RESEARCH REPORT
SWINE HEALTH

Title: Feed transmission of PEDV to neonatal pigs**, NPB Project #13-266**

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Date Submitted: 06/19/2014

Industry Summary:
The objective of this research was to evaluate the potential transmission of porcine epidemic diarrhea virus (PEDV) to neonatal pigs from PEDV-contaminated feed components. Feed components retained at manufacturing facilities shortly after the emergence of PEDV in the United States were collected and submitted to the Iowa State University Veterinary Diagnostic Laboratory and tested positive for PEDV by polymerase chain reaction (PCR). A feed suspension was subsequently fed to PRRSV- and PEDV-negative neonatal pigs for 7 consecutive days during which time the pigs were monitored for development of clinical signs that included vomiting and/or diarrhea. Rectal swabs were collected daily to evaluate PEDV shedding in feces. None of the pigs fed the feed components submitted by manufacturers developed diarrhea and PEDV was not detected from rectal swabs collected from challenged piglets. However, piglets administered complete feed spiked with PEDV which was isolated in the laboratory (positive control pigs) developed diarrhea and shed PEDV as detected by PCR testing. Additionally, the positive control pigs were the only group that exhibited microscopic evidence of viral enteritis. Although the PEDV-contaminated feed components retained by manufacturers did not cause clinical disease or induce shedding of PEDV under the conditions of this study, the clinical findings and laboratory testing of the positive control pigs confirmed that feed spiked with a live PEDV cell-culture isolate can serve as a vehicle for transmission of virus leading to the development of clinical disease in neonatal piglets.

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

Scientific Abstract: Porcine epidemic diarrhea virus (PEDV) was detected in swine in the United States (US) for the first time in May 2013 and since that time has caused significant economic losses for the swine industry. The main objective of this research was to determine by bioassay whether feed or feed components containing PEDV could transmit virus to neonatal pigs resulting in clinical disease and viral shedding. Complete feed, feed pre-mix and dried porcine plasma samples retained by feed manufacturers and collected shortly after the emergence of PEDV in US swine tested positive for PEDV by polymerase chain reaction (PCR) and were utilized in this bioassay. Additionally, PEDV-negative complete feed was spiked with PEDV isolated in the laboratory to serve as a positive control. Five-day-old, PRRSV- and PEDV-negative, neonatal pigs were fed a suspension of the PEDV-positive retained feed components and the positive control feed for 7 days. Positive control pigs first appeared thin and exhibited diarrhea 3 days post-infection (dpi) and continued to have diarrhea until the end of the trial. None of the pigs in the groups fed a suspension of PEDV PCR-positive feed components or the negative control group developed diarrhea throughout the duration of the trial. PEDV was detected in the feces by PCR in all pigs in the positive control group from day 3 dpi and feces remained PEDV PCR positive through the end of the trial. Additionally, the positive control pigs were the only group to exhibit microscopic evidence of atrophic enteritis consistent with PEDV infection. These results confirm that feed containing live PEDV can serve as a vehicle for transmission of this virus leading to the development of clinical disease in neonatal pigs. However, retained PEDV PCR-positive feed components collected by manufacturers shortly after PEDV was confirmed in the US did not transmit virus to neonatal piglets under the conditions of this study.

Introduction: Porcine epidemic diarrhea virus (PEDV) is a highly contagious and enteropathogenic alphacoronavirus of pigs and is the causative agent of porcine epidemic diarrhea (PED). Historically, PEDV was first identified as an enteric disease in feeder and fattening pigs in the United Kingdom in 1971.[3,5] Subsequently, the virus spread to several Asian countries including Korea, China, Japan, the Philippines, and Thailand where it is considered endemic in many areas [3,4]; however, severe outbreaks are still reported and have resulted in high mortality rates in piglets with significant economic losses particularly in the Philippines, South Korea and China.[3,6,7] PEDV infection and clinical disease resembles transmissible gastroenteritis virus (TGEV)infection. Both are characterized by anorexia, depression, vomiting and watery diarrhea without blood frequently resulting in high mortality rates particularly in neonatal piglets which are often less than 10 days of age.[2,3,8] In naïve herds, clinical signs may be observed in swine of all ages.[2] Macroscopic lesions are confined to the small intestines which are typically thin-walled and transparent and contain yellow, watery feces.[2,3,8]

PEDV was detected for the first time in the United States (US) in May 2013. According to a recent National Animal Health Laboratory Network (NAHLN) report, 30 states have confirmed cases of PEDV infection in pigs; over 5500 positive cases have been reported since the virus was first detected in the US; and the majority of the positive cases have occurred in suckling and nursery pigs.[1] Mortalities in suckling and nursery pigs infected with this virus have been substantial [2,3,4] underlying the significant economic impact of this virus. In spite of stringent biosecurity measures by swine producers, PEDV continues to infect herds through methods or fomites that remain poorly understood or are unknown. Therefore, determining how PEDV may have entered the US and understanding the epidemiology of this virus are paramount to improving measures to better control its spread.

It remains unknown how PEDV entered the US swine population or how it was initially transmitted so quickly throughout the US. A report characterizing the origin and evolution of PEDV through temporal and geographical evidence suggests the US strain of PEDV likely originated in China's Anhui Province.[5] Veterinary diagnosticians, swine veterinarians and producers have hypothesized that PEDV may have entered the US and been transmitted in feed or feed components. Recently, preliminary reports from Canada [9, 10] and research reported on the University of Minnesota PEDV webpage (http://www.cvm.umn.edu/sdec/SwineDiseases/pedv/) may have confirmed feed as a potential

source of PEDV transmission; however, it remains unknown if feed was the source of the PEDV outbreak in the US. The objective of this research was to characterize feed components contaminated with PEDV that was collected by manufacturers and stored shortly after PEDV was confirmed in the US as a potential source of transmission to naïve swine populations.

Objective:
Determine if retained PEDV PCR-positive feed components stored at -80°C for 130 days can transmit the virus to neonatal piglets and cause disease as determined by clinical signs, shedding of virus in feces and microscopic intestinal lesions with antigen detection

Materials & Methods:
Animals
Twenty-five, domestic, cross-bred neonatal piglets approximately 5-days old, from a PEDV- and TGEV-free herd and known to be negative for porcine reproductive and respiratory syndrome virus (PRRSV), were purchased and delivered to Iowa State University (ISU); and received an antibiotic injection (Excede ®, Pfizer) per labeled directions upon arrival. Piglets were confirmed negative for PEDV by PCR testing prior to initiation of the study.

Confirmation of PEDV(+) feed samples from manufacturers
Three feed samples, one each of complete feed, feed pre-mix and dried porcine plasma, retained by feed manufacturers since April and May 2013, were received by the ISU VDL in July and August. Samples were processed for PEDV N-gene rRT-PCR by combining 40ml of PBS with 10g of feed and incubating this solution at 4°C overnight. Following incubation, the feed solution was then centrifuged at 4200 x *g* for 10 minutes; and the supernatant was collected and saved separately from the remaining feed pellet. An aliquot of the supernatant was further processed to extract RNA (MagMax Viral RNA Extraction, Life Techologies, Carlsbad, CA) for testing by PEDV N-gene rRT-PCR as described previously.[11] The supernatants from all 3 feed samples tested positive for PEDV and these results were confirmed by PCR testing of the feed supernatants at the National Veterinary Services Laboratory (NVSL; Table 1). The remaining portions of the submitted feed components were stored at -80°C until the start of the experiment.

Table 1. *Confirmation of PEDV(+) feed components retained from feed manufacturers. PEDV PCR results from supernatants of feed components submitted to ISU VDL with corroborating PCR results from testing completed by NVSL*

Groups	Feed sample ID	ISU VDL PEDV N-gene rRT-PCR[a]	NVSL PEDV nRT-PCR[b,c]
A	Pre-mix 13-24271 #2	positive Ct=34.2	positive
B	Dried porcine plasma 13-28250 #10	positive Ct=30.0	positive
C	Complete feed 13-28250 #16	positive Ct=33.8	positive

[a] realtime reverse transcription PCR; rRT-PCR
[b] nested reverse transcription PCR; targets N-gene and S-gene; nRT-PCR
[c] PCR product was confirmed as PEDV by sequencing

PEDV (+) control and PEDV (–) control feed preparation
A complete feed which tested negative by PEDV N-gene rRT-PCR (PEDV-negative feed) was utilized to generate the positive and negative control feeds. A PEDV isolate (strain USA/NC/2013/35140 P3) cultured from a confirmed case of PED enteritis in neonatal piglets was used to generate the positive control feed. The virus stock had a titer of 4x10^5 $TCID_{50}$/ml (based on CPE and immunofluorescence staining 2 days post-titration). Two hundred eighty microliters of the PEDV virus stock

USA/NC/2013/35140 P3 was spiked into 140 g PEDV-negative feed in 560 ml PBS and this solution was then incubated at 4°C overnight. Following incubation, the solution was centrifuged at 4200 x *g* for 10 minutes and the supernatant was collected [PED(+) supernatant] and saved separately from the remaining feed pellet [PED(+) feed pellet]. Both sample types were stored at -80°C until used for inoculation. Prior to storage, an aliquot of the PED (+) 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 same procedure as above, except that PEDV isolate was not added to the PBS prior to its addition to the PEDV-negative feed.

Study design

Following a day of acclimation, piglets were randomly divided into five groups with five piglets in each group. Piglet groups were housed in separate, temperature-controlled rooms. Piglets were offered milk replacer (Esbilac; Pet-AG, Hampshire, IL) mixed with plain yogurt three times daily (morning, noon and evening) and water ad libitum. Once daily, piglets were given 10ml of feed supernatant by oral-gastric gavage utilizing an 8 gauge French catheter; and once daily 10g of processed PEDV-positive feed pellets were added to the combined milk replacer/yogurt mixture (Table 2). Treatments were continued for 7 consecutive days (Day 0-7).

Table 2. *Experimental design. Five day-old piglets were randomized into 5 treatment groups and fed known PEDV(+) and PEDV(-) feeds*

Groups	*n*	Treatment	Feeding schedule
1	5	PEDV(-) control feed	AM: milk/yogurt Noon: milk/yogurt, top dress feed pellets PM: milk/yogurt & gavage 10ml feed suspensio
2	5	PEDV (+) Feed sample A[a]	AM: milk/yogurt Noon: milk/yogurt, top dress feed pellets PM: milk/yogurt & gavage 10ml feed suspensio
3	5	PEDV (+) Feed sample B[b]	AM: milk/yogurt Noon: milk/yogurt, top dress feed pellets PM: milk/yogurt & gavage 10ml feed suspensio
4	5	PEDV(+) Feed sample C[c]	AM: milk/yogurt Noon: milk/yogurt, top dress feed pellets PM: milk/yogurt & gavage 10ml feed suspensio
5	5	PEDV(+) control fee	AM: milk/yogurt Noon: milk/yogurt, top dress feed pellets PM: milk/yogurt & gavage 10ml feed suspensio

[a] Feed A = pre-mix
[b] Feed B = dried porcine plasma
[c] Feed C = complete feed

Sample collection

Rectal swabs were collected from all piglets prior to inoculation and once daily thereafter for the course of the study. Colonic contents, serum, and tissues (small intestine and colon) were collected at necropsy from all pigs. Formalin-fixed sections of small intestine were examined by light microscopy and evaluated by immunohistochemistry (IHC) for PEDV.

Results:

Neither clinical diarrhea nor vomiting was noted in the negative control piglets or the piglets which were inoculated with feeds A, B or C for the duration of the study. The positive control piglets developed diarrhea at 3 days post-inoculation (dpi); and diarrhea in this group continued until the study was terminated. Vomiting was not observed and all positive control piglets were alive at the termination of the study.

At necropsy, the positive control piglets were thin, somewhat dehydrated and fecal staining of the perineal region was evident. The small intestines were segmentally thin-walled and the spiral colons contained yellow, watery contents. Neither the negative control piglets nor the piglets inoculated with feeds A, B or C had external evidence of diarrhea; and at necropsy their colons contained formed feces.

Pooled rectal swabs from all piglet groups were negative for PEDV by rRT-PCR prior to inoculation. Fecal viral shedding of PEDV was not detected in the negative control piglets nor the piglets inoculated with feeds A, B or C at 1, 3, 5 or 7 dpi. PEDV fecal shedding was first detected in a single piglet in the positive control group at 1dpi; and by 3 dpi, fecal shedding of PEDV was detected in all piglets and continued until necropsy at 7 dpi in all of the piglets in the positive control group. Microscopic evidence of villous atrophy was observed within the small intestines in 3/5 of the positive control piglets; and PEDV was detected within the small intestines by IHC in 5/5 of the piglets in this group at the termination of the study (Table 3). Villous atrophy was not evident in piglets in the negative control group or in any of the piglets in the groups which were inoculated with feeds A, B or C; and PEDV was also not detected by IHC within the small intestines of any of the piglets in these groups.

Table 3. *Numeric scoring of atrophic enteritis lesions and IHC staining of sections of small intestines in the piglets fed PEDV(+) control feed (i.e. positive control group)*

Piglet number	Atrophic enteritis – H&E stained sections[d]		IHC sections[e]
	Jejunum (3 sections)	Ileum (1 section)	Ileum
46	0	0	2
47[f]	1	1	1
48	0	0	2
49	1	1	1
50	1	1	1

[d] atrophic enteritis scoring; 0=negative, 1=mild, 2=moderate, 3=severe
[e] IHC scoring; 0=negative, 1=positive at 10%, 2=positive at 10-50%, 3=positive at >50%
[f] first piglet to shed PEDV in feces at 1 dpi

Discussion:
The objective of the present study was to determine if a bioassay could prove that feed contaminated with PEDV could cause clinical manifestations of PED and viral shedding in neonatal piglets. Unfortunately, the PEDV PCR-positive feed components retained by manufacturers and utilized in this study did not cause PED in the inoculated neonatal piglets; and evidence of fecal viral shedding was not detected. There could be several reasons for this. First, viability of the PEDV present in these feed samples was not confirmed; and therefore the virus present may not have been infective and able to cause clinical disease. It is possible that the extended storage of these feed samples may have had an adverse effect on the viability of the PEDV detected by PCR. It is difficult to perform virus isolation for PEDV regardless of sample type and isolation attempts would have remained inconclusive if negative as well. Second, a low amount of PEDV was present in these feed samples (as indicated by the high Ct values from the rRT-PCR), which may not correlate with a sufficient amount of virus to cause infection even in neonates. Lastly, since feed is not a completely uniform matrix, there may have been variability in the amount of PEDV present in the suspensions and feed pellets that were orally administered to the piglets.

However, this study confirmed by bioassay that feed spiked with a known viable cell culture isolate of PEDV can act as a vehicle for viral transmission with development of clinical PED and result in fecal viral shedding when such feed is administered to susceptible pigs shortly after preparation. Although the route by which this virus entered the US is still unproven, confirmation that feed can support transmission of PEDV suggests that greater scrutiny of feed components and feed by-

products imported into this country may be warranted to prevent the entry and further transmission of PEDV and other transboundary diseases not currently in the US. Additionally, confirmation of feed as a potential vehicle for viral transmission helps to clarify how this virus may have been transmitted so readily and quickly among swine farms across the US despite adequate on-farm biosecurity measures.

Acknowledgments
The authors would like to thank the American Association of Swine Veterinarians for funding this project. We also thank the Iowa State University Laboratory Animal Resources staff and numerous veterinary students for their assistance with animal care.

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