

## SWINE HEALTH

**Title:** Assessing PEDV infectivity of samples collected from a PEDV contaminated feed mill  
– NPB #15-211

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**Industry Summary:** Introduction of porcine epidemic diarrhea virus (PEDV) into swine herds not only had a significant impact on animal health, but continues to be significant for pork producers and the feed manufacturing industry since it appears to be the first widely transmissible virus to cause disease via transmission in animal feed. Previous research has demonstrated that cross contamination of subsequent batches of feed can occur after PEDV contaminated feed is made. Feed batch sequencing is a proposed mitigation technique to decrease PEDV cross-contamination during feed manufacturing where negative feed is sequenced through the feed mill after potentially contaminated feed. This study aimed to investigate the infectivity of retained samples collected during a previously funded NPB study from samples collected after the mixer on sequence 3 and 4 following a batch of PEDV-inoculated feed. Additionally, the study aimed to investigate infectivity of sequenced feed treatments collected from batches after being transported through a bucket elevator feed conveyor. Additionally, fluid from swabs collected from the contaminated feed manufacturing surfaces were evaluated. Our results indicated that challenging pigs with feed from the mixer sequence 3, mixer sequence 4, and bucket elevator feed conveyor sequence 1 to sequence 4 did not result in PEDV infectivity. Furthermore, challenging pigs with dust collected from the conveyor surfaces did not result in PEDV

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infectivity. It is unknown if this lack of infectivity is due to insufficient replication, sampling and storage methodology, or a true effect; so the hypothesis that animal food-contact surface dust is infectious is still conceivable and remains to be proven

**Keywords:** PEDV, feed sequencing, contamination, dust, swab, swine

**Scientific abstract:** The objective of this experiment was to evaluate the infectivity of sequenced feed and the infectivity of swabs collected from PEDV contaminated feed manufacturing equipment. All samples were collected from our previous PEDV study (NBP #14-273). In the previous study, PEDV-free swine feed was manufactured to represent the negative control. Feed was mixed for 5 min then sampled, then discharged for 10 min into a conveyor and sampled again upon exit. Next, a 500 mL aliquot of PEDV isolate (USA/IN/2013/19338 P8) with a quantitative real-time PCR (qPCR) cycle threshold of 11 was used to inoculate 49.5 kg of PEDV-free feed to form the positive control. The positive control was mixed, conveyed and sampled similar to the negative control. Next, 4 sequence treatments (sequence 1 to 4) were formed by adding a 50 kg batch of PEDV negative feed to the mixer after the prior batch was mixed and conveyed; all sequences were mixed, conveyed, and sampled as previously described. None of the equipment was cleaned between treatments. This process was replicated 3 times. Designated feed manufacturing equipment surfaces were swabbed after each feed treatment. Bioassay for samples collected from the mixer during sequence 3 and 4, all conveyor feed treatments, and swabbed conveyor dust was conducted and consisted of 54 mixed sex ( $3.18 \pm 0.79$  kg BW) initially 10 d old pigs. Pigs were confirmed negative for PEDV and were allocated to 1 of 18 treatment rooms. Control pigs remained PEDV negative for the study. All pigs from the conveyor positive treatment (3/3) were qPCR positive on fecal swabs by the end of the study. No PEDV infectivity was found in feed samples from sequence 3 and 4 collected from the mixer and samples collected from the bucket elevator conveyor during sequence 1 to sequence 4. Finally, bioassay surface samples from the conveyor did not result in infectivity. It is unclear if storing these samples for 11 months before initiation of the bioassay contributed to the lack of infectivity that was observed.

**Introduction:** Feed has been confirmed as one of the many routes of PEDV-transmission and has led to investigations into identifying ways to mitigate infectivity of contaminated diets or feed ingredients (Schumacher et al., 2016; Dee et al., 2014; Cochrane, 2015). Previous work from our group (NBP #14-273) evaluated feed batch sequencing as a mitigation technique to reduce the risk of PEDV cross-contamination. In that study, 4 sequences of uncontaminated feed were made following the production of a PEDV contaminated batch. Feed was sampled after each sequence at the mixer and after being conveyed in a bucket elevator. Environmental samples were also obtained after each sequence. That study demonstrated infectious PEDV cross-contamination of feed can occur after 2 sequenced batches of feed sampled from the mixer (Woodworth, 2015). However, feed collected from the mixer from sequence 3 and 4 and after all 4 sequences were conveyed through the bucket elevator conveyor were not evaluated by bioassay.

Detection of PEDV and porcine delta coronavirus RNA in feed manufacturing facilities (Greiner, 2016) has been reported. Similarly, during our study (NBP #14-273) PEDV RNA was detected on feed contact surfaces after feed batch sequencing even when the feed was PEDV PCR negative (Woodworth, 2015). Neither of these studies evaluated infectivity. During our study (NBP #14-273) we retained samples of feed and environmental samples. Thus, the objective for the current study was to evaluate the infectivity of these retained samples.

**Objective:**

1. Exp. 1: Evaluate PEDV infectivity from feed sequence 3 and 4 samples retained from the mixer and all feed samples collected after exiting the bucket elevator conveyor for sequences 1 to 4.
2. Exp. 2: Investigate the PEDV infectivity of retained environmental samples collected from the conveyor, an animal feed-contact surface, during feed manufacture.

**Materials and Methods:**

The feed inoculation and manufacturing portion of the experiments was replicated three times and was previously conducted at the Kansas State University Cargill Feed Safety Research Center and has been described in detail in a previous report (NBP #14-273; Woodworth, 2015). The PEDV stock virus

isolation, propagation, and titration used in the Woodworth et. al (2015) study were performed in Vero cells (ATCC CCL-81) at Iowa State University (Ames, IA) as described elsewhere (Chen et al., 2014a). All animal work from the current study was conducted at the Iowa State University Veterinary Medical Research Institute (Ames, IA).

#### *Background on Exp. 1: Feed sequencing*

Briefly, in Exp. 1, 50 kg of swine diet was spiked with U.S. PEDV prototype strain cell culture isolate USA/IN/2013/19338 cell passage 8 to form a positive feed treatment with an estimated  $4.5 \times 10^4$  TCID<sub>50</sub>/g that was mixed, discharged, and conveyed following the negative control as previously described (Woodworth, 2015). Subsequent 50 kg batches of PEDV-negative swine diet was similarly mixed, discharged, and conveyed to form sequence 1 to sequence 4. No equipment was cleaned in-between sequences to mimic commercial feed manufacturing conditions. Feed samples collected from the mixer from sequence 3 and sequence 4 were processed for bioassay (as described below), however these samples were then stored at -80°C for 11 months while awaiting bioassay. Feed samples collected from the conveyor from all treatments (negative, positive, sequence 1 to sequence 4) were likewise immediately processed for bioassay (as described below); however, these samples were also stored at -80°C for 11 months while awaiting bioassay.

#### *Conveyor animal feed-contact surface contamination*

Animal feed-contact surface contamination from the conveyor was evaluated while manufacturing feed in the sequencing study. Environmental sampling, baseline environmental decontamination, evaluation of swabs, and contamination of the facility is described elsewhere (Woodworth, 2015). Briefly, swabs each containing 2 mL of sterile PBS, were used to aseptically collect surface contamination from the plastic interior of 4 randomly chosen conveyer buckets (one swab each) and 4 belt areas (one swab each) adjacent to the chosen buckets per treatment (negative, positive, sequence 1 to sequence 4) per replicate. Swabs were then immediately transported for qPCR and results

reported in (Woodworth, 2015). Supernatant from swab tubes were transferred to 96-deep well plates and stored frozen at -80°C for 11 months while awaiting bioassay.

#### *Processing, qPCR and handling of bioassay samples*

Feed samples were processed for determining infectivity via bioassay. Briefly, 100 g feed was added to 400 mL of PBS in 500 mL bottles, thoroughly shaken by hand to mix, and stored at 4°C overnight to form the supernatant. Twenty mL aliquots of supernatant from each bottle were then harvested for bioassay using sterile serologic pipettes with a pipette controller (Pipetboy; Integra Biosciences, Hudson, NH). A 4 mL aliquot of the feed suspension and supernatant sampled directly from the swab tubes were evaluated using a PEDV spiked gene-based qPCR assay (J. Bai, unpublished data). Additionally, a 4 mL feed supernatant aliquot and remaining swab supernatant after bioassay challenge was evaluated at the Iowa State University Veterinary Diagnostic Laboratory using a PEDV N-gene-based qPCR assay (Chen et al., 2014b).

#### *Bioassay*

The pig bioassay portion of the current study experiments and experimental protocols were approved by the Iowa State University Institutional Animal Care and Use Committee and adhered to the ethical and humane use of animals for research. Fifty-four 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 known prior exposure to PEDV. Upon arrival at the Iowa State University Laboratory Animal Facility in late March 2016, pigs were ear tagged, weighed, and administered a dose of ceftiofur (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 qPCRs at the ISU VDL. To further confirm PEDV negative status, serum was collected and confirmed serologically negative for PEDV and TGEV. 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. Prior to challenge, inoculum from swabs were allowed to

thaw at room temperature, then were pooled according to replicate and treatment for each pig and finally stored overnight at 4°C until used for bioassay the next day. Inoculum from feed treatments were allowed to thaw overnight at 4°C until used for bioassay the next day. Bioassay procedures were similar to those previously described (Madson et al., 2014a; Thomas et al., 2015). 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 supernatant (amounts described below) by orogastric gavage using an 8 gauge French catheter on d 0 post-inoculation (0 dpi).

*Feed sequencing bioassay design:*

A total of 12 rooms (36 pigs) were assigned to feed treatment groups with 1 negative control room and 11 challenge rooms.

The control and feed treatment challenge rooms were designated as:

- Room 1) Replicates 1, 2, and 3 Negative Conveyor Controls (30 mL/pig)
- Room 2) Replicate 1, Mixer Sequence 3 (10 mL/pig)
- Room 3) Replicate 2, Mixer Sequence 3 (10 mL/pig)
- Room 4) Replicate 3, Mixer Sequence 3 (10 mL/pig)
- Room 5) Replicate 1, Mixer Sequence 4 (10 mL/pig)
- Room 6) Replicate 2, Mixer Sequence 4 (10 mL/pig)
- Room 7) Replicate 3, Mixer Sequence 4 (10 mL/pig)
- Room 8) Replicate 1, 2, and 3 Positive Conveyor Control (30 mL/pig)
- Room 9) Replicate 1, 2, and 3 Conveyor Sequence 1 (30 mL/pig)
- Room 10) Replicate 1, 2, and 3 Conveyor Sequence 2 (30 mL/pig)
- Room 11) Replicate 1, 2, and 3 Conveyor Sequence 3 (30 mL/pig)
- Room 12) Replicate 1, 2, and 3 Conveyor Sequence 4 (30 mL/pig)

*Conveyor animal food-contact bioassay design:*

A total of 6 rooms (18 pigs) were assigned to evaluate environmental sampling with 1 negative control room and 5 challenge rooms. Each pig from the negative control room was given eight, 1

mL aliquots of inoculum from swabbed conveyor surfaces (composed of 4 conveyor bucket and 4 conveyor belt surfaces) derived from the same replicate for a total of 8 mL. Each pig within a room therefore represented replicate 1, 2, or 3. Similar to the negative control room, each pig from the subsequent rooms were given eight, 1 mL aliquots of inoculum swabbed from conveyor surfaces (composed of 4 conveyor bucket and 4 conveyor belt surfaces) and each pig within a room represented replicate 1, 2, or 3 of each treatment.

The environmental sample challenge rooms were designated as:

Room 13) Replicates 1, 2, and 3 Negative Conveyor Controls (8 mL/pig)

Room 14) Replicates 1, 2, and 3 Positive Conveyor Controls (8 mL/pig)

Room 15) Replicates 1, 2, and 3 Sequence 1 (8 mL/pig)

Room 16) Replicates 1, 2, and 3 Sequence 2 (8 mL/pig)

Room 17) Replicates 1, 2, and 3 Sequence 3 (8 mL/pig)

Room 18) Replicates 1, 2, and 3 Sequence 4 (8 mL/pig)

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 qPCR. Pigs were humanely euthanized at 7 dpi and an aliquot of cecal content was evaluated for PEDV qPCR.

## Results

### *Feed sequencing*

Table 1 summarizes mixer sequence 3 and 4 and conveyor bioassay results. None of the pigs from mixer sequence 3 and sequence 4 shed virus on rectal swabs throughout the study nor had detectible PEDV RNA in cecum contents at 7 dpi. All pigs from the negative conveyor feed treatment were qPCR negative on rectal swabs collected before inoculation and for the duration of the study. One pig from the positive conveyor feed treatment was qPCR positive on rectal swab at 2 dpi and by 4 dpi, 3 of 3 pigs had qPCR positive rectal swabs and shed virus to the end of the study and in 7 dpi cecum

contents. None of the pigs from conveyor feed sequence 1 to sequence 4 shed PEDV on fecal swabs nor had detectible PEDV in cecum contents at 7 dpi.

*Bucket elevator feed conveyor animal-food contact surface contamination*

Suspensions from the bucket elevator feed conveyor animal-food contact surfaces were challenged in pigs and did not result in PEDV infectivity.

**Discussion:**

The potential for PEDV contamination in feed manufacturing facilities is a concern since research has confirmed feed and feed ingredients are vectors for PEDV transmission (Dee et al., 2014; Pasick et al., 2014). Since little is known about the infectivity of virus cross-contamination during animal feed production or the infectivity of contaminated feed manufacturing surfaces, the purpose of this study was to evaluate infectivity from feed batch sequence 3 and 4 collected from mixer, all sequences collected after the conveyor and evaluate swabbed surfaces from knowingly PEDV contaminated conveyor equipment by use of the 10-d-old bioassay model. Virulence of the PEDV isolate used was established by previous studies (Schumacher et al. 2016; Thomas et al., 2015) and in the current study all pigs challenged with positive feed collected from the conveyor exhibited signs of infectivity by 7 dpi.

Sequencing after virally contaminated feed is based on procedures demonstrated to reduce carryover contamination of medicated feed. Sequencing a batch of medicated-free feed has been shown to reduce significant drug carryover in medicated feed manufacturing (Herrman et al., 1995; Martinez-Kawas, 2008). The initial sequencing study (NBP #14-273; Woodworth, 2015) confirmed that feed batch sequencing is effective at reducing, but not eliminating, PEDV cross contamination.

Although we demonstrated infectivity in the PEDV contaminated feed from the bucket elevator feed conveyor, we failed to demonstrate infectivity in the third and fourth batch of sequenced feed collected from the

mixer. Furthermore, feed samples collected after bucket elevator feed conveyor feed sequence 1 and conveyor feed sequence 2 were bioassay negative even though the feed was previously demonstrated to be qPCR positive.

One factor that might influence this lack of infectivity in the presence of qPCR positive samples is extended storage time. Bioassay experiments challenging pigs with stored PEDV samples have been previously performed (Alonso et al., 2014; Schneider et al., 2015), however the duration was not specified. Although most samples containing any type of virus are routinely stored frozen at low temperatures to maintain infectivity (Block et al., 1978), specimens containing low titers from other viruses have been documented to not retain viability as long as high titer samples when stored long-term at low temperatures (Gould, 1999). Therefore, it is possible that sequenced conveyor feed treatments did not contain enough viable PEDV that survived during the 11 month storage.

Supernatant from swabbed conveyor surfaces following production of PEDV inoculated feed was also not infectious. Again, it is possible that storage had an effect on supernatant infectivity. Although we were unsuccessful at finding evidence of infectivity, the hypothesis that surface contamination after sequencing PEDV inoculated feed is still conceivable and remains to be demonstrated. Additional research is needed to further minimize the risk of infectious viral pathogen contamination during feed manufacturing.

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**Table 1. Pig bioassay results from manufactured and batch sequenced porcine epidemic diarrhea virus (PEDV) inoculated feed collected from mixing and conveying equipment<sup>1</sup>**

Item	Fecal swabs <sup>2</sup>					7 dpi Cecum content
	0 dpi	2 dpi	4 dpi	6 dpi	7 dpi	
Feed from mixer, Positive pigs/Total <sup>3</sup>						
Sequence 3	0/9	0/9	0/9	0/9	0/9	0/9
Sequence 4	0/9	0/9	0/9	0/9	0/9	0/9
Feed from conveyor, Positive pigs/Total						
Negative	0/3	0/3	0/3	0/3	0/3	0/3
Positive	0/3	1/3	1/3	3/3	3/3	3/3
Sequence 1	0/3	0/3	0/3	0/3	0/3	0/3
Sequence 2	0/3	0/3	0/3	0/3	0/3	0/3
Sequence 3	0/3	0/3	0/3	0/3	0/3	0/3
Sequence 4	0/3	0/3	0/3	0/3	0/3	0/3

<sup>1</sup>Tissue culture fluid containing  $4.5 \times 10^6$  TCID<sub>50</sub>/ml of PEDV was inoculated into 49.5 kg of PEDV negative feed to form the positive treatment. For each negative, positive and sequence batch, feed was mixed for 5 min and sampled, then discharged for 10 min into the conveyor and sampled upon exit. Equipment was not cleaned between treatments. Sequences were formed by sequentially adding 50 kg of PEDV negative feed to the mixer after the prior batch was processed. This process was replicated 3 times. For bioassay, pigs were initially 10 d old and 3.2 kg BW. Feed from the mixer was inoculated into pigs in 3 rooms with 3 pigs per room. For the samples collected after the conveyor, each pig in each room was inoculated from a separate replicate resulting in 1 room per treatment with each pig representing a separate replicate. Bioassay for sequence 3 and 4 and conveyor samples was conducted 11 mo after sample collection.

<sup>2</sup>Fecal swabs and cecum contents were analyzed for PEDV by qPCR on 0, 2, 4, 6, and 7 days post inoculation (dpi) and necropsied at d 7 where cecum contents and tissues were collected.

<sup>3</sup>Count of pigs shedding detectable PEDV RNA/number of pigs analyzed.