

Experimental inoculation of neonatal piglets with feed naturally contaminated with porcine epidemic diarrhea virus

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Summary

Piglets did not develop diarrhea when fed porcine epidemic diarrhea virus polymerase chain reaction-positive feed that had been retained by manufacturers in early 2013. The virus was detected in feces of positive-control piglets, which exhibited clinical signs and histologic evidence of infection.

Keywords: swine, porcine epidemic diarrhea virus, neonatal pigs, feed transmission

Received: January 30, 2015

Accepted: June 16, 2015

Resumen - Inoculación experimental de lechones neonatales con alimento contaminado naturalmente con el virus de la diarrea epidémica porcina

Los lechones no desarrollaron diarrea cuando fueron alimentados con alimento positivo a la reacción en cadena de polimerasa al virus de la diarrea epidémica porcina de que había sido guardado por los fabricantes a principios del 2013. El virus fue detectado en heces de lechones control positivos, los cuales exhibieron signos clínicos y evidencia histológica de infección.

Résumé - Inoculation expérimentale de porcelets nouveau-nés avec de la nourriture contaminée naturellement par le virus de la diarrhée épidémique porcine

Des porcelets ne développèrent pas de diarrhée lorsque nourri avec de la nourriture positive par réaction d'amplification en chaîne par la polymérase pour le virus de la diarrhée épidémique porcine qui était retenue par le fabricant depuis le début de l'année 2013. Le virus fut détecté dans les fèces de porcelets témoins positifs, qui démontrèrent des signes cliniques et des évidences histologiques d'infection.

Porcine epidemic diarrhea virus (PEDV), a highly contagious and enteropathogenic alphacoronavirus of pigs, is the causative agent of porcine epidemic diarrhea (PED). Porcine epidemic diarrhea manifests as anorexia, depression, vomiting, and watery diarrhea without blood. High mortality rates are common in piglets less than 10 days of age.¹⁻³ Weaned pigs also develop PED, but mortality rates are lower.⁴ Porcine epidemic diarrhea virus was initially detected in US swine in April 2013 and has caused significant economic losses for the swine industry.

According to a recent US Department of Agriculture (USDA) Swine Enteric Coronavirus Disease Situation Report,⁵ thirty-four states have confirmed cases of PEDV infection in pigs. Deaths in suckling pigs infected with this virus have been substantial

in the United States, which highlights its devastating impact.² It remains unknown how PEDV entered the US swine population. Reports from Canada⁶ and the United States⁷ suggest feedstuffs contaminated with PEDV may be a route of transmission. In early 2013, feed samples retained by manufacturers were submitted to the Iowa State University (ISU) Veterinary Diagnostic Laboratory (VDL) and contained PEDV RNA as detected by polymerase chain reaction (PCR) testing. However, it was unknown if this feed contained live virus and could transmit PEDV to pigs, or if this feed was a source of the initial PEDV outbreak in the United States. The main objective of this research was to determine if the feed samples collected and retained by feed manufacturers shortly after PEDV emerged in the United States and known to contain

PEDV RNA could be a source of transmission to PEDV-naive neonatal piglets.

Materials and methods

Confirmation of PEDV-positive retained feed samples from manufacturers

Three feed samples, one each of complete feed, feed pre-mix, and dried porcine plasma, retained in sealed plastic bags and stored at room temperature (18.3°C to 21.1°C) by feed manufacturers since April and May 2013, were received at the ISU-VDL in July and August 2013. Ten grams of feed were mixed with 40 mL of phosphate buffered saline (PBS; pH 7.2), agitated by vortexing for 15 seconds, and incubated at 4°C overnight. After incubation, the feed suspension was centrifuged at 4200g for 10 minutes, and the supernatant from the 20% suspension was collected. An aliquot of the supernatant was further processed to extract RNA (MagMax Viral RNA Extraction; Life Technologies, Carlsbad, California) for PEDV N-gene real-time reverse transcription (rRT)-PCR as described previously.⁸ The supernatants from all three feed samples were PCR-positive for PEDV at the ISU-VDL and were confirmed PCR-positive by additional testing at the National

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This article is available online at <http://www.aasv.org/shap.html>.

Pillatzki AE, Gauger PC, Madson DM, et al. Experimental inoculation of neonatal piglets with feed naturally contaminated with porcine epidemic diarrhea virus. *J Swine Health Prod.* 2015;23(6):317-320.

Table 1: PEDV-positive status of feed sample supernatants utilized in a bioassay in neonatal piglets, with PEDV-positive status of manufacturer-provided feeds confirmed by testing at NVSL*

Feed	Feed sample ID	ISU-VDL PEDV N-gene rRT-PCR	Genomic copies/mL†	NVSL PEDV nRT-PCR‡
A	Pre-mix #2	Positive, Ct = 34.2	7.0×10^3	Positive
B	Dried porcine plasma #10	Positive, Ct = 30.0	1.21×10^5	Positive
C	Complete feed #16	Positive, Ct = 33.8	9.18×10^3	Positive
D	Positive-control feed§	Positive, Ct = 25.5	2.55×10^6	ND

* A 20% suspension of each feed sample in phosphate buffered saline was incubated overnight and centrifuged. The supernatant was retained for rRT-PCR testing for PEDV RNA, with Ct values < 40 considered positive.

† Based on standard curves established at the ISU-VDL.

‡ nRT-PCR targets N-gene and S-gene. The PCR product was confirmed as PEDV by sequencing.

§ A PEDV cell-culture isolate (strain USA/NC/2013/35140 P3) from a confirmed field case of PEDV enteritis in neonatal piglets⁹ was used to generate the positive-control feed.

PEDV = porcine epidemic diarrhea virus; NVSL = National Veterinary Services Laboratory; ISU-VDL = Iowa State University Veterinary Diagnostic Laboratory; rRT-PCR = real-time reverse transcription polymerase chain reaction; nRT-PCR = nested reverse transcription polymerase chain reaction; Ct = cycle threshold; ND = not done.

Veterinary Services Laboratory (Table 1). The remaining portions of the feed samples were stored at -80°C at the ISU-VDL until the start of this experiment.

PEDV-positive and PEDV-negative control feed preparation

A complete feed that tested negative by PEDV N-gene rRT-PCR was utilized to generate the positive- and negative-control feeds. For the PEDV N-gene rRT-PCR used, a cycle threshold (Ct) value of < 40 was considered positive. A PEDV cell-culture isolate (strain USA/NC/2013/35140 P3) from a confirmed field case of PEDV enteritis in neonatal piglets⁹ was used to generate the positive-control feed. The virus stock had a titer of 4×10^5 median tissue culture infectious doses (TCID₅₀). Feed negative for PEDV (140 g feed in 560 mL PBS) was spiked with 280 µL of the PEDV virus stock (USA/NC/2013/35140 P3), and this suspension was then incubated at 4°C overnight. After incubation, the suspension was centrifuged at 4200g for 10 minutes, and the supernatant (PED-positive supernatant) was collected and saved separately from the remaining feed pellet (PED-positive feed pellet). On the basis of the dilution factor and the titer of the virus stock utilized, the PED-positive supernatant (20% suspension) theoretically contained PEDV at 160 TCID₅₀ per mL. Both samples were stored at -80°C for approximately 1 month until used for inoculation. Prior to storage, an aliquot of the PED-positive supernatant was processed to extract RNA for testing by

PEDV N-gene rRT-PCR, which confirmed its positive status (Ct = 25.5).

Negative-control feed was generated by the described procedure, except that the PEDV isolate was not added to the PBS prior to its addition to the PEDV-negative feed.

Study design

This experimental protocol was reviewed and approved by the ISU Institutional Animal Care and Use Committee.

Twenty-five domestic cross-bred neonatal piglets, approximately 5 days old, from a herd free of PEDV and transmissible gastroenteritis virus and negative for porcine reproductive and respiratory syndrome virus, were delivered to the ISU Laboratory Animal Resources unit. Upon arrival, piglets received an intramuscular injection of ceftiofur at a dosage of 5 mg per kg (Excede; Zoetis, Kalamazoo, Michigan) per labeled directions. Piglets were confirmed negative for PEDV by PCR testing of fecal swabs, as described, prior to initiation of the study. After a day of acclimation, piglets were randomly assigned numbers by drawing ID tags from a container and were divided into five groups with five piglets per group (Table 2). Piglet groups were housed in separate temperature-controlled rooms. Piglets were offered a mixture composed of approximately two-thirds milk replacer (Esbilac; Pet-AG, Hampshire, Illinois) mixed with one-third plain yogurt three times daily at approximately 8-hour intervals. Water was available ad libitum. Once daily, piglets were

given 10 mL of feed supernatant by oral-gastric gavage utilizing an 8-gauge French catheter, and once daily, 10 g of processed PEDV-positive feed pellets were added to the combined milk replacer-yogurt mixture (Table 2). Treatments were continued for 7 consecutive days (0 to 7 days post inoculation [DPI]). At 7 DPI, all piglets were humanely euthanized by an overdose of pentobarbital, and complete necropsy examinations were performed.

Rectal swabs were collected from all piglets prior to inoculation and once daily for the course of the study. Colonic contents and sections of proximal, middle, and distal small intestine and colon were collected at necropsy from all piglets. Fecal swabs and colonic contents were tested for PEDV by PCR as described. Formalin-fixed sections of small intestine were evaluated by light microscopy for villus atrophy by a veterinary pathologist (AEP) who was blinded to the treatment groups at the time of evaluation. Immunohistochemistry (IHC) slides of ileum were prepared utilizing a monoclonal antibody specific for the spike protein of PEDV,^{2,4} and IHC slides were evaluated by the same veterinary pathologist for positive immunoreactivity to PEDV antigen.

Results

Neither clinical diarrhea nor vomiting was observed in the negative-control piglets (Group 1) or piglets in groups 2, 3, or 4 for the duration of the study. The positive-control piglets (Group 5) developed diarrhea

Table 2: Treatment groups and daily feeding regimes of piglets administered via oral gavage suspensions of manufacturer-provided feeds containing PEDV, as confirmed by PEDV rRT-PCR testing*

Group	Treatment	Feeding schedule
1 n = 5	PEDV-negative control feed	AM: milk-yogurt† Noon: milk-yogurt, top-dress feed pellets PM: milk-yogurt and gavage 10 mL feed suspension
2 n = 5	PEDV-positive pre-mix	AM: milk-yogurt Noon: milk-yogurt, top-dress feed pellets PM: milk-yogurt and gavage 10 mL feed suspension
3 n = 5	PEDV-positive dried plasma	AM: milk-yogurt Noon: milk-yogurt, top-dress feed pellets PM: milk-yogurt and gavage 10 mL feed suspension
4 n = 5	PEDV-positive complete feed	AM: milk-yogurt Noon: milk-yogurt, top dress feed pellets PM: milk-yogurt and gavage 10 mL feed suspension
5 n = 5	PEDV-positive control feed	AM: milk-yogurt Noon: milk-yogurt, top-dress feed pellets PM: milk-yogurt and gavage 10 mL feed suspension

* Preparation of feed suspensions described in Table 1.

† Milk-yogurt mixture composed of approximately two-thirds milk replacer and one-third plain yogurt.

PEDV = porcine epidemic diarrhea virus; rRT-PCR = real-time reverse transcription polymerase chain reaction.

without vomiting at 3 DPI, and diarrhea continued until the study was terminated at 7 DPI. All Group 5 piglets were alive at termination of the study.

At necropsy, the Group 5 piglets were thin and mildly dehydrated, and varying amounts of fecal material were adhered to the perineal region. The small intestines were segmentally thin-walled, and the ceca and spiral colons contained yellow, watery contents. Neither the negative-control piglets nor piglets in groups 2, 3, and 4 had evidence of diarrhea, and their colons contained formed feces.

Pooled rectal swabs from all piglet groups were negative for PEDV by PCR prior to inoculation. Porcine epidemic diarrhea virus was not detected in fecal swabs from the piglets in groups 1, 2, 3, or 4 for the duration of this study. Fecal shedding of PEDV was first detected in a single piglet in Group 5 at 1 DPI, and by 3 DPI, PEDV RNA was detected in fecal swabs from all piglets in this group and continued until necropsy.

Mild to moderate villus atrophy was observed within sections of ileum in the positive-control piglets, and PEDV was detected within the ileum by IHC in all

piglets in this group. Villus atrophy was not observed in piglets in the negative-control group or in piglets in groups 2, 3, or 4, and PEDV was not detected by IHC in any of the piglets in these groups.

Discussion

The objective of this study was to determine if a bioassay could prove that PEDV PCR-positive complete feed and feed components retained by feed manufacturers shortly after PEDV emerged in the United States could cause infection, clinical signs of PED, and PEDV shedding in neonatal piglets. The PEDV PCR-positive feed retained by manufacturers and utilized in this study did not cause evidence of infection or clinical PED in the orally inoculated neonatal piglets, and PEDV shedding was not detected. These results are similar to those reported from a bioassay conducted by Bowman et al¹⁰ utilizing RT-PCR PEDV-positive pelleted commercial feed obtained from an unopened feed bag that was delivered directly to a farrow-to-finish swine production site, coinciding with a PED outbreak at that facility. One reason for the lack of clinical signs and PEDV shedding in the current study and in the study by Bowman et

al¹⁰ may be that the nucleic acid detected by PCR in the feed samples did not represent infectious virus. Inactivation of PEDV in porcine plasma by the spray-drying process has been reported;^{11,12} however, conflicting results about whether spray-dried porcine plasma can transmit infectious PEDV have also been reported by another investigator.⁶ Preliminary work by Schumacher et al¹³ concluded that PEDV PCR-positive feed (Ct = 37) provided the minimum infectious dose of PEDV to cause viral shedding in piglets as tested in a bioassay. The feed samples retained by manufacturers and utilized in this study had lower Ct values, indicating the quantity of PEDV present should have been adequate to cause clinical disease if infectious virus were present. Additionally, extended storage time of these feed samples under varying conditions may have reduced or eliminated the infectivity of the PEDV detected by PCR. Additional research has demonstrated that PEDV can be inactivated by several disinfectants,¹⁴ and preliminary results reported by Cochrane et al¹⁵ indicate enhanced degradation of PEDV within feed under varying conditions of time and chemical treatment. However, the effectiveness of treatments on inactiva-

tion of virus varied by feed matrix, and in vivo infectivity was not tested by bioassay. It is difficult to perform virus isolation for PEDV to prove infectivity regardless of sample type, and in vitro isolation attempts in this study would have remained inconclusive even if cell culture results had been determined negative from the submitted feed samples. Therefore, a neonatal piglet bioassay was necessary to confirm infectivity. Lastly, it is possible that the retained feed samples submitted by manufacturers may not have been representative of the overall concentration of PEDV in the entire batch of feed from which they were obtained, since feed is not a uniform matrix.

This study did confirm by bioassay and supports the findings of previous work by Dee et al.,⁷ that feed spiked with a known viable cell-culture isolate of PEDV can act as a vehicle for virus transmission with development of clinical PED, and can result in PEDV fecal shedding in susceptible piglets. Although mortality is generally high in suckling piglets infected with PEDV,^{2,16} there were no piglet deaths in the positive-control group of the current study, even though piglets were inoculated daily for 7 days, developed clinical signs of diarrhea, and shed virus. The daily gastric gavage of the piglets in the positive-control group may have alleviated the severe dehydration which occurs with clinical PED, resulting in the zero mortality observed in this study. However, the viability of the PEDV detected in the inoculum and administered to the positive-control piglets may have also been poor. Potential causes for poor virus viability in the positive-control feed could include the environment of the feed matrix itself, storage of the positive-control feed inoculum prior to usage, virus passage in cell culture, or a combination of these factors. The relative virulence of the PEDV utilized in the positive-control feed was not assessed and was beyond the scope of this study.

A notable difference between the PEDV PCR-positive feed samples utilized for this bioassay and those utilized for other bioassays^{7,10} is that the feed samples used in the current study came directly from the manufacturers and had never been delivered to a swine production facility. Although the route by which PEDV entered the United States is still unproven, confirmation that feed can support transmission of PEDV suggests that greater scrutiny of feed components and feed by-products may be warranted to prevent further spread of PEDV and entry of other transboundary diseases into the United States. Additionally, confirmation of feed as a vehicle for virus transmission suggests

contaminated feed may have contributed to the initial rapid dissemination of PEDV among US swine farms despite adequate on-farm biosecurity. Further studies are necessary to better understand the effects of length of storage time, environmental conditions, chemical mitigation, and feed matrix composition on the viability and transmission of PEDV in swine.

Implication

Under the conditions of this study, feed contaminated with infectious PEDV can serve as a vehicle for PEDV transmission.

Acknowledgments

The authors would like to thank the American Association of Swine Veterinarians for funding this project (NPB project #13-266). We also thank the ISU-VDL staff for their assistance with testing and the Laboratory Animal Resources staff and numerous veterinary students for their assistance with animal care and animal procedures.

Conflict of interest

None reported.

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