RESEARCH REPORT
SWINE HEALTH

Title: Utilizing feed sequencing as a biosecurity intervention for preventing PEDV cross-contamination in the feed manufacturing process. – **NPB #14-273**

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INDUSTRY SUMMARY

The contamination of feed and feed ingredients with Porcine Epidemic Diarrhea Virus (PEDV) is a major concern to the swine industry and knowing feed or feed ingredients can support PEDV transfer, feed mills have become a potential source of cross contamination. This study aimed to utilize the only known pilot feed mill facility approved for pathogenic feed agent use in the United States to 1) determine if sequencing feed batches could minimize the risk of PEDV cross-contamination; 2) evaluate the effect of conditioning PEDV-contaminated feed below traditional pelleting temperatures on PEDV infectivity as measured by PCR and bioassay; and 3) evaluate the effect of manufacturing PEDV-contaminated feed on subsequent feed mill environmental surface contamination. The results suggest feed batch sequencing can reduce the concentration of PEDV genetic material in feed; however, feed can still be infectious and environmental surfaces, including equipment, remain contaminated. Pigs challenged with feed conditioned at less than 130°F resulted in PEDV infection. While these temperatures are below typical conditioning temperatures, feed is frequently manufactured below optimal temperatures at times during the feed manufacturing process, such as during start-up or resolution of a pellet mill plug. This contaminated pelleted feed could then lead to cross-contamination in the cooler or grain handling equipment; potentially infecting PEDV-free feed. All sampled feed mill surfaces were contaminated with PEDV RNA after production of the positive treatment and remained contaminated after batches of feed. Since the virus was ubiquitous,

decontamination of the facility proved challenging and required dry physical cleaning, wet chemical cleaning, and dry heat for complete decontamination. Proper decontamination in a large-scale commercial feed mill would be nearly impossible and therefore it is important to prevent the entry of PEDV contamination into the mill. This research helps raise awareness about the impact of producing PEDV-contaminated feed in a mill, the importance of feed mill biosecurity, and potential mitigation solutions to reduce the risk of infectivity of PEDV in animal feed.

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Keywords: PEDV, feed, pellet mill, bioassay, sequencing, swab

SCIENTIFIC ABSTRACT

This study aimed to utilize the only known pilot feed mill facility approved for pathogenic feed agent use in the United States to 1) determine if feed sequencing could minimize the risk of PEDV cross-contamination; 2) evaluate the effect of conditioning PEDV-contaminated feed below traditional pelleting temperatures on PEDV as measured by PCR and bioassay; and 3) evaluate the effect of manufacturing PEDV-contaminated feed on subsequent feed mill environmental surface contamination. Two simultaneous experiments were conducted to achieve these objectives. In Exp. 1, a PEDV-free corn, soybean meal-based swine diet was manufactured in a 0.11 m^3 electric paddle mixer to represent the negative control. Feed was discharged from the mixer, conveyed through a pilot-scale bucket elevator, and discharged into segregated biohazard containers. Negative control samples were collected at the mixer prior to discharge and at the bucket elevator during discharge. Next, a 500 ml aliquot of PEDV isolate (USA/IN/2013/19338 P7) with a PCR cycle threshold value (Ct) of 11 was uniformly mixed into 4.5 kg of the complete swine diet using a manual stainless steel benchtop paddle mixer. The PEDV-inoculated feed was then added to 45 kg of complete swine diet and mixed using the 0.11 m^3 electric paddle mixer to create the PEDV-contaminated feed treatment that served as the positive control. The positive control was then sampled and discharged similar to that described for the negative control. Four subsequent 50-kg batches (Sequence 1 to 4) of the PEDV-free swine diet were sequenced through the electric mixer, bucket elevator, and discharged with samples collected from each Sequence as previously described. All samples were analyzed for presence of PEDV genetic material by quantitative PCR (qPCR). The samples from the positive control, Sequence 1, and Sequence 2 were then mixed with PBS, stored overnight, and supernatant was extracted for use in bioassay. In Exp. 2, the positive

control treatment was pelleted using a pilot-scale pellet mill previously heated to a 71°C conditioning temperature for one hour. After the positive control feed was added to the hopper, steam was turned off until the conditioner temperature dropped below 37°C to simulate temperature change during the resolution of a pellet die plug. Steam was then added, and five pelleted samples were collected at targeted conditioner temperatures of 37, 46, 54, 62 and 71°C (± 1.2°C) using a 30 to 35 second conditioning time. After production was complete, the pellet mill was swabbed for presence of PEDV RNA detected by qPCR. The pelleted feed was then mixed with PBS overnight before extraction of the supernatant that was subsequently used for bioassay. Both experiments were replicated three times with decontamination of the feed mill and all equipment between replications and at the start and end of the study. As in our previous studies, there was a loss of approximately 10 Ct values when PEDV-cultured media was added to unprocessed mash feed, and an additional 3 Ct loss when feed was diluted 1:1,000. In Exp. 1, there was no PEDV detected from feed samples collected from the mixer during or after Sequence 2 or from the bucket elevator discharge during or after Sequence 3, but all equipment and environmental surfaces remained positive with detected PEDV genetic material obtained by swabbing, even after Sequence 4. Infectivity resulted from at least one bioassay room per treatment when pigs were challenged with inoculum from the positive control and both Sequence 1 and 2, despite the fact that Sequence 2 feed did not have PEDV RNA detected by qPCR. Additionally, PEDV infection resulted when pigs were fed feed pelleted at the two lowest conditioning temperatures, 37 and 46°C. Environmental swabs indicated widespread surface contamination of the equipment and work area after a PEDV contaminated batch of feed is processed. In summary, sequencing batches of feed should be considered a PEDV risk mitigation strategy, and not a risk elimination strategy. Introduction of PEDV-infected feed into a feed mill will likely result in ubiquitous contamination of equipment and surfaces, even after several batches of PEDV-free feed are produced. If targeted pellet mill conditioning temperatures are not reached, there is risk of transmitting PEDV through contaminated feed. Most concerning, feed without detectible PEDV RNA was demonstrated to be infective. More research is needed to determine if contaminated surfaces are infective and how to best decontaminate a PEDV-contaminated feed mill.

INTRODUCTION

Some previous reports of Porcine Epidemic Diarrhea Virus (PEDV) outbreaks in the U.S. and Canada were suspected to be caused by consumption of PEDV-contaminated feed or feed ingredients (Pasick et al., 2014). Feed has

since been confirmed as one of the many routes of PEDV-transmission, which has led to investigations into identifying ways to mitigate infectivity in contaminated diets or feed ingredients (Dee et al., 2014a; Dee et al., 2014b; Jones, 2014). Woodworth et al. (2014; NPB #14-159) confirmed PEDV cross-contamination could occur in feed. The resulting unintentionally-contaminated feed had PEDV RNA detected by quantitative real-time PCR (qPCR), but infectivity was not confirmed via bioassay. Furthermore, results from the previous study demonstrated that pelleting PEDV-contaminated feed at conditioning temperatures of 155°F (68.3°C) and above resulted in detection of PEDV RNA by qPCR, but rendered the feed non-infectious when tested by bioassay. However, we are unaware of any data that establishes viral inactivity at conditioning temperatures below 155°F (68.3°C). Although it would be highly uncommon to set target pellet mill conditioning temperatures below 155°F (68.3°C), it is possible that feed may be produced below these operating limits. This commonly would occur during start-up of the pellet mill or during a pellet mill die plug, which is often resolved without manual intervention by turning off steam to the conditioner in an effort to unclog the die. This can lead to significant quantities of pelleted feed being produced that has not reached the target conditioning temperature. The resulting feed conditioned at a lower temperature may still have infectious PEDV particles and/or potentially contaminate the pellet cooler, post-pellet feed handling equipment and bins at the feed mill, trucks, and bins, feed lines, and feeders at the farm.

Recent work by Greiner (2014) has began to shed light on PEDV and swine delta corona virus (SdCV) feed mill contamination. Mills feeding PEDV- or SdCV-contaminated herds had a greater likelihood for an environmental sample to test positive or suspect for PEDV particles. Thus, more knowledge is needed to help feed mill managers strengthen biosecurity protocols and reduce the risk of transmission. This can be achieved by utilization of environmental sampling in the feed mill following production of infected feed. Therefore, the objectives are as follows:

OBJECTIVES

1. Determine the efficacy of sequencing batches of feed to minimize the risk of PEDV cross-contamination in a pilot scale feed mill as measured by qPCR and bioassay.
2. Determine the efficacy of increasing pellet mill conditioning temperature on PEDV-survivability as measured by qPCR and bioassay.

3. Evaluate the potential for environmental PEDV contamination in a feed mill after a PEDV-positive batch of feed is produced by utilizing qPCR.

MATERIALS AND METHODS

The feed manufacturing portion of the experiments 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. Each experiment was replicated three times with decontamination before and after each replicate confirmed by the absence of PEDV-RNA in the feed, equipment, and environment as measured by qPCR.

PEDV Virus Isolate

Virus isolation, propagation, and titration were performed in Vero cells (ATCC CCL-81) as described by Chen et al. (2014). The U.S. PEDV prototype strain cell culture isolate USA/IN/2013/19338, passage 8 (PEDV19338) was used to inoculate feed. The stock virus titer contained 4.5 x 10^6 $TCID_{50}$/ml and was divided into three 500 mL aliquots that were stored at -80ºC prior to use, with one aliquot used in each replication.

Baseline Environmental Decontamination, Evaluation, and Containment

Before and/or after each replication, the FSRC was decontaminated following a standard protocol approved by the Kansas State University Institutional Biosafety Committee (Huss et al., 2015). Briefly, the FSRC was physically cleaned using compressed air and sweeping, chemically cleaned using a two-step process: 1). 1:256 dilution of ammonium glutaraldehyde blend (Synergize; Preserve International, Reno, NV) and 2). 10% sodium hypochlorite solution. The facility was then heated to 60°C for at least 24 hours and cooled to room temperature before baseline swabs (World Bioproducts, Mundelein, IL) were collected from designated surfaces to ensure no detectible PEDV genetic material was present. Prior to pathogen entry and until decontamination was confirmed, the facility was held in containment mode with

negative air pressure and High-Efficiency Particulate Arrestance filters preventing contaminated air from leaving the facility.

Feed Manufacturing

A standard corn-soybean meal-based swine diet (Table 1) was manufactured at the Kansas State University O.H. Kruse Feed Technology Innovation Center in Manhattan and served as the negative control feed for this experiment. A subsample of the feed was obtained prior to inoculation for each replication and confirmed negative by qPCR for the presence of PEDV RNA at the Kansas State University Research Park Molecular Diagnostics Development Laboratory in Manhattan. The negative control treatment was formed by mixing a 50 kg batch of the swine diet in a 0.11 m^3 electric paddle mixer (H. C. Davis Sons Manufacturing, model# SS-L1; Bonner Springs, KS) that was previously validated to obtain a mixer efficiency using a standard protocol (McCoy, 2005). A mixing time of 5 minutes was determined to achieve a uniform mix with a coefficient of variation of less than 10%. Feed was mixed for 5 minutes prior to collecting a 500 g sample by subsampling five equally spaced locations within the mixer. The feed was then discharged at a rate of approximately 4.5 kg/min into a bucket elevator (Universal Industries, model# SC97278, Cedar Falls, IA) that carried 74 buckets (each 737 cm^3); feed was then discharged through a downspout and was collected in biohazard containers.

A 500 mL aliquot of stock PEDV was then mixed into a 4.5 kg batch of swine feed using procedures established in a prior experiment (Woodworth et al., 2014). Briefly, feed and virus were mixed using a manual, bench-top laboratory scale stainless steel paddle mixer previously validated for mixer efficiency. The stock virus was slowly added during mixing, and a wet mix time of 2.5 minutes was used to confirm a consistent PEDV-contaminated mix, which served as the PEDV feed inoculum.

The PEDV inoculum (4.5 kg of feed + 500 ml of stock virus) was added to 45 kg of PEDV-free swine diet to form the positive control in the same 0.11 m^3 electric paddle mixer and the entire batch of positive control feed was then mixed for

5 minutes, discharged for 10 minutes into biohazard containers, with samples collected at the mixer and bucket elevator as previously described, and finally held at -2°C until the start of the thermal processing portion of the study.

Exp. 1: Feed Batch Sequencing

After the positive control feed was manufactured and discharged from the bucket elevator, four 50 kg batches of PEDV-free feed were sequenced through the mixer and bucket elevator (Sequence 1 to Sequence 4), and discharged into biohazard containers. Each Sequence was added to the electric paddle mixer, mixed for 5 minutes, sampled using procedures described above, and discharged for 10 minutes. All feed samples were analyzed for the presence of PEDV RNA by qPCR and the negative control, positive control, Sequence 1, and Sequence 2 were processed and utilized for bioassay.

Exp. 2: Thermal Processing

After Exp. 1 was complete, PEDV-free feed was pelleted using a pilot-scale single pass conditioner and pellet mill (Model CL5, CPM, Waterloo, IA). The pellet mill was heated by pelleting negative control feed with a conditioning temperature of 71°C for 60 minutes. The steam valve was then turned off until the conditioning temperature dropped below 37°C to mimic procedures commonly used to resolve a feed plug. Next, PEDV-inoculated feed was removed from the freezer and added to the pellet mill hopper. Once PEDV-inoculated feed started passing through the pellet mill, steam was slowly added, and five pelleted samples were collected at targeted hot mash conditioner temperatures of 37, 46, 54, 62, and 71°C (± 1.2°C) with feed processed using a 30 second conditioning time. These conditioning temperatures were selected based on a previously-determined prediction equation for the specific pellet mill to result in hot pellet temperatures of 40, 52, 63, 73, and 80°C, respectively.

Environmental Sampling

The same large foam tipped swabs used for the baseline PEDV-determination were also used to swab designated locations after discharge of the negative control, positive control, and Sequences 1 through 4 for each replicate (Figure 1). Swabs were categorized by sampled material or type of environmental surface from specific locations. Briefly, concrete surfaces included a floor drain, high foot traffic, and low foot traffic area. Metal surfaces included a vertical garage door surface and horizontal table ledge. A rubber surface was the bottom tread of a boot worn during the experiment. Equipment surfaces included metal feed mixer paddles, metal mixer bottom, metal interior of the mixer lid, plastic interior of a bucket elevator, and the rubber bucket elevator belt adjacent to sampled buckets. After Sequence 5, the pellet mill was allowed to cool, disassembled, and swabs collected at the metal interior of pellet mill hopper, metal interior of the mash conditioner, metal feeder screw, metal die interior rim, and metal die exterior shroud. All swabs were again collected from the same designated surfaces after physical cleaning, Step 1 and Step 2 of chemical cleaning, and heat-up.

The environmental swabbing sampling areas were outlined in heat-stable marker when possible to form 5 subsampled areas. Subsampled areas within a location were equal in size; however, the areas ranged from approximately 38 cm^2 to 107 cm^2 between locations. Subsampled areas within location were randomly allocated to be sampled after the negative control, positive control, and Sequence 1 to 4 feed treatments were manufactured. Thus, one subsampled area was swabbed per treatment and no subsampled area was sampled twice during each replication. Prior to the manufacturing of the negative control and after Step 2 of chemical cleaning and heat-up, the entire surface of all 5 areas were swabbed to establish and confirm baseline levels. During Replication 2 and 3, an additional 3 locations had 4 subsampled areas (metal vertical garage door, metal interior of the mixer lid, and concrete high foot traffic area) covered before the experiment began and removed in subsequent steps to evaluate the risk of cross-contamination after the manufacturing of each of Sequence 1 to 4. These swabs were categorized as covered swab areas.

To collect samples, a clean pair of disposable gloves was worn and a swab opened aseptically. The foam swab tip was rubbed across the designated surface area in a left to right, and up to down manner to swab the entire designated subsample area. Each swab was then placed in 2 mL of phosphate buffered saline (pH 7.4; 1X; Life Technologies Corp. Grand Island, NY) until analysis.

Sample Transportation and Processing

Feed samples were sealed in sterile sampling bags (Fisher Scientific Company, Ottawa, Ontario) and swabs were tightly capped before transportation out of the FSRC by approved exit protocols. Briefly, samples were sprayed with or submerged into 70% ethanol, placed into two sealed 7.6-L secondary containers (SC Johnson, Racine, Wisconsin), and placed on ice inside a clean transport cooler. The outer surface of the cooler was disinfected with 70% ethanol and removed from the feed mill by a designated individual following a dedicated pathway. Immediately afterwards, the floor along the cooler transportation pathway was disinfected with 1:256 of ammonium glutaraldehyde blend.

Three 100 g subsamples of each feed treatment per replicate were processed for detection of PEDV RNA via qPCR and determining infectivity by bioassay. Briefly, 100g of feed was added to 400 mL of PBS in 500 mL bottles, thoroughly shaken by hand to mix, and stored at 4ºC 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 using a PEDV spiked gene-based qPCR assay (J. Bai, unpublished data) utilized in previous experiments (Woodworth et al., 2014). Additionally, a 4 mL aliquot was evaluated at the Iowa State University Veterinary Diagnostic Laboratory using a PEDV N-gene-based qPCR assay (Chen et al., 2014). There were also 20 mL aliquots harvested from negative control, positive control, Sequence 1, and Sequence 2 treatments, which were stored for less than 1 month at -20ºC until use in the bioassay. Frozen aliquots were transported to the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL) by currier transport in accordance with an approved USDA Export Protocol. A 30 mL aliquot from each of the same treatments was stored at -80ºC for long-term storage and subsequent potential analyses or experiments.

Bioassay

The Iowa State University Institutional Animal Care and Use Committee reviewed and approved the pig bioassay protocol. Seventy-eight crossbred, 10 d old mixed sex pigs, similar to those utilized by Thomas et al. (2015), were sourced

from a single commercial farrow-to-wean herd with no prior exposure to PEDV. Upon arrival at the Iowa State University Laboratory Animal Facility, pigs were ear tagged, weighed, and administered a dose of cefitiofur (Excede, Zoetis, Florham Park, NJ). Fecal swabs were also obtained at arrival, and all pigs were confirmed negative for PEDV, porcine delta corona virus (PDCoV), and transmissible gastroenteritis virus (TGEV) using virus-specific rRT-PCRs at the ISU VDL. To further confirm PEDV negative status, serum was collected and confirmed negative for PEDV antibody by an indirect fluorescent antibody assay (Madson et al., 2014) and TGEV antibody by ELISA. Three pigs were randomly allocated into separate, but identical bioassay rooms, and a 2-day adaptation period was allowed prior to the beginning of the bioassay.

Bioassay procedures were similar to those previously described (Madson et al., 2014a; Thomas, 2014a). Briefly, rooms each had independent ventilation systems with solid flooring that was minimally rinsed to reduce PEDV aerosols. Pigs were fed liquid milk replacer twice daily and offered a commercial pelleted swine diet *ad libitum* with free access to water. Each pig was administered 10 mL of the aliquot by orogastric gavage using an 8 gauge French catheter on d 0 post-inoculation (0 dpi).

Exp. 1: Feed Batch Sequencing

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.

The control and challenge rooms were designated as:

Room 1) Replicates 1, 2, and 3 Negative Controls
Room 2) Replicate 1, Positive Control
Room 3) Replicate 1, Sequence 1
Room 4) Replicate 1, Sequence 2

Room 5) Replicate 2, Positive Control
Room 6) Replicate 2, Sequence 1
Room 7) Replicate 2, Sequence 2
Room 8) Replicate 3, Positive Control
Room 9) Replicate 3, Sequence 1
Room 10) Replicate 3, Sequence 2

Exp. 2: Thermal Processing

A total of 16 rooms (48 pigs) were assigned to treatment groups with 1 negative control room and 15 challenge rooms. Again, 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. Again, 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. The control and challenge rooms were designated as:

Room 1) Replicates 1, 2, and 3 Negative Controls
Room 2) Replicate 1, 37°C Conditioning Temperature
Room 3) Replicate 1, 46°C Conditioning Temperature
Room 4) Replicate 1, 54°C Conditioning Temperature
Room 5) Replicate 1, 62°C Conditioning Temperature
Room 6) Replicate 1, 71°C Conditioning Temperature
Room 7) Replicate 2, 37°C Conditioning Temperature
Room 8) Replicate 2, 46°C Conditioning Temperature
Room 9) Replicate 2, 54°C Conditioning Temperature
Room 10) Replicate 2, 62°C Conditioning Temperature
Room 11) Replicate 2, 71°C Conditioning Temperature
Room 12) Replicate 3, 37°C Conditioning Temperature
Room 13) Replicate 3, 46°C Conditioning Temperature
Room 14) Replicate 3, 54°C Conditioning Temperature
Room 15) Replicate 3, 62°C Conditioning Temperature
Room 16) Replicate 3, 71°C Conditioning Temperature

In both experiments, rectal swabs were collected on d -2, 0, 2, 4, 6, and 7 days post inoculation (dpi) from all pigs and tested for PEDV RNA by quantitative RT-PCR (qPCR). Pigs were humanely euthanized at 7 dpi, and fresh small intestine, cecum, and colon samples were collected at necropsy along with an aliquot of cecal content. One section of formalin-fixed proximal, middle, distal jejunum and ileum was collected per pig for histopathology (Madson et al., 2014). Infectivity of cecal content was evaluated for PEDV by qRT-PCR.

Histopathology and Immunohistochemistry

Tissues were routinely processed and fixed in neutral buffered formalin, embedded, sectioned, and stained with hematoxylin and eosin stain. One section of proximal, middle and distal jejunum and three serial sections from the piece of ileum for each pig were evaluated. In each section, one full-length villous 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 6 values per pig. The three values per jejunum and three values per ileum were averaged into one value per pig for calculating the villous: crypt ratio. Porcine epidemic diarrhea virus immunohistochemistry slides were prepared on the sections of ileum (Madson et al., 2014b). 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 (> 50 to 100%).

Statistical analysis

Data of the effects of feed batch sequencing and conditioner temperature 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, qPCR (feed, fecal, and environmental) results were summarized using descriptive statistics.

RESULTS

Feed Manufacturing

Exp. 1: Feed Batch Sequencing

No PEDV RNA was detected by qPCR after the negative control treatment was manufactured (Table 2). However, all feed samples and equipment swabs had detectible PEDV RNA after the positive control was manufactured, with the associated Ct of the feed and swab samples being approximately 30. Interestingly, only 77.8% of mixer or bucket elevator feed samples had detectable PEDV RNA after Sequence 1, yet 100% of the equipment swabs had detectable PEDV RNA. There was no detectible 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, 66.7% of mixer swabs and 100% of bucket elevator swabs had detectible PEDV RNA. After Sequence 3 and 4, none of the feed samples had detectible PEDV RNA, yet 44.4% of the mixer swabs and 100% of the bucket elevator swabs had detectible PEDV RNA. As the batches of feed progressed from the positive control to Sequence 4, there was little change in Ct values from swab samples. Thus, a similar quantity of PEDV RNA was detected throughout the experiment after the initial positive feed batch was manufactured.

Exp. 2: Thermal Processing

When PEDV-inoculated feed was processed at five different conditioning temperatures (37, 46, 54, 62, and 71°C), the respective mean cycle threshold (Ct) values as detected by qPCR were 32.5, 34.6, 37.0, 36.5, and 36.7, respectively (Table 5). All 9 of the feed samples conditioned at 37, 46 or 54°C had detectible PEDV RNA; whereas 8 of the 9 processed at 62 or 71°C had detectible PEDV RNA. It was observed that Ct value increased as conditioning temperature increased to 54°C, with little change thereafter.

Bioassay

Exp. 1 Feed Batch Sequencing

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 (Room 9, Replicate 3) yielded detectible PEDV RNA at 2 dpi, and all three pigs in Room 9 had fecal swabs and cecum contents with detectible viral particles by 4 through 7 dpi. A fecal swab from another pig gavaged with aliquot from Sequence 1 (Room 6, Replicate 2) yielded detectible PEDV RNA at 7 dpi, but no detectible PEDV RNA was observed from cecum contents. One pig gavaged with aliquot from Sequence 2 (Room 7, Replicate 2) had a fecal swab with detectible RNA at 2 dpi, although no PEDV RNA was detected by qPCR in the inoculum. By 4 dpi, all 3 pigs in Room 7 had detectible 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 villous 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 lower ($P < 0.05$) 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 villous 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.

Exp. 2: Thermal Processing

As in Exp. 1, fecal shedding of PEDV was not detected in rectal swabs or cecum contents from pigs fed the PEDV-negative control for the duration of the study (Table 6). Of the nine total pigs gavaged with aliquot from the PEDV-positive diet conditioned at 37°C, a fecal swab from one pig (Room 7, Replicate 2) yielded detectible PEDV RNA at 2 dpi, and all three pigs in Room 7 had fecal swabs and cecum contents with detectible viral particles by 4 through 7 dpi. In addition, three pigs gavaged with aliquots from the treatment conditioned at 46°C had detectible fecal PEDV RNA at 2 to 7 dpi, and all pigs were in the same room (Room 8, Replicate 2). No pig

challenged with feed conditioned at or above 54°C had detectible PEDV RNA in fecal swabs or cecum content for the duration of the study.

The pigs challenged with the feed conditioned at 37°C had shorter ($P < 0.05$) villous heights than pigs challenged with any other treatment (Table 7). Furthermore, pigs challenged with feed conditioned at 37, 54, or 62°C had deeper ($P < 0.05$) crypt depths than pigs challenged with the negative control or feed conditioned at 71°C. This led to pigs challenged with feed conditioned at 37°C to have a lower ($P < 0.05$) villus height: crypt depth ratio than pigs challenged with the negative control or feed conditioned at 54, 62, or 71°C. Porcine epidemic diarrhea virus IHC immunoreactivity was visible in the cytoplasm of villous enterocytes of pigs challenged with the two lowest processed temperatures: 37 and 46°C when harvested at 7 dpi.

Environment swabs. All baseline swabs had no detectible PEDV RNA (Table 8), which confirmed that the environment was PEDV-free at the initiation of each replication. After the negative control was manufactured, 22% (2 of 9) and 33% (1 of 3) rubber surfaces had detectible PEDV RNA, with all positive samples occurring during Replicate 2. After both the positive control and Sequence 1 were manufactured, 66.7% of metal, 100% of concrete, and 100% of rubber surfaces had detectible PEDV RNA. After Sequence 2, 83.3% of metal and 100% of concrete or rubber surfaces had detectible PEDV RNA. After Sequence 3, 66.7% of metal and 100% of concrete and rubber surfaces had detectible PEDV RNA. Detectible PEDV RNA contamination on metal surfaces increased to 83.3% after Sequence 4, but 100% of all concrete and rubber surfaces contained detectible PEDV RNA, similar to Sequence 2.

After chemical cleaning Step 1 with a glutaraldehyde blend, all environmental swabs were negative for PEDV RNA except Replicate 2, when 16.7% (1 out of 6) of metal surfaces and 11.1% (1 out of 9) of concrete surfaces had detectible RNA. Still, no swabs had detectable PEDV RNA after Step 2 of chemical cleaning with sodium hypochlorite. After pelleting the positive control and subsequent sequences, 100% of swabs sampled from the pellet mill had detectible PEDV RNA (Average Ct = 30.8). After Steps 1 and 2 of chemical cleaning with ammonium glutaraldehyde blend and sodium hypochlorite, all environmental swabs were negative for PEDV RNA except Replicate 2, where 13.3% of swabs had

detectible PEDV RNA. For this replicate, both steps of chemical cleaning were repeated and the equipment and facility re-swabbed, after which no further PEDV RNA was detected (data not shown).

Covered swab areas indicated the ability of dust to cross-contaminate equipment (Table 9). After the positive control was manufactured, 100% of swab areas from equipment and concrete surfaces, and 33.3% of metal surfaces had detectible PEDV RNA. Notably, 100% of the equipment and concrete covered swab areas had detectible PEDV RNA after Sequence 1 and 2, but none of the metal environmental covered swab areas. After Sequence 3, 50% of the equipment, 100% of concrete, and 0% of metal environmental covered swab areas had detectible PEDV RNA. After Sequence 4, 50% of the equipment, 100% of concrete, and 50% of metal environmental covered swab areas had detectible PEDV RNA

DISCUSSION

We have again demonstrated that feed can be one of the potential vectors of PEDV transmission in pigs by producing infection when pigs were challenged with PEDV-inoculated feed, similar to our previous research (Woodworth et. al., 2014). In addition, this research reports breakthrough evidence that PEDV infectivity may occur when qPCR does not detect PEDV RNA, and for the first time confirms the hypotheses that batch-to-batch carryover from PEDV-infected feed can result in subsequent infectious cross-contamination of feed and that infectivity in one pig can lead to subsequent infectivity of other pigs housed in the same environmental conditions.

As noted from previous PEDV studies we have conducted, the feed samples in this experiment had a predictable Ct value difference of approximately 13 (4 logs) when the PEDV-inoculated batch was used to form the positive control treatment, which equates to a 10,000-fold difference in the quantity of PEDV RNA that was detected (data not shown). More studies should be conducted to investigate possible viral degradation of PEDV RNA when added to feed or binding of the viral particles of RNA to feed.

One key finding is that sequencing feed batches can serve as a potential method to reduce the risk of PEDV and may be a valuable consideration in a feed mill biosecurity plan. Detectible PEDV genetic material was observed in the mixer (Sequence 1) and bucket elevator discharge (Sequence 1 and 2) after manufacturing knowingly infected feed. Thus, sequencing feed batches appears to be one potential method to minimize the likelihood of occurrence of detectible PEDV RNA, most likely due to a dilution effect and the ability for PEDV-free feed to push out the remaining contaminated particles from within the equipment. During Exp. 1, the mixer and bucket elevator were not cleaned or disinfected between treatments, so sequences mimicked the subsequent feed manufacturing process in a commercial mill. Notably, the risk of infectivity appears to be reduced, but not eliminated. After Sequence 1, 77.8% (7/9) of the feed samples still had detectible PEDV RNA, and 11.1% (1/9) pigs became infected compared to 100% (9/9) that were infected with the positive control. Therefore, feed batch sequencing should be considered to minimize the risk of PEDV contamination when manufacturing with higher risk ingredients, but other preventive measures should also be employed.

In accordance with our previous research (Woodworth et al., 2014), when pigs did become infected, they generally did so by 2 dpi and continued to shed virus until 7 dpi. There was only one instance where a pig (Room 6, Sequence 2) had detectible RNA in a fecal swab at 7 dpi, but lacked detectible RNA in cecum contents at necropsy. Although the fecal swab contained PEDV viral particles, the Ct value was high; indicating the quantity of viral RNA detected was relatively low. There were no other signs of infectivity in the room or at necropsy to suggest the pig was truly infected. In other studies, early coronavirus infections have resulted in detectable genetic material before any clinical signs or histopathological lesions have developed (R. Hesse, personal communication), but we cannot confirm this is the case.

Surprisingly, feed that had no detectible virus as determined by qPCR was found to be infectious. In the present study, infection was confirmed with positive IHC in 2 of the 3 pigs at 7 dpi. Thus, the combination of undetected viral particles and possible susceptibility of one pig to PEDV may explain why infection occurred after challenge with an inoculum that had no detectible RNA. As expected, once the pig was infected, the virus transmitted to the other two pigs inside the room within 48 hours. While is the suspected mode of transmission that causes epidemic outbreaks within barns, this report is to

our knowledge the first documentation of this transmission. Pigs continued to shed virus to the end of the study, and those infected later in the study (4 dpi) were IHC positive, which further confirmed PEDV infection.

The PEDV infectivity of a pig when inoculated with negative inoculum according to qPCR was surprising because qPCR is known to have high sensitivity for presence of genetic material. Still, this phenomenon has been reported in other published research, and we suspect that infectious genetic material was indeed present below the threshold of detection and not the result of experimental or analytical error. Other investigators have reported similar findings below levels of detection using intestinal scrapings and tissue culture fluid as challenge material. For example, Goyal et al. (2014) demonstrated when clarified intestinal homogenates harvested from PEDV-infected pigs were serially diluted until no longer detectible by qPCR, pigs inoculated with the dilutions had diarrhea, and mucosal samples had Ct values as low as 16. A study by Thomas et al. (2015) described similar results, where infectivity resulted even when the sample was below the limit of detection (Ct > 45). Thus, the minimum infectious dose of PEDV may be below the detectible threshold of the high sensitivity of qPCR analysis. Still, qPCR is a valuable tool to reduce the likelihood of infectivity.

Interestingly, none of the pigs challenged with the positive control had detectible viral antigen in small intestine tissues as examined by IHC at 7 dpi. Immunoreactivity is dependent on stage of infection and quantity of detectible antigen expression within tissues (Ramos-Vara, 2005). All the pigs challenged with the positive control inoculum showed evidence of infection earlier in the study. By 7 dpi, the pigs began to recover and shed lower quantities of detectible virus as measured by qPCR, which resulted in higher fecal swab and cecum content Ct values. Fecal PEDV Ct values of 30 and above have been correlated with virus detection without evidence of lesions or immunoreactivity (Taplett and Burrough, 2015). This may explain why pigs challenged with the positive treatment were IHC negative at 7 dpi and pigs inoculated with aliquots from Sequence 1 or 2 that became infected later were IHC-positive at termination of the study.

One of our previous experiments (Woodworth et al., 2014) evaluated the minimum infectious dose to infect pigs with PEDV and used low and high PEDV concentrations in feed to test the ability of a pellet mill to mitigate viral contamination. Tested conditioning temperatures were 68.3, 79.4, and 90.6°C for 45, 90, and 180 s conditioner retention

times. In the low PEDV dose, Ct values of the processed feed ranged from 37 to 40 and in the high dose, values were 30 to 31 Ct. In each sample, the PEDV RNA was detectable; however, the bioassay was negative. Thus, even though PEDV RNA was detected, it did not lead to infectivity.

Processing temperatures, like the ones used in the study by Woodworth et al. (2014) are not always reached due to the startup of equipment or during times when the pellet mill is plugged and the steam is turned off to force the lodged material out of the die. In this study, the temperature range reflects the conditioning temperature that potentially occurs immediately after unplugging a pellet mill by stopping the steam application without manual assistance. This is a common practice in feed mills producing pelleted feeds. Once cleared, the feed material passing through the pellet mill may not be conditioned at the target temperature, thus potentially allowing for PEDV-contaminated pelleted feed to be produced and contaminate the cooler, post-pellet grain handling equipment, delivery trucks, farm bins, feed lines, and feeders with viral particles. For this study, the scenario was simulated by first heating the pellet mill and die for one hour to ensure the equipment was operating at normal processing temperature and the metal was thoroughly heated. Then, the steam valve was turned completely off to drop the conditioner temperature to below 35°C. The steam was then turned back on, and samples were collected at the respective target experimental conditioning temperatures. Feed samples were collected based on conditioner temperature (hot mash temperature) because this value is typically set in commercial production and is easily measured and adjusted by the pellet mill operator in comparison to the impracticality of measuring and adjusting hot pellet temperatures during production. In accordance with our previous study, there was still detectable PEDV RNA at each experimental conditioning temperature. The present study showed the lowest processing temperature had the lowest PEDV Ct value, which suggests the greatest quantity of PEDV RNA present. Thus, infected feed can survive low conditioner temperatures and become a risk of infection for pigs. Finally, thermal processing of the positive control treatment did not decontamination pellet mill surfaces as detectible PEDV RNA was consistently found at relatively similar Ct values on all sampled surfaces.

It was observed that as inoculated feed was mixed and conveyed, widespread dust contamination of feed mill surfaces ensued. Even when sequenced feed samples lacked detectible PEDV RNA, equipment surfaces remained positive with

detectible viral particles, with no significant change in Ct value for the duration of the study. This is likely because aerosolized viral particles mixed with feed dust coated the environment after feed treatments were mixed and exited the bucket elevator spout. Porcine epidemic diarrhea virus particles have been documented to become aerosolized on dust and can be infective (Alonso et al., 2014). The infectivity of the dust in the present study, however, is unknown. It is concerning that once a surface in a feed mill is contaminated; it appears to have the ability to harbor PEDV. Future research should address the quantification of this risk and how to best decontaminate a PEDV-contaminated feed mill. Nonetheless, this research clearly indicates the feed mill itself, in addition to manufactured feed, can be a potential vector for PEDV transmission.

Unexpectedly, PEDV RNA was detected from a pair of boot bottoms and the concrete floor after manufacturing the negative control in 1 replicate. We hypothesize this genetic material remained on the boot due to inadequate cleaning after a previous replicate, and was tracked and detected on the concrete floor. Due to the heating of the room prior to each replicate, the viral material should not have been infective. However, we believe contaminated rubber boot bottoms helped track and spread the virus as genetic material was consistently detected on concrete floor surfaces, thus highlighting the importance of foot traffic biosecurity in any facility, including feed mills. Important implications of this finding are that foot traffic should be limited across receiving pits or in hand-add areas that have direct access to the mixer, and boots should be regularly cleaned.

One limitation from the environmental contamination portion of this research is the lack of bioassay to evaluate infectivity. Our hypothesis is that swabs from environmental samples containing PEDV RNA are infectious because there had been no chemical or heat steps applied to inactivate the PEDV. This hypothesis remains to be proven, but we recommend that biosecurity protocols consider including procedures to minimize and control dust in feed mills as it may be a vector of potentially infectious PEDV. Therefore, material collected from dust collection systems and sweepings should be stored and disposed of instead of added back into product flow per traditional measures (McDaniel, 2005). Dust is not only a potential harborage of bacteria, but also fungal organisms and endotoxins (Smid et al., 1992; Awad, 2007). Thus, it is our recommendation that feed mills consider accepting the potential disadvantage of shrink by disposing of dust

because feed mills are nearly impossible to decontaminate properly, particularly those with detectible PEDV particles (Vestby et al., 2009).

Because there is limited knowledge available that documents effective disinfection of PEDV-contaminated feed mills, it is important to describe the procedures utilized in this experiment. However, it should be recognized that most facilities do not have the ability to wet clean with chemicals or effectively heat to temperatures facilitating PEDV decontamination. Thus, further research is necessary to evaluate the effectiveness of dry chemical cleaning methods and other modes of decontamination. The ammonium glutaraldehyde blend and concentration used in this experiment were selected because previous research had demonstrated its ability to inactivate PEDV at a concentration of 1:256 or greater (Thomas, 2014b). Upon detection of PEDV genetic material after chemical disinfection in Replicate 2, an investigation revealed there was a dilution and application error of the disinfectant through the power washer. The problem was resolved, chemical cleaning was restarted, and full decontamination resulted. This experience illustrates the importance of using the correct concentrations of disinfectant for proper decontamination of PEDV.

Sodium hypochlorite, or household bleach, has also been shown to be effective at the inactivation and destruction of PEDV genetic material, and was used after chemical cleaning with the glutaraldehyde blend (OIE, 2014). Chemical cleaning with sodium hypochlorite was effective in all but 1 replicate, where 2 swabs had detectible PEDV genetic material from pellet mill surfaces. This could be due to inadequate disinfection of hard-to-clean areas within the pellet mill. After a second cleaning with bleach, all surfaces were decontaminated and lacked detectible PEDV RNA. While presence of genetic material does not necessarily correlate with infectivity, this illustrates the difficulty of feed mill decontamination once a feed mill has been contaminated with a virus.

In conclusion, the results of the present study suggest that a feed batch sequencing protocol can be used to reduce, but not eliminate PEDV carryover risk between batches of feed. Feed that has a low risk of contamination should be manufactured before feed manufactured for high-risk pigs, such as early nursery pigs or lactating sows, to reduce carryover risk. Concerning findings from this study are as follows: 1) even though feed may not have detectable RNA, the

equipment surfaces contained viral particles for several sequences which could lead to cross-contamination; 2) feed without detectable PEDV genetic material can be infective; 3) a PEDV-contaminated feed mill environment has the potential to provide a route of PEDV infection into farms even after several batches of PEDV-negative feed are produced. We fully acknowledge this risk will be difficult to quantify and document under field conditions in commercial settings. However, we speculate this may be a potential explanation for some of the sporadic PEDV infections observed in the field. Furthermore, 4) once a feed mill is contaminated with PEDV, viral particles in dust continue to be detected on most surfaces until chemically cleaned, 5) thermal processing is one step to reduce the risk of PEDV transmission; however, if target processing temperatures are not reached, such as during resolution of a plugged pellet mill, there is a risk of transmitting PEDV through contaminated pelleted feed. Our data suggest that feed should be conditioned at temperatures above 54.4°C in order to minimize the risk of PEDV transfer. Thus, care should be taken if suspected PEDV-contaminated feed is conditioned below the target temperature to prevent downstream cross-contamination. The practicality of decontaminating a PEDV infected feed mill or minimizing contaminated feed during processing is one of many new challenges in the feed manufacturing industry where biosecurity is an evolving concept since feed has been demonstrated to be a vehicle of PEDV transmission. Additional research is needed to further define the best ways to improve the biosecurity in our commercial feed mills to minimize the risk of biological pathogen contamination.

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Table 1. Diet composition

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, ground	1.15
Salt	0.50
L-Thr	0.03
Trace mineral premix[1]	0.15
Sow add pack[2]	0.50
Vitamin premix[3]	0.25
Phytase[4]	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]Each kilogram contains 26.4 g Mn, 110 g Fe, 110 g Zn, 11g Cu, 198 mg I, and 198 mg Se.

[2]Each kilogram contains 220,000 mg choline, 88 mg biotin, 660 mg folic acid, 1,980 mg pyridoxine.

[3]Each kilogram contains 4,400,000 IU vitamin A, 660,000 IU vitamin D_3, 17,600 IU vitamin E, 1,760 mg menadione, 3,300 mg riboflavin, 11,000 mg pantothenic acid, 19,800 mg niacin, 15.4 mg vitamin B_{12}.

[4]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 and equipment

	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)
Equipment swabs, %						
Mixer[5]	- (0/9)	100.0 (9/9)	100.0 (9/9)	66.7 (6/9)	44.4 (4/9)	44.4 (4/9)
Bucket elevator[6]	- (0/24)	100.0 (24/24)	100.0 (24/24)	100.0 (24/24)	100.0 (24/24)	100.0 (24/24)
Feed, Ct[7]						
Mixer	-	31.7	38.1	-	-	-
Bucket elevator spout	-	30.9	37.8	39.0	-	-
Equipment swabs, Ct						
Mixer	-	29.2	33.9	34.9	35.4	34.8
Bucket elevator	-	30.8	31.8	32.9	32.5	32.1

[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 x 3 repetitions. Feed was mixed for 5 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 detectible PEDV RNA (Ct > 45).

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

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

[5]Values represent the mean of 3 swabs from inside the mixer per treatment x 3 repetitions.

[6]Values represent the mean of 8 swabs from inside the bucket elevator per treatment x 3 repetitions.

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

Table 3. Effect of sequencing batches of porcine epidemic diarrhea virus (PEDV) inoculated feed on quantitative real-time (qPCR) cycle threshold (Ct) of feed, fecal swabs and cecum contents of pigs[1]

			Fecal swabs, Ct					
Item	Feed inoculum tested at KSU	Feed inoculum tested at ISU	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)	- (0/3)
Positive	100.0 (9/9)	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)	66.7 (6/9)	- (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)	- (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	29.2	-	23.0	17.2	18.2	21.5	26.3
Sequence 1	38.1	38.9	-	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 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. An aliquot of inoculum was analyzed by KSU and ISU for PEDV qPCR. Pigs were initially 10 d old and 9.3 kg BW. There were 3 pigs per room that received inoculum from one feed sample per repetition which was divided into three aliquots, except for the negative control pigs which 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. Pigs were necropsied at day 7 and cecum content and tissues were collected.

[2]Day post inoculation.

[3]No detectible PEDV RNA (Ct > 45).

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

[5]Mean cycle threshold (Ct) value of samples with detectible 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	398.9±30.9	159.6±8.8	2.5 ±0.16[b]	0
Positive	368.0±17.8	173.6 ± 5.1	2.1±0.09[a]	0
Sequence 1	388.9 ±17.8	161.6±5.1	2.4±0.09[b]	0.6
Sequence 2	378.8 ± 17.8	153.8±5.1	2.5±0.09[b]	0.8

[1]500 mL of issue 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 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.

[a,b]Means within column lacking a common superscript are different ($P < 0.05$).

Table 5. Effect of increasing pelleting temperature on porcine epidemic diarrhea virus (PEDV) infected feed as analyzed by PEDV quantitative real-time PCR (PEDV qPCR)[1]

	Mash conditioner temperature				
	37.8°C	46.1°C	54.4°C	62.8°C	71.1°C
% positive[2]	100.0 (9/9)	100.0 (9/9)	100.0 (9/9)	88.9 (8/9)	88.9 (8/9)
Cycle threshold (Ct)[3]	32.5	34.6	37.0	36.5	36.7

[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 which was mixed for 5 minutes, discharged into the leg of the bucket elevator, collected upon exiting the end spout, and subsequently processed. One feed sample per temperature per replicate × 3 replicates was collected, divided into three aliquots and diluted in PBS to form supernatants. Each value represents the mean of 3 replicates × 3 repetitions. Pellet mill die was warmed-up for approximately 1 hour before processing positive treatment. The PEDV-inoculated feed was added to the hopper after temporarily being chilled in a deep freezer. Shortly thereafter, the steam was turned off until the mash conditioner temperature dropped to below 37.8°C. Steam was slowly added and the five processed samples were collected at mash condition temperatures of 37.8, 46.1, 54.4, 62.8, and 71.1°C (± 1.2°C) with a condition time of approximately 30-40 seconds.
[2]Means represent the percent of samples that had detectible RNA by PEDV qPCR analysis.
[3]Mean cycle threshold (Ct) value of samples with detectible PEDV RNA below 45.

Table 6. Influence of increasing processing temperatures of porcine epidemic diarrhea virus (PEDV) inoculated feed on quantitative real-time qPCR cycle threshold (Ct) of feed, fecal swabs and cecum contents of pigs[1]

			Fecal swabs, Ct					
Item	Feed inoculum tested at KSU	Feed inoculum tested at ISU	0 dpi[2]	2 dpi	4 dpi	6 dpi	7 dpi	7 dpi Cecum content, Ct
Processed feed, %								
Negative	-[3]	-	-		-	-	-	-
37.8°C (100°F)	100.0 (9/9)[4]	100.0 (9/9)	-	11.1 (1/9)	33.3 (3/9)	33.3 (3/9)	33.3 (3/9)	33.3 (3/9)
46.1°C (115°F)	100.0 (9/9)	88.9 (8/9)	-	33.3 (3/9)	33.3 (3/9)	33.3 (3/9)	33.3 (3/9)	33.3 (3/9)
54.4°C (130°F)	100.0 (9/9)	55.6 (5/9)	-	-	-	-	-	-
62.8°C (145°F)	88.9 (8/9)	88.9 (8/9)	-	-	-	-	-	-
71.1°C (160°F)	88.9 (8/9)	100.0 (9/9)	-	-	-	-	-	-
Processed feed, Ct[5]								
Negative	-	-	-	-	-	-	-	-
37.8°C (100°F)	32.5	30.8	-	15.8	27.5	16.6	17.8	16.9
46.1°C (115°F)	34.6	32.4	-	24.5	15.2	15.4	17.9	18.8
54.4°C (130°F)	37.0	36.0	-	-	-	-	-	-
62.8°C (145°F)	36.5	36.3	-	-	-	-	-	-
71.1°C (160°F)	36.7	35.2	-	-	-	-	-	-

[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 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. An aliquot of inoculum was analyzed by KSU and ISU for PEDV qPCR. Pigs were initially 10 d old and 9.3 kg BW. There were 3 pigs per room that received inoculum from one feed sample per repetition which was divided into three aliquots, except for the negative control pigs which one of three pigs received an aliquot from one of three repetitions. Thus, 48 pigs were divided into 16 treatment rooms. Fecal swabs were collected throughout the study. Pigs were necropsied at day 7 and cecum content and tissues were collected.

[2]Day post inoculation.

[3]No detectible PEDV RNA (Ct > 45).

[4]Means represent the percent of samples that had detectible RNA by PEDV qPCR analysis with numbers in parenthesis being the number with detectible PEDV and total number of samples collected.
[5]Mean cycle threshold (Ct) value of samples with detectible PEDV RNA below 45.

Table 7. Morphologic and immunohistochemistry evaluation of small intestine from pigs that were challenged with porcine epidemic diarrhea virus (PEDV) inoculated feed processed at increasing temperatures[1]

	Morphology[2]			
Item	Villus height, μm	Crypt depth, μm	Villus height to crypt depth ratio	Immunohistochemistry (IHC)[3]
Processed feed				
Negative	395.3±27.1[a]	245.1±18.3[a]	2.1±0.2[b]	0
37.8°C (100°F)	299.8±15.6[b]	188.2±10.6[b]	1.6±0.1[a]	0.7
46.1°C (115°F)	374.4±15.6[a]	217.0±10.[6ab]	1.7±0.1[ab]	0.6
54.4°C (130°F)	380.3±15.6[a]	190.9±10.6[b]	2.0±0.1[b]	0
62.8°C (145°F)	387.3±15.6[a]	199.5±10.6[b]	1.9±0.1[b]	0
71.1°C (160°F)	385.9±15.6[a]	218.8±10.6[a]	1.8±0.1[b]	0

[1]500 mL of issue 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 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 45 pigs for the remaining treatments (10 ml per pig). Thus, each value represents the mean of three pigs per negative treatment and nine pigs conditioning temperature treatment 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 mean from 9 pigs per remaining treatments.

[a,b]Means within column lacking a common superscript are different ($P < 0.05$).

Table 8. Effect of sequencing batches of feed on porcine epidemic diarrhea virus (PEDV) contamination of environmental surfaces as determined by quantitative real-time PCR (qPCR)

	Treatment[1]							
Item	Baseline[2]	After negative	After positive	After sequence 1	After sequence 2	After sequence 3	After sequence 4	After chemical cleaning with ammonium glutarald-ehyde blend[3]	After chemical cleaning with sodium hypocholo-rite[4]
Swab location, %									
Metal[5]	-[6] (0/8)[7]	- (0/6)	66.7 (4/6)	66.7 (4/6)	83.3 (5/6)	66.7 (4/6)	83.3 (5/6)	16.7 (1/6)	- (0/6)
Concrete[8]	-(0/12)	22.2 (2/9)	100.0 (9/9)	100.0 (9/9)	100.0 (9/9)	100.0 (9/9)	100.0 (9/9)	11.1 (1/9)	- (0/9)
Rubber[9]	-(0/4)	33.3 (1/3)	100.0 (3/3)	100.0 (3/3)	100.0 (3/3)	100.0 (3/3)	100.0 (3/3)	- (0/3)	- (0/3)
Pellet mill[10]	- (0/20)	NC[11]	100.0 (15/15)	NC	NC	NC	NC	33.3 (5/15)	13.3 (2/15)
Swab location, Ct[12]									
Metal	-	-	31.3	31.7	33.4	32.0	33.2	35.3	-
Concrete	-	38.4	33.7	33.9	33.0	32.6	33.2	36.5	-
Rubber	-	38.3	31.9	32.3	33.0	32.7	32.4	-	-
Pellet mill	-	NC	30.8	NC	NC	NC	NC	35.7	36.8

[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 a 10 minute discharge of the previous treatment into the leg of the bucket elevator which then exited the end spout before locations were sampled.

[2]Baseline represents the initial set of swabs to ensure there was no detectible PEDV RNA before each repetition and one set of swabs after completion of the study.

[3]Diluted to 1:256 with potable water.

[4]Diluted to 5-10% with potable water.

[5]Metal includes a sample from the garage door and stainless steel table ledge per treatment × 3 repetitions.

[6]No detectible PEDV RNA (Ct > 45).

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

[8]Concrete includes a sample from the drain, low traffic and high traffic floor area per treatment × 3 repetitions.

[9]Rubber includes a sample from the bottom of a pair of rubber boots per treatment × 3 repetitions.

[10]Only the positive treatment was processed. 5 samples were taken from the pellet mill after processing the positive treatment × 3 repetitions.

[11]Sample not collected.

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

Table 9. Effect of covering or not covering sample site during sequencing batches of porcine epidemic diarrhea virus (PEDV) infected feed as analyzed by PEDV quantitative real-time PCR (PEDV qPCR)

	Treatment[1]				
Item	After positive	After sequence 1	After sequence 2	After sequence 3	After sequence 4
Sample type, %					
Equipment					
Mixer lid, not covered[2]	100.0 (3/3)[3]	100.0 (3/3)	100.0 (3/3)	100.0 (3/3)	100.0 (3/3)
Mixer lid, covered[4]	NC[5]	100.0 (2/2)	100.0 (2/2)	50.0 (1/2)	50.0 (1/2)
Concrete surface					
High traffic floor area, not covered	100.0 (3/3)	100.0 (3/3)	100.0 (3/3)	100.0 (3/3)	100.0 (3/3)
High traffic floor area, covered	NC	100.0 (2/2)	100.0 (2/2)	100.0 (2/2)	100.0 (2/2)
Metal surface					
Garage door, not covered	33.3 (1/3)	33.3 (1/3)	66.7 (2/3)	33.3 (1/3)	66.7 (2/3)
Garage door, covered	NC	-[6] (0/2)	- (0/2)	- (0/2)	50.0 (1/2)
Sample type, Ct[7]					
Equipment					
Mixer lid, not covered	30.3	32.5	32.8	34.2	33.6
Mixer lid, covered	NC	36.6	38.8	36.7	39.0
Concrete surface					
High traffic floor area, not covered	30.8	31.7	31.8	31.1	32.4
High traffic floor area, covered	NC	34.4	34.5	34.4	34.8
Metal surfaces					
Garage door, not covered	37.9	39.0	38.8	39.0	38.1
Garage door, covered	NC	-	-	-	39.1

[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 a 10 minute discharge of the previous treatment into the leg of the bucket elevator.

[2] Not covered refers to an open area on the mixer lid, a high traffic floor, a low traffic floor, and a garage door area that was routinely sampled. One area was sampled per treatment × 3 replicates.

[3] Means represent the percent of samples that had detectible RNA by PEDV qPCR analysis with numbers in parenthesis being the number with detectible PEDV and total number of samples collected. Swabs were sampled after each feed treatment was mixed for 5 minutes, discharged for 10 minutes, carried through the bucket elevator, and had exited the bucket elevator and spout. None of the equipment was cleaned in-between treatments.

[4] Covered refers to one area per treatment on the mixer lid, high traffic floor area, low traffic floor area, and garage door area that was covered at the beginning of the experiment. One covered area on the mixer lid, high traffic floor area, low traffic floor area, and garage door was uncovered before

each sequence and then sampled after discharge of the current sequence. Thus, dust specific to the feed treatment was sampled and one sample was taken per treatment × 2 replicates.

[5] Sampled not collected.

[6] No detectible PEDV RNA (Ct>45).

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

Figure 1. Arrangement of the first floor of the Kansas State University Cargill Feed Safety Research Center. Designated areas swabbed for PEDV qPCR analysis include high and low foot traffic areas, drain, garage door, pellet mill, table ledge, bucket elevator, and feed mixer. Not shown are rubber boot bottoms.