RESEARCH REPORT
SWINE HEALTH

Title: The pathogenesis and characterization of porcine epidemic diarrhea virus (PEDV) and porcine enteric deltacoronavirus (PdCV) in neonatal gnotobiotic (Gn) swine – **NPB #14-188**

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Industry Summary:

Porcine epidemic diarrhea virus (PEDV) and porcine enteric deltacoronavirus (PdCV) emerged recently in the United States and caused significant economic loss in the swine industry. Currently, the minimum *in vivo* viral infectious doses for PEDV and PdCV are not known. This information is important for the determination of infectious virus loads and for assessing feeds/environments as sources of infectious virus. The objective of this proposal is to identify the minimal titratable infectious dose of PEDV and PdCV in gnotobiotic piglets. Ten-fold dilutions (10^6, 10^4, 10^3, 10^2, 10^1 PFU) of PEDV and PdCV were performed and orally inoculated into each piglet in each group of 10-day-o

ld gnotobiotic piglets (n=5). For PEDV, inoculation doses of 10^6, 10^4, 10^3, and 10^2 PFU/piglet caused severe clinical signs (profuse watery diarrhea, vomiting, and dehydration) and gross and histologic lesions in small intestine. Piglets inoculated with 10^1 PFU of PEDV caused no to mild clinical signs and histologic lesions in small intestine. For PdCV, doses above 10^3 PFU per piglets caused severe clinical signs and pathological lesions whereas no to mild lesions observed for piglets inoculated with 10^2 PFU of PdCV. Thus, under our experimental conditions, the minimal infectious dose for PEDV and PdCV in gnotobiotic piglets is 10^1 PFU and 10^2 PFU, respectively. The finding that low infectious particles were sufficient to cause an infection in piglets highlights the need to develop effective measures to ensure complete inactivation of PEDV and PdCV in farm and environment.

Keywords:

Porcine epidemic diarrhea virus; porcine deltacoronavirus; pathogenesis; infectious dose; gnotobiotic pig

Scientific Abstract:

Porcine epidemic diarrhea virus (PEDV) and porcine enteric deltacoronavirus (PdCV) are enteric coronaviruses and caused significant economic loss in the swine industry in the US. Currently, the pathogenesis of PEDV and PdCV in piglets was poorly understood and the minimum *in vivo* viral infectious doses for PEDV and PdCV are not known. Here, we characterized the pathogenesis of PEDV and PdCV Ohio strains and determined the minimal titratable infectious dose of PEDV and PdCV in gnotobiotic piglets. To do this, ten-fold dilutions (10^6, 10^4, 10^3, 10^2, 10^1 PFU) of PEDV and PdCV were performed and orally inoculated into each piglet in each group of 10-day-old gnotobiotic piglets (n=5). For PEDV, inoculation doses of 10^6, 10^4, 10^3, and 10^2 PFU/piglet caused severe clinical signs including profuse watery diarrhea, vomiting, and dehydration. Histologically, severe villous atrophy of the duodenum, jejunum and ileum was observed in these groups. Immunohistochemical (IHC) analysis showed that a large number of PEDV antigens were found on the mucosal epithelia of intestinal tissues using polyclonal antisera raised against PEDV. In contrast, piglets inoculated with 10^1 PFU of PEDV caused no to mild clinical signs and histologic lesions in small intestine. We found for the first time that the PdCV US strains caused severe diarrhea, vomiting, and dehydration, clinically indistinguishable from PEDV. Histologically, the PdCV caused severe lesions in stomach and small intestine, and mild interstitial pneumonia in gnotobiotic piglets. For PdCV, doses above 10^3 PFU per piglets caused severe clinical signs and histologic lesions whereas no to mild lesions observed for piglets inoculated with 10^2 PFU of PdCV. Thus, under our experimental conditions, the minimal infectious dose for PEDV and PdCV in gnotobiotic piglets is 10^1 PFU and 10^2 PFU, respectively. The finding that low infectious particles were sufficient to cause an infection in piglets highlights the need to develop effective measures to ensure complete inactivation of PEDV and PdCV in farm and environment.

Introduction

The minimum *in vivo* viral infectious doses for PEDV and PdCV are not known. This information is important for the determination of infectious virus loads and for assessing feeds/environments as sources of infectious virus. Gnotobiotic (Gn) piglets are ideal subjects to conduct titration studies because they are germ-free and susceptible for PEDV and PdCV infection.

Objectives:

The objectives of this proposal is to identify the minimal titratable infectious dose needed to cause diarrheal disease in Gn piglets and to collect data relative to length/amount of virus shedding from day of inoculation (5-days of age) to 35 days of age (age limits in units). Objectives will be met by completion of: **Aim 1:** To perform 10-fold *in vivo* infectious PEDV titrations in groups (n=3-4) of Gn piglets and, **Aim 2:** To perform 10-fold *in vivo* infectious PdCV titrations in groups (n=3-4) of Gn piglets.

Materials & Methods:

For **Aim 1**, groups of Gn piglets will be derived from date-mated gravid sows and raised in germfree isolation units (n=3-4 piglets/unit). Ten-fold dilutions (10^6, 10^4, 10^3, 10^2, 10^1 PFU) of PEDV will be performed, aliquots of each saved for RT-qPCR and these dilutions (in 2.0 ml of tissue culture medium/piglet) will be orally inoculated into each piglet in each group of Gn piglets. Uninfected controls will receive 2.0 ml sterile culture medium. In

Aim 2, the same dilution and challenge experiments will be performed with PdCV. By necessity, these challenge experiments will not be performed simultaneously but rather in a sequentially in a series of litters (n=12 piglets/litter). Prior to infection and at daily intervals through PID 10, rectal, nasal and vaginal swabs will be collected; blood (2.0ml in citrate tubes) for determination of viremia will be collected twice weekly. After PID 10, swabs will be collected every three days (blood for viremia twice weekly) until moribund or 35 days of age (the longest interval permitted by IACUC regulations). At termination (35 days of age) or when moribund (3 days PI and beyond), piglets will be sedated, removed from the units and humanely terminated with *Euthol*^{R} solution. Photographs will be taken and duplicate samples for virology (one in *RNAlater*^{R} and one for virus isolation) collected from the stomach (cardia, fundus, antrum), duodenum, jejunum, ileum, caecum, spiral colon, terminal colon, spleen, liver, kidney, mesenteric lymph nodes, and lungs. Adjacent samples are collected into 10% formalin for histopathology, *in situ* hybridization and immunohistochemistry. All procedures are routine procedures in virology and pathology laboratories.

Results: Report your research results by objective.

Objective 1: PEDV pathogenesis and minimal infectious dose

(1) **We have successfully characterized the pathogenesis of PEDV Ohio strain VBS1 and VBS2 in gnotobiotic (Gn) piglets.** Groups of 10-day-old Gn piglets (n=5) were orally inoculated with 10^4 PFU of PEDV Ohio strain VBS1 or VBS2. After challenge, the piglets were observed and evaluated daily for weight and body temperature changes, and clinical sign of PEDV infection. Daily rectal mucosal/fecal swabs were collected from each piglet and diarrhea/fecal consistency score was assigned to each piglet. We found that all Gn piglets inoculated with PEDV VBS1 or VBS2 strain developed sudden-onset, severe, persistent and watery diarrhea at 24 post-infection. Gross findings were similar in all piglets. The perineum, ventral abdomen and hind legs were coated with yellow adherent diarrheic feces. Histologically, severe villous atrophy of the duodenum, jejunum and ileum was observed. Immunohistochemistry (IHC) analysis showed that a large number of PEDV antigens were found on the mucosal epithelia of intestinal tissues using polyclonal antisera raised against PEDV. In addition, high levels of viral RNA copies (10^{10} -10^{11} genomic RNA copies/g or ml) were detected in feces and intestinal tissue segments whereas moderate levels of viral RNA (10^4 -10^6 genomic RNA copies/g or ml) were detected in blood, kidney, liver, and spleen. Taken together, these results demonstrated that PEDV VBS1 and VBS2strains were highly virulent in Gn piglets.

(2) **We have determined the minimal infectious dose of PEDV VBS2 in gnotobiotic (Gn) piglets.** 10-day-old Gn piglets (n=5) were orally inoculated with 10^6, 10^4, 10^3, 10^2, and 10^1 PFU of cell culture adapted, plaque purified PEDV VBS2 strain. Gn piglets were also challenged with 5 ml of MEDM and served as uninfected controls. After virus challenge, the piglets were observed and evaluated daily for body weight and temperature changes, and clinical signs of PEDV infection. Daily rectal fecal swabs were collected from each piglet for virus detection. The results showed that Gn piglets infected with 10^6, 10^4, 10^3 and 10^2 PFU of PEDV VBS2 developed similar clinical symptoms of PEDV infection including watery diarrhea, vomiting, and dehydration at PID 1. There was no significantly difference in clinical signs among these doses. Interestingly, Gn piglets infected with 10^1 PFU of PEDV VBS2 had no to mild diarrhea for 1 day, and quickly recovered. At the termination date, duodenum, jejunum, ileum, transverse colon, spiral colon, descending colon, liver, spleen, lung, kidney, and blood were collected from each piglet. High levels of PEDV RNA (8-12 log RNA copies/g) were detected in intestinal tissues and feces of infected piglets in Gn piglets inoculated with 10^6, 10^4, and 10^3 PFU of PEDV VBS2. Viral RNA shedding in 10^2 PFU group was relatively lower (6-8 log RNA copies/g). Gn piglets infected with 10^1 PFU of PEDV VBS2 had low level of RNA shedding (1-4 log RNA copies/g). Consistent with viral RNA shedding and clinical signs,

gross and histologic lesions in small intestine were severe in Gn piglets inoculated with 10^6, 10^4, and 10^3PFU of PEDV VBS2. Gn piglets inoculated with 10^2PFU of PEDV VBS2 had moderate lesions in small intestine whereas only mild lesions were observed in piglets inoculated with 10^1PFU of virus. Therefore, the minimal infectious dose of PEDV VBS2 strain is 10^1 PFU per Gn piglet.

Objective 2: PdCV pathogenesis and minimal infectious dose

(1) We successfully reproduced porcine deltacoronavirus (PdCV)-associated diseases in gnotobiotic (Gn) piglets. We found that 10-day-old Gn piglets inoculated with 10^6 genomic RNA copies of PdCV Ohio CVM1 developed sudden-onset, severe, persistent and watery diarrhea. Vomiting was observed in the 48- and 72h-PdCV-infected piglets. Core body temperatures remained within normal limits. Respiratory signs (coughing and nasal discharge) were not observed. At day 3 post-infection, Gn piglets were terminated. Dilated gas and fluid-filled small intestine with thin translucent walls were observed, and both the stomach and small intestine contained coagulated liquid milk replacement diet. The cecum, spiral colon and terminal colon were dilated and filled with liquid yellow intestinal fluids. In addition to the intestinal changes, ascites, hydrothorax and thymic atrophy were detected in the piglet. At histological level, severe villous atrophy of the duodenum, jejunum and ileum was apparent. The villous changes were associated with extensive intestinal epithelial degeneration and necrosis. In addition, focal areas of gastric epithelial cell degeneration and necrosis were observed in the gastric pits within the cardia, greater curvature of the fundus and antrum of the stomach. In addition, PdCV caused mild interstitial pneumonia in Gn piglets.

(2) Immunohistobiochemical (IHC) staining showed that a large number of PdCV antigens were detected in epithelial cells of duodenum, jejunum and ileum. Finally, high levels of PdCV were shed in pig feces. These results demonstrated that PdCV Ohio CVM1 is highly virulent to Gn piglets.

(3) We showed that a cell culture-adapted, plaque purified PdCV Michigan (MI) strain caused similar diseases in Gn piglets compared to PdCV Ohio CVM1. The finding that PdCV is a significant enteric disease of swine highlights the need to develop effective measures to control this disease.

(4) We successfully determined the minimal infectious dose of PdCV MI strains in Gn piglets. Briefly, groups of 10-day-old Gn piglets (n=5) were orally inoculated with 10^6, 10^4, 10^3, and 10^2 PFU of PdCV MI strain. After virus challenge, the piglets were observed and evaluated daily for body weight and temperature changes, and clinical signs of PdCV infection. We found that Gn piglets infected with 10^4 PFU and 10^6 PFU of PdCV developed similar clinical symptoms of PdCV infection including watery diarrhea, vomiting, and dehydration at PID 1. Gn piglets infected with 10^3 PFU had mild diarrhea. Gn piglets infected with 10^2 PFU had only soft stools and no obvious diarrhea was observed. Therefore, the severity of disease was correlated with the inoculation dose. Similarly, Gn piglets inoculated with 10^6 and 10^4 PFU of PdCV MI strain had high level of viral RNA shedding ($10^{9\text{-}10}$ genomic RNA copies/g feces) and had severe histologic lesion in small intestine. However, Gn piglets infected with 10^3 PFU of virus had low level of viral RNA shedding ($10^{3\text{-}7}$ genomic RNA copies/g feces) and had mild to moderate histologic lesion in small intestine. Gn piglets infected with 10^2 PFU of virus had very low level of viral RNA shedding ($10^{1\text{-}3}$ genomic RNA copies/g feces) and had no or mild to histologic lesion in small intestine. Together, the minimal infectious dose of PdCV MI strain is 10^2 PFU per piglet.

<u>Discussion:</u>

(1) **The minimal infectious dose for PEDV is lower than that of PdCV in gnotobiotic piglets.** In this study, we found that the minimal infectious dose for PEDV and PdCV in gnotobiotic piglets is 10^1 PFU

and 10^2 PFU, respectively. This suggests that the infectious dose for PEDV is lower than that of PdCV. This seems correlated with the epidemiology, morbidity, and mortality rate of PEDV and PdCV in field. PEDV is highly infectious and the mortality rate can reach 80-100% in suckling piglets. However, it was reported that PdCV associated diseases are relatively less severe, and mortality rate is about 30-60%.

(2) **PdCV in Gn piglets: similarities with PEDV and TGEV.** We have shown that both PdCV Ohio CVM1 and MI strains induce significant disease in Gn pigs. The overall clinical disease associated with PdCV infection is very similar to that induced by TGEV and PEDV except that vomiting is more commonly seen in PdCV-infected piglets and the mortality rates are reported to be lower. Similar to TGEV and PEDV, PdCV replicates extensively in the small intestine. High levels of genomic RNAs were detected in feces, intestinal contents and tissues. A large number of antigen-positive cells were detected in all sections of small intestine. Intestinal gross and histologic findings in PdCV-infected piglets are similar to those described by PEDV and TGEV. Finally, moderate levels of PdCV-RNAs were detected in blood and extra-intestinal tissues, consistent with viremic dissemination similar to what has been observed in TGEV and PEDV-infected piglets.

(3) **Unique features of PdCV infection in Gn piglets:** Our study found that PdCV has two unique features of infection that are distinct from TGEV and PEDV. First, PdCV infection causes epithelial lesions in the glandular pits of the stomach. To our knowledge, involvement of gastric mucosal epithelial cells is not reported for either TGEV or PEDV. There is one report documenting gastric lesions associated with coronavirus RNAs in SARS-convalescent human patients, establishing a precedent for findings. Second, PdCV infection is associated with mild interstitial pneumonia, evident in formalin-inflated lungs. In this regard, mild and ill-defined interstitial lesions in pulmonary parenchyma suggest that PdCV may have an unrecognized PRCV-like respiratory component. Modest amounts PdCV viral RNAs were found in lung tissue homogenates and PdCV antigen was detected in bronchial mucosal epithelial cells. Pathologic changes in the lung have not been reported for PEDV and TGEV.