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Utilizing Feed Sequencing to Decrease the Risk of Porcine Epidemic Diarrhea Virus (PEDV) Cross-contamination During Feed Manufacturing

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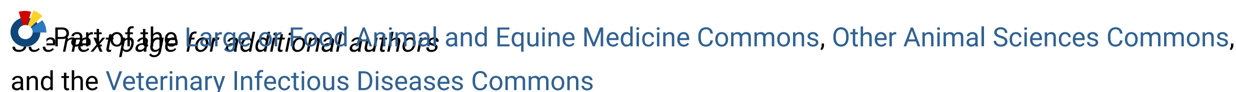

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Utilizing Feed Sequencing to Decrease the Risk of Porcine Epidemic Diarrhea Virus (PEDV) Cross-contamination During Feed Manufacturing

Abstract

Understanding key points of potential cross-contamination during the feed manufacturing process is important to developing efficacious methods to control or prevent transmission of pathogens into swine diets. In this study, an experiment was conducted involving 30 crossbred 10-d-old pigs that were used as a bioassay model for Porcine Epidemic Diarrhea Virus (PEDV) to determine the effects of feed batch sequencing on PEDV cross-contamination and subsequent infectivity. PEDV with a PCR cycle threshold value (Ct) of 11 was uniformly mixed into 4.5 kg of swine diet using a stainless steel bench top mixer validated for mixing efficiency. The inoculated feed was then added to 45 kg of swine diet and mixed using a 4 ft^3 electric paddle mixer validated for mixing efficiency to form the positive experimental treatment. Feed was discharged, carried into a bucket elevator, and exited through a downspout. Subsequent treatment batches were formed when 50 kg of PEDV-free swine diet was sequenced immediately after the PEDV-inoculated batch without cleaning the equipment to replicate the batching process used in commercial feed mills. The subsequent sequence batches (1-4) mixed, discharged, and sampled similar to the PEDV-positive batch. Feed samples were analyzed for the presence of PEDV using PCR and bioassay. Pigs were then orally challenged with harvested supernatant. Fecal swabs were collected for PEDV PCR testing. At seven days after challenge, all pigs were necropsied. Cecum contents, ileum, and jejunum were collected for PCR, histologic, and immunohistochemistry (IHC) evaluation. Overall, the results indicate that sequencing reduced but did not eliminate the risk of PEDV transmission. All pigs (9/9) challenged with the positive treatment were infected with PEDV with feed that had a Ct mean of 31.7. The discharge for the first sequence had a Ct value of 38.1 and infected pigs were noted in pigs from one of three rooms used to bioassay the feed. The second sequence did not have detectable PEDV RNA by using PCR. Interestingly, feed from the second sequence was infectious as verified by infection in pigs from one of three rooms used for bioassay. This study is the first to demonstrate feed without detectable PEDV RNA can be infective but is similar to other research using tissue homogenates and cell culture as bioassay material. In summary, feed batch sequencing should be considered a risk mitigation strategy that can be incorporated into feed mill biosecurity programs but should not be considered a risk elimination strategy.

Keywords

bioassay, feed, PCR, PEDV, sequencing, swine

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Utilizing Feed Sequencing to Decrease the Risk of Porcine Epidemic Diarrhea Virus (PEDV) Cross-contamination During Feed Manufacturing[1]

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Summary

Understanding key points of potential cross-contamination during the feed manufacturing process is important to developing efficacious methods to control or prevent transmission of pathogens into swine diets. In this study, an experiment was conducted involving 30 crossbred 10-d-old pigs that were used as a bioassay model for Porcine Epidemic Diarrhea Virus (PEDV) to determine the effects of feed batch sequencing on PEDV cross-contamination and subsequent infectivity. PEDV with a PCR cycle threshold value (Ct) of 11 was uniformly mixed into 4.5 kg of swine diet using a stainless steel bench top mixer validated for mixing efficiency. The inoculated feed was then added to 45 kg of swine diet and mixed using a 4 ft^3 electric paddle mixer validated for mixing efficiency to form the positive experimental treatment. Feed was discharged, carried into a bucket elevator, and exited through a downspout. Subsequent treatment batches were formed when 50 kg of PEDV-free swine diet was sequenced immediately after the PEDV-inoculated batch without cleaning the equipment to replicate the batching process used in commercial feed mills. The subsequent sequence batches (1-4) mixed, discharged, and sampled similar to the PEDV-positive batch. Feed samples were analyzed for the presence of PEDV using PCR and bioassay. Pigs were then orally challenged with harvested supernatant. Fecal swabs were collected for PEDV PCR testing. At seven days after challenge, all pigs were necropsied. Cecum contents, ileum, and jejunum were collected for PCR, histologic, and immunohistochemistry (IHC) evaluation.

[1] Funding, wholly or in part, was provided by the National Pork Checkoff.

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Overall, the results indicate that sequencing reduced but did not eliminate the risk of PEDV transmission. All pigs (9/9) challenged with the positive treatment were infected with PEDV with feed that had a Ct mean of 31.7. The discharge for the first sequence had a Ct value of 38.1 and infected pigs were noted in pigs from one of three rooms used to bioassay the feed. The second sequence did not have detectable PEDV RNA by using PCR. Interestingly, feed from the second sequence was infectious as verified by infection in pigs from one of three rooms used for bioassay.

This study is the first to demonstrate feed without detectable PEDV RNA can be infective but is similar to other research using tissue homogenates and cell culture as bioassay material. In summary, feed batch sequencing should be considered a risk mitigation strategy that can be incorporated into feed mill biosecurity programs but should not be considered a risk elimination strategy.

Key words: bioassay, feed, PCR, PEDV, sequencing, swine

Introduction

Previous work conducted by our team has demonstrated PEDV cross contamination of feed during feed manufacturing can occur (Woodworth et al., 2014)[5]. The resulting unintentionally contaminated feed batch had less detectable PEDV RNA by PCR than the original batch yet raised questions whether it could be infectious. Due to the lack of additional data detailing PEDV cross contamination during feed manufacturing, we hypothesize that strategically sequenced diets during feed production may reduce the risk of PEDV cross contamination. Therefore, the objective of this experiment was to determine the efficacy of feed batch sequencing to minimize the risk of PEDV cross contamination as measured by PCR and bioassay.

Procedures

The feed manufacturing portion of this experiment was conducted at the Kansas State University Cargill Feed Safety Research Center (FSRC; Manhattan, KS), a 3-story biosafety level 2 biocontainment laboratory containing pilot scale mixers, conveying equipment, and pellet mills. The experiment was replicated three times with decontamination before and after each replicate confirmed by the absence of PEDV-infected particles in the feed, equipment, and environment as measured by PCR.

The virus used to inoculate feed was U.S. PEDV prototype strain cell culture isolate USA/IN/2013/19338, passage 8 (PEDV19338), and contained 4.5×10^6 tissue culture infectious particles/ml ($TCID_{50}$/ml). The virus was divided into three, 500 ml aliquots with one aliquot used in each replication.

The feed (Table 1) used was a corn and soybean meal-based diet manufactured at the Kansas State University O.H. Kruse Feed Technology Innovation Center (Manhattan, KS). A subsample of the feed was obtained prior to inoculation for each repetition and

[5] Woodworth, J. C., C. R. Stark, R. A. Hesse, R. G. Main, J. Zhang, M. D. Tokach, P. C. Gauger, and S. S. Dritz. 2014. Determining the impact of conditioning time and temperature in pelleted diets on Porcine Epidemic Diarrhea Virus (PEDV) survivability in complete swine diets- NPB#14-159. http://research.pork.org/FileLibrary/ResearchDocuments/14-159-WOODWORTH-KSt.pdf (Accessed 14 March 2015).

was confirmed negative. Prior to inoculation of feed, 50 kg of swine diet was mixed in a 4 ft^3 electric paddle mixer (H. C. Davis Sons Manufacturing; Bonner Springs, KS) that was previously validated for mixing efficiency using a standard protocol (McCoy, 2005)[6]. The feed was mixed for five minutes before a 400 to 500 g sample was collected by subsampling five equally spaced locations within the mixer. The feed was then discharged at a rate of approximately 10 lb/min into the leg of the bucket elevator (Universal Industries, Cedar Falls, IA) that carried 74 buckets (each 45 in^3) of feed that exited through a downspout and was collected.

The PEDV inoculum was established by mixing a 500 ml aliquot of stock virus into a 4.5 kg batch of the swine diet; this was done using procedures established in a prior experiment (Woodworth et. al, 2014)[6]. The PEDV inoculum (4.5 kg of feed + 500 ml of stock virus) was added to 45 kg of swine diet to form the positive experimental treatment and was mixed for five minutes, sampled, and discharged for 10 minutes. Four subsequent feed batch sequence treatments (Sequence 1 to Sequence 4) were mixed and discharged following the PEDV-inoculated feed treatment. Each sequence consisted of 50 kg of PEDV-free swine diet that was added to the mixer, mixed for five minutes, sampled and discharged for 10 minutes. After mixing, feed samples were collected from all batches and were analyzed by Kansas State University (KSU) for the presence of PEDV RNA by PCR and processed for bioassay.

Three subsamples (100 g per sample) of each feed treatment per replicate were processed for detection of PEDV RNA via PCR and for infectivity using bioassay. Briefly, a 100 g sample of feed was added to 400 ml of PBS in 500 ml bottles, thoroughly mixed and stored at 40°F for approximately twelve hours. Aliquots were then collected using sterile serologic pipettes with a pipette controller (Pipetboy; Integra Biosciences, Hudson, NH). A 4 ml aliquot of the feed suspension was evaluated by KSU using a PEDV spiked gene-based PCR assay. Additionally, aliquots were harvested from negative, positive, sequence 1, and sequence 2 treatments and temporarily frozen at -4°F until use in the pig bioassay within 1 month of collecting samples.

The Iowa State University Institutional Animal Care and Use Committee approved the pig bioassay protocol. A total of 10 rooms (30 pigs) were assigned to treatment groups with 1 negative control room and 9 challenge rooms. Each pig from the negative control room was given a 10 mL aliquot of inoculum created from the negative control feed collected from the electric mixer during each of Replicate 1, 2, and 3. Different from the negative control room, each pig in each challenge room was given an aliquot of inoculum from the same replicate, and one room was representative of a treatment in a single replicate.

Briefly, pigs from each experimental treatment were housed in separate rooms that each had independent ventilation systems. Rooms had solid flooring that was minimally rinsed to reduce PEDV aerosols. Each pig was administered 10 ml of the PBS feed suspension supernatants by orogastric gavage using an 8 gauge French catheter (0 dpi). Rectal swabs were collected on d -2, 0, 2, 4, 6, and 7 dpi from all pigs and analyzed by Iowa State University for PEDV RNA by PCR. Fresh small intestine, cecum, and colon

[6] McCoy, R. A. 2005. Mixer testing. In: E. Schofield, editor, Feed manufacturing technology V. American Feed Industry Association, Arlington, VA. p. 620-622.

were collected at necropsy at 7 dpi along with an aliquot of cecal content. One section of formalin-fixed proximal, middle, distal jejunum, and ileum was collected for histopathology. Cecal contents were evaluated for PEDV by PCR.
Tissues were routinely processed and fixed in neutral buffered formalin, embedded, sectioned, and stained with hematoxylin and eosin stain. Three serial sections from a piece of ileum from each pig was evaluated. In each of the sections, one full-length villus and crypt were measured, based on tissue orientation, using a computerized image system (Nikon Eclipse TI-U microscope with NIS-Elements imaging software, basic research version 3.3, Nikon Instruments Inc., Melville, NY). Thus, one crypt and villi was measured per section of intestine for a total of 3 values per pig. The three values per ileum were averaged into one value per pig for calculating the villus/crypt ratio.

PEDV immunohistochemistry (IHC) slides were prepared on the sections of ileum. Antigen detection was scored based on the following criteria: No signal (0), mild (1 to 10% signals), moderate (11 to 25% signals), abundant (26 to 50% signals), and diffuse (51 to 100%).

Data of the effects of batch sequencing on villus height, crypt depth, and villus height to crypt depth ratio were analyzed as a completely randomized design using PROC MIXED in SAS (SAS Institute, Inc., Cary, NC) with pig as the experimental unit by a pairwise comparison. Results for treatment criteria were considered significant at $P \leq 0.05$ and marginally significant from $P > 0.05$ to $P \leq 0.10$. Swabs, IHC, PCR (feed, fecal, and environmental) results were summarized using descriptive statistics.

Results and Discussion

No PEDV RNA was detected by PCR after the negative control treatment was manufactured (Table 2). However, all feed samples had detectable PEDV RNA after the positive control was manufactured, with the associated feed Ct being approximately 30. A total of 77.8% of mixer and bucket elevator feed samples had detectable PEDV RNA after Sequence 1. There was no detectable PEDV RNA from the feed samples collected from the mixer after Sequence 2; however, 22.2% of the feed samples collected from the bucket elevator spout had detectable PEDV RNA. After Sequence 3 and 4, none of the feed samples had detectable PEDV RNA. Still, it should be noted that the boot pit depth of the bucket elevator used for our experimental purposes was very shallow compared to the cross-contamination area present in most feed mills, where boot pits are typically several feet deep.

As expected, fecal shedding of PEDV was not detected in rectal swabs or cecum contents from pigs gavaged with the PEDV-negative control for the duration of the study (Table 3). Also, pigs in all three rooms gavaged with the positive control from each replicate indicated infection via the detection of viral particles in fecal swabs from 2 to 7 dpi. Of the nine total pigs gavaged with aliquot from Sequence 1, a fecal swab from one pig yielded detectable PEDV RNA at 2 dpi, and all three pigs had fecal swabs and cecum contents with detectable viral particles by d 4 through 7 after challenge. A fecal swab from another pig gavaged with aliquot from Sequence 1 yielded detectable PEDV RNA at 7 dpi, but no detectable PEDV RNA was observed from cecum contents. One pig gavaged with aliquot from Sequence 2 had a fecal swab with detectable RNA at 2 dpi, although no PEDV RNA was detected in the inoculum. By 4 dpi, all 3 pigs had

detectable PEDV RNA, and continued to shed virus through the end of the study at 7 dpi.

The pigs challenged with the positive control, Sequence 1 and Sequence 2 treatments had numerically shorter villus heights and deeper crypt depths than pigs challenged with PEDV-negative control (Table 4). This led to pigs challenged with the positive control to have a numerically lower villus height:crypt depth ratio than pigs challenged with the negative control, Sequence 1, or Sequence 2. Porcine Epidemic Diarrhea Virus IHC immunoreactivity was not visible in the cytoplasm of villus enterocytes of pigs challenged with the positive control when harvested at 7 dpi. Immunohistochemistry was, however, positive for pigs challenged with either Sequence 1 or Sequence 2.

In conclusion, the results of the present study suggest that a sequencing protocol can be used to reduce but not eliminate PEDV carryover risk between batches of feed. Concerning findings from this study revealed that feed without detectable PEDV genetic material can be infective. The use of a pilot scale feed mill effectively illustrated that a contaminated feed mill has the potential to be a source of infection and route into farms even after two batches of negative feed were produced. Additional research is needed to further define the best ways to increase biosecurity to control or prevent virus transmission in our commercial feed mills.

Table 1. Diet composition (as-fed)

Item	Negative control
Ingredient, %	
Corn	79.30
Soybean meal, 46.5 CP	15.70
Choice white grease	1.00
Calcium phosphate (monocalcium)	1.40
Limestone	1.15
Salt	0.50
L-Thr	0.03
Trace mineral premix	0.15
Sow add pack	0.50
Vitamin premix	0.25
Phytase[1]	0.02
Total	100.00
Chemical analysis, %	
DM	91.4
CP	17.1
Crude fiber	3.7
Ca	0.78
P	0.52
Fat	3.5

[1] High Phos 2700 GT, DSM Nutritional Products, Parsippany, NJ.

Table 2. Effect of sequencing batches of feed on Porcine Epidemic Diarrhea Virus (PEDV) contamination of feed.

	Treatment[1]					
Item	After negative	After positive	After sequence 1	After sequence 2	After sequence 3	After sequence 4
Feed, %						
Mixer	-[2] (0/9)[3]	100.0 (9/9)	77.8 (7/9)	- (0/9)	- (0/9)	- (0/9)
Bucket elevator spout[4]	- (0/9)	100.0 (9/9)	77.8 (7/9)	22.2 (2/9)	- (0/9)	- (0/9)
Feed, Ct[5]						
Mixer	-	31.7	38.1	-	-	-
Bucket elevator spout	-	30.9	37.8	39.0	-	-

[1] 500 ml of tissue culture containing 4.5×10^6 $TCID_{50}$/ml of PEDV was inoculated into a 4.5 kg batch of feed, then added to 45kg of PEDV negative feed to form the positive treatment. Sequences were formed by sequentially adding 50 kg of PEDV negative feed to the mixer after the previous batch was discharged from the mixer, through the bucket elevator, and exited the spout. One feed sample from the mixer and bucket elevator per treatment was collected, divided into three aliquots and diluted in PBS to form supernatants. Each value represents the mean of 3 replicates × 3 repetitions. Feed was mixed for five minutes per treatment, sampled from the mixer, then discharged for 10 minutes into the bucket elevator and exited the end spout. None of the equipment was cleaned in between treatments.

[2] No detectable PEDV RNA (Ct > 45).

[3] Means represent the percent of samples that had detectable RNA by PEDV PCR analysis with numbers in parenthesis being the number with detectable PEDV and total number of samples collected.

[4] Once feed exited the end spout, one sample was collected per treatment and repetition.

[5] Mean cycle threshold (Ct) value of samples with detectable PEDV RNA below 45.

Table 3. Influence of sequencing and Porcine Epidemic Diarrhea Virus (PEDV) inoculated feed on quantitative reverse transcription (PCR) cycle threshold (Ct) of feed, fecal swabs and cecum contents of pigs[1]

		Fecal swabs, Ct					
Item	Feed inoculum, Ct	0 dpi[2]	2 dpi	4 dpi	6 dpi	7 dpi	7 dpi Cecum content, Ct
Feed from mixer, %							
Negative	-[3] (0/3)[4]	- (0/3)	- (0/3)	- (0/3)	- (0/3)	- (0/3)	- (0/3)
Positive	100.0 (9/9)	- (0/9)	100.0 (9/9)	100.0 (9/9)	100.0 (9/9)	100.0 (9/9)	100.0 (9/9)
Sequence 1	77.8 (7/9)	- (0/9)	11.1 (1/9)	33.3 (3/9)	33.3 (3/9)	44.4 (4/9)	33.3 (3/9)
Sequence 2	- (0/9)	- (0/9)	11.1 (1/9)	33.3 (3/9)	33.3 (3/9)	33.3 (3/9)	33.3 (3/9)
Feed from mixer, Ct[5]							
Negative	-	-	-	-	-	-	-
Positive	31.7	-	23.0	17.2	18.2	21.5	26.3
Sequence 1	38.1	-	18.8	27.1	25.3	26.4	19.5
Sequence 2	-	-	15.8	22.7	16.4	17.2	19.8

[1] Tissue culture containing 4.5×10^6 $TCID_{50}$/ml of PEDV was inoculated into a 4.5 kg batch of feed, then added to 45 kg of PEDV negative feed to form the positive treatment. Sequences were formed by sequentially adding 50 kg of PEDV negative feed to the mixer after a 10 minute discharge of the previous treatment into the leg of the bucket elevator. One feed sample per treatment per replicate × 3 replicates was collected, divided into three aliquots and diluted in PBS to form supernatants. Thus, three replicates were analyzed in triplicate and each feed supernatant value represents the mean. An aliquot of inoculum was analyzed by KSU for PEDV PCR. Pigs were initially 10-d-old and 9.3 kg BW. Three pigs per room received inoculum from one feed sample per repetition that was divided into three aliquots. For negative control pigs, one of three pigs received an aliquot from one of three repetitions. Thus, 30 pigs were divided into 10 treatment rooms. Fecal swabs were collected throughout the study and analyzed by ISU for PEDV PCR. Pigs were necropsied at day 7 and cecum content and tissues were collected.
[2] Day post inoculation.
[3] No detectable PEDV RNA (Ct > 45).
[4] Means represent the percent of samples that had detectable RNA by PEDV PCR analysis with numbers in parenthesis being the number with detectable PEDV and total number of samples collected.
[5] Mean cycle threshold (Ct) value of samples with detectable PEDV RNA below 45.

Table 4. Morphologic and immunohistochemistry evaluation of small intestine from pigs that were challenged with Porcine Epidemic Diarrhea Virus (PEDV) inoculated feed and sequenced feed[1]

	Morphology[2]			
Item	Villus height, μm	Crypt depth, μm	Villus height to crypt depth ratio	Immunohistochemistry (IHC)[3]
Feed from mixer				
Negative	372.2±41.1	167.34±12.5	2.2 ±0.2	0
Positive	354.0±23.7	170.7 ± 7.2	2.1±0.12	0
Sequence 1	366.2 ±23.7	165.3±7.2	2.2±0.12	0.6
Sequence 2	365.8 ± 23.7	157.0±7.2	2.3±0.12	0.8

[1] 500 mL of issue culture containing 4.5 × 106 TCID50/ml of PEDV was inoculated into a 4.5 kg batch of feed, then added to 45kg of PEDV negative feed to form the positive treatment. Sequences were formed by sequentially adding 50 kg of PEDV negative feed to the mixer after a 10 minute discharge of the previous treatment into the leg of the bucket elevator. One feed sample per treatment per replicate x 3 replicates was collected, divided into three aliquots and diluted in PBS to form supernatants. Thus, three replicates were analyzed in triplicate and each feed supernatant value represents the mean. The supernatant was administered one time via oral gavage on d 0 to each of three pigs for the negative treatment and 9 pigs for the remaining treatments (10 ml per pig). Thus, each value represents the mean of three pigs per negative treatment and nine pigs per positive, sequence 1, and sequence 2 treatments and 6 villi and 6 crypts measured per pig. Pigs were initially 10 d old and 9.3 kg BW.

[2] Intestinal cross-sections were fixed in formalin and stained with hematoxylin and eosin (H&E) for evaluation.

[3] Three sections of ileum were evaluated and averaged into one categorical value per pig. Categorical values were assigned for each pig (0=no signal, 1=mild, 2=moderate, 3=abundant, 4=diffuse) and reported as the mean from 3 pigs per negative treatment and from 9 pigs per remaining treatments.

Figure 1. Charged boot from bucket elevator from this study demonstrates a potential area of cross-contamination. Sequences mimicked the subsequent feed manufactured in a commercial mill. Notably, the boot pit in this bucket elevator was shallow compared to that in most commercial systems, where the cross-contamination area can have a depth of multiple feet.