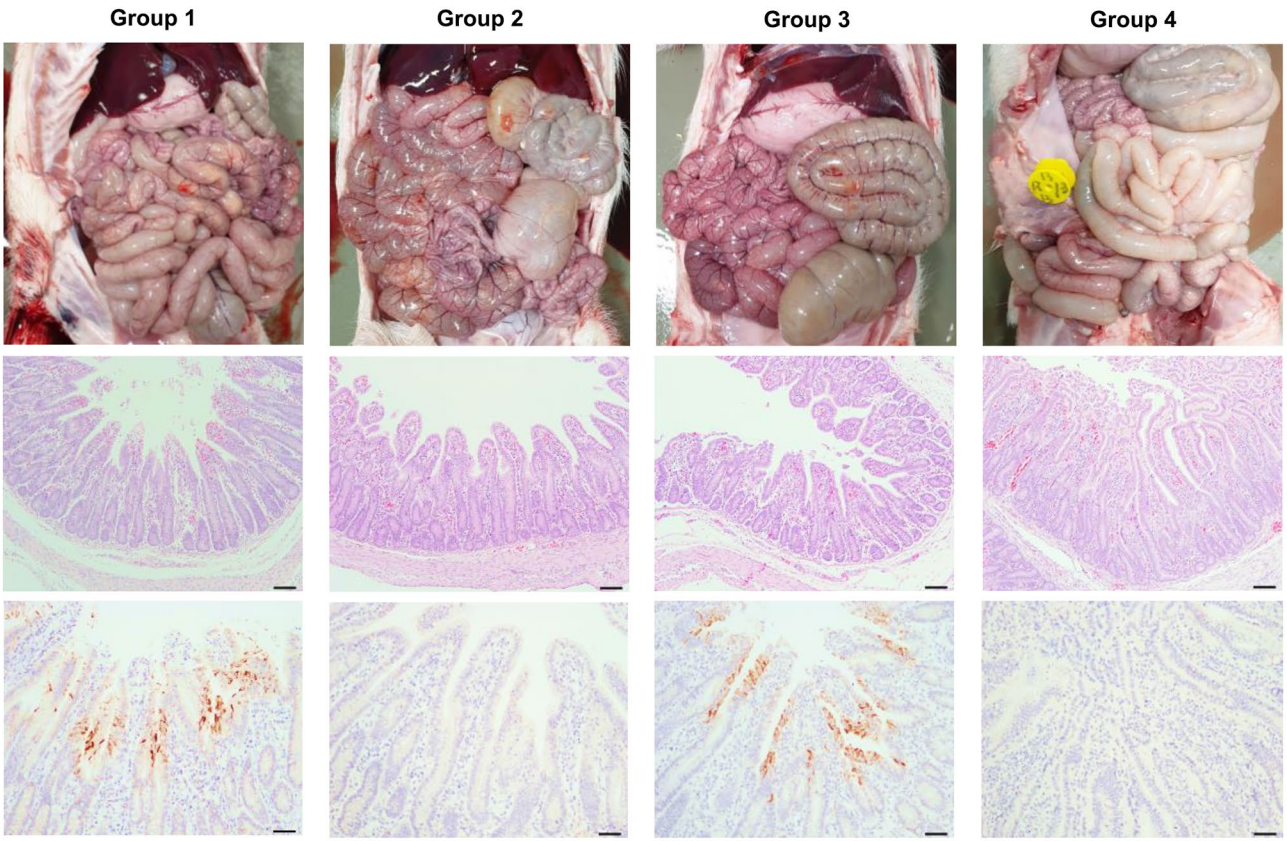


**Fig. 4** Macroscopic and microscopic small intestine lesions in piglets after inoculation with PEDV (group 1), CPA (group 2), or PEDV/CPA (group 3), and in sham control animals (group 4). Small intestines of individual piglets from each group were examined for gross lesions. Representative necropsy images are presented at the top of each panel. Hematoxylin- and eosin-stained tissue sections of the proximal jejunum from representative piglets in each group are shown at the middle of each panel (100× magnification, scale bar = 100 μm). IHC analysis results showing PEDV antigens in jejunal tissue sections from representative pigs in each group are presented at the bottom of each panel (200× magnification, scale bar = 50 μm). Immunostaining of the PEDV N proteins (brown staining) was detected in the epithelial cells of the proximal jejunum in piglets inoculated with PEDV alone and in those co-inoculated with CPA and PEDV. No PEDV antigen was identified in the small intestine of the CPA single-inoculated and mock-inoculated piglets.

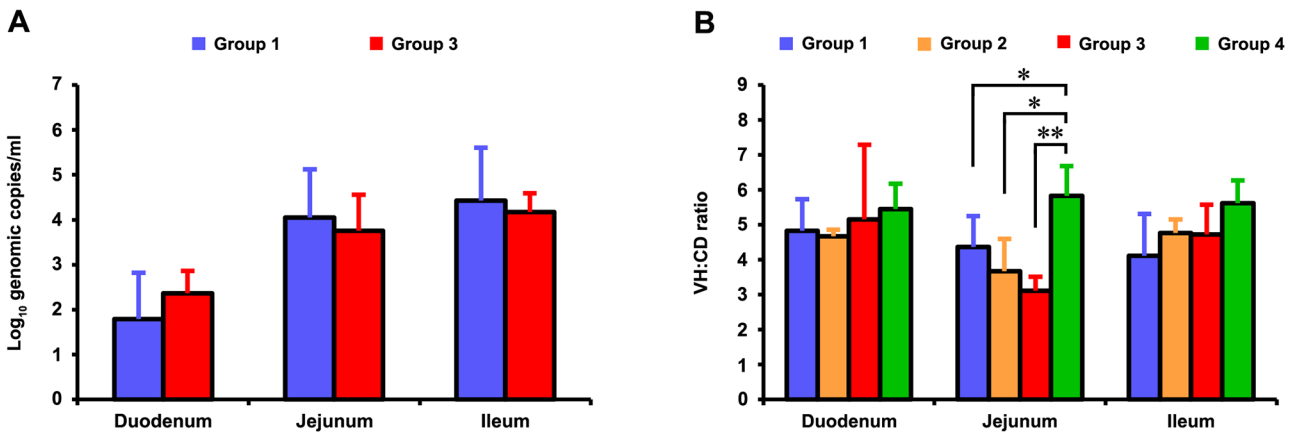


**Fig. 5** The viral load and villous height:crypt depth (VH:CD) ratios in the small intestinal tissues of piglets from each group. (A) PEDV RNA loads ($log_{10}$ genomic copies/ml) in each intestinal tissue collected at necropsy were determined using rRT-PCR. There were no significant differences between the groups. (B) Five villi and crypts of each small intestine section were measured. The mean VH:CD ratios of individual small intestine sections in each group are presented, and the error bars indicate the SDM. *, $P = 0.001–0.05$; **, $P < 0.001$

(4.36 ± 0.89) groups, and the highest ratio was observed in the uninoculated control group (5.82 ± 0.86).

## Discussion

Porcine diarrheal disease is a leading cause of neonatal mortality and reduced weight gain, resulting in significant economic losses. The primary enteric pathogens include bacteria such as *Escherichia coli* and *C. perfringens* as well as coronaviruses and rotaviruses. In South Korea, PEDV is the most widespread agent of diarrhea, with high neonatal mortality that has devastated domestic pig-producing farms for the last three decades. Furthermore, endemic PEDV causes post-weaning diarrhea in older pigs, which shed the virus continuously and can act as a source of virus circulation and recurrence on affected farms, increasing the risk of viral transmission to contiguous farms. Although PEDV infection causes less-severe disease and fewer deaths in weaned pigs than in nursing piglets, coinfection with other bacterial or viral pathogens may enhance viral virulence, thereby worsening the disease severity and increasing morbidity and mortality in weaned pigs. Furthermore, PEDV-infected weaned pigs have reduced ADG and ADFI, leading to a slow growth rate [9]. In this regard, the production of post-weaning pigs with single PEDV infections or coinfections that do not reach a suitable slaughter weight at a certain age is not favorable for the swine industry. *C. perfringens* is a part of the pig intestinal microbiome and is considered an enteric bacterial pathogen in pre- and post-weaning piglets [3, 23, 24]. However, whether *C. perfringens* is involved in diarrheal disease on domestic pig farms is still unclear. To provide insights into the epidemiological status of *C. perfringens* in South Korea, the present study was performed to investigate the prevalence and types of *C. perfringens* circulating in pig populations.

We found a relatively high prevalence (32.0%) of *C. perfringens* in diarrheal samples collected from 41% of the pig farms that were sampled. The *C. perfringens* isolates in 64 out of 65 samples belonged to the CPA toxinotype. More than 80% of the CPA isolates that contain the *cpa* (α-toxin) and *cpb2* (β2-toxin) genes can be virulent or potentially virulent. Consistent with recent studies [3, 23, 24], our data show that the toxin-producing CPA was the most frequently detected type of *C. perfringens* in pre- and post-weaning diarrhea cases. By contrast, CPC is less commonly associated with neonatal diarrhea, although it causes fatal necrohemorrhagic enteritis [4]. We identified only one CPC case, suggesting that it plays a minor role in diarrhea. Testing for bacterial and viral pathogens on 54 farms revealed simultaneous infections with at least two pathogens (e.g., CPA and PEDV) in nearly 40% of the farms, indicating that diarrhea in weaning piglets infrequently has only a single causative agent. It is generally acknowledged that CPA can cause diarrhea, but is often a part of a multifactorial disease, which must be considered when implementing prophylactic and therapeutic measures [25, 26]. Interestingly, we found that 50% of the CPA-positive samples tested positive for PEDV, showing CPA/PEDV coinfection to be the second most frequently detected combination of pathogens in diarrheic pigs. Although its role in the pathogenesis of diarrhea is not well understood, CPA can increase disease severity and aggravate growth retardation in PEDV-infected pigs. To corroborate these results, we tested the effect of coinfection on diarrhea in weaned pigs and found a synergistic pathogenic relationship between CPA and PEDV.

In the present study, 21-day-old weaned piglets were inoculated with HP-PEDV KNU-141112 or/and CPA, and the clinical manifestations were observed and evaluated daily. Our previous studies showed the high enteropathogenicity of KNU-141112, with 100% mortality in 5-day-old neonatal piglets [17, 18]. Considering the strong virulence of KNU-141112 and its high lethality in neonatal piglets, 21-day-old piglets were used in this study to reflect the authentic clinical features of weaned piglets caused by HP-PEDV infection. The weaned pigs infected with HP-PEDV KNU-141112 showed either no diarrhea or mild diarrhea with no deaths, and the disease was self-limiting, confirming the age-dependent resistance to PEDV [6]. The clinical presentation of CPA, including creamy or mucoid diarrhea, has been described in neonatal piglets [19, 27]. In this study, singly infected weaned pigs exhibited limited diarrheal symptoms that lasted 4–5 days, but the coinfected animals in the HP-PEDV/CPA group showed more-severe disease, mainly characterized by a longer duration of diarrhea and a higher level of PEDV shedding when compared to the single CPA- or HP-PEDV-inoculated groups. In addition, the average body weight of the piglets in the coinfection group was significantly lower than in the single-infection group, indicating that CPA has a detrimental effect on the growth performance of pigs that are coinfected with PEDV. Together, these data suggest a confirmed synergistic pathogenic effect of PEDV and CPA coinfection.

The HP-PEDV single-infection pigs had no macroscopic lesions typical of PEDV at necropsy. This observation could be due to the rapid turnover of enterocytes and the high proliferation rate and numbers of crypt stem cells in weaned pigs, which are essential for efficient digestion and adsorption of milk or water to prevent severe dehydration and contribute to recovery from disease in older piglets upon PEDV infection [10]. Likewise, no gross lesions indicative of CPA were observed in the CPA single-infection pigs at necropsy, as shown previously [19, 27]. Consequently, there were no significant differences in macroscopic lesions among the

experimental groups. Although CPA infection results in villous tip necrosis associated with heavy colonization of bacillary bacteria in close contact with injured enterocytes, which could be used for CPA diagnosis, such microscopic findings are rarely detected, and lesions are frequently not noticed [19]. We did not observe CPA-associated microscopic changes, except for jejunal villous atrophy, in the CPA single-infection and HP-PEDV/CPA coinfection groups under our experimental conditions. In addition, PEDV viral RNA titers were similar in the duodenum, jejunum, and ileum of the HP-PEDV/CPA-coinoculated pigs and PEDV single-infection pigs. Consistent with these results, IHC showed that the amount of PEDV antigen in the jejunum was similar between the HP-PEDV singly infected and HP-PEDV/CPA coinfected piglets. However, the VH:CD ratio, measuring the severity of villous atrophy, was lower in the jejunum of the HP-PEDV/CPA co-inoculated pigs than in HP-PEDV or CPA singly infected pigs. Moreover, our animal experiments showed that the PEDV titers in feces were significantly higher in the HP-PEDV/CPA-co-inoculated pigs than in the pigs with PEDV infection alone. Therefore, we can deduce that CPA promoted infection and replication of PEDV in the co-inoculated weaned pigs.

In conclusion, this is the first report of the prevalence of CPA associated with piglet diarrhea in South Korea and the disease outcomes of coinfection with HP-PEDV and CPA in weaned piglets. Our PCR-based survey indicated that HP-G2b PEDV was the predominant pathogen detected in pre- and post-weaning diarrheic samples, reflecting the current situation in which the virus continues to affect domestic pig farms endemically. Second, the prevalence of CPA single infection and coinfection with HP-G2b PEDV was similar in weaning pigs from diarrheal farms. Although HP-PEDV or CPA alone caused no or limited diarrheal diseases in weaned piglets, coinfection with HP-PEDV and CPA significantly elevated the disease severity in older piglets. The synergistic pathogenic consequences are associated with enhanced replication of PEDV in the presence of CPA. Given that coinfection with enteric pathogens is associated with post-weaning diarrhea in South Korea, where PEDV is endemic, the monitoring and prophylaxis or therapy of CPA in PEDV-affected farms should lessen the impact of disease caused by PEDV and CPA. Our data will advance our understanding of the synergistic pathogenic mechanisms triggered by porcine enteric pathogens and help control PEDV on infected or endemic farms.

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## Declarations

**Ethical declarations** All animal procedures were carried out in accordance with the guidelines established by the Institutional Animal Care and Use Committee.

**Conflict of interest** The authors declare that they have no conflict of interest.

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