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## Cover Page Footnote

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## Evaluating the Effect of Manufacturing Porcine Epidemic Diarrhea Virus (PEDV)-Contaminated Feed on Subsequent Feed Mill Environmental Surface Contamination<sup>1</sup>

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### Summary

This study aimed to utilize the only known pilot feed mill facility approved for pathogenic feed agent use in the United States to evaluate the effect of manufacturing Porcine Epidemic Diarrhea Virus (PEDV)-contaminated feed on subsequent feed mill environmental surface contamination. In this study, PEDV inoculated feed was manufactured and conveyed on equipment along with four subsequent batches of PEDV-free feed. Equipment and environmental surfaces were sampled using swabs and analyzed for the presence of PEDV RNA by PCR. The experiment was replicated three times with decontamination of the feed mill and all equipment between replications. Overall, environmental swabs indicated widespread surface contamination of the equipment and work area after a PEDV contaminated batch of feed was processed. There was little difference in environmental sample cycle threshold (Ct) values after manufacturing each of the subsequent PEDV-negative feed batches. In summary, introduction of PEDV-infected feed into a feed mill will likely result in widespread contamination of equipment and surfaces, even after several batches of PEDV-free feed are produced. Eliminating the PEDV RNA from the feed mill environment was challenging and required procedures that are not practical to apply on a regular basis in a feed mill. This data suggests that it is extremely important to prevent the introduction of PEDV-contaminated feed, ingredients, or other vectors of transmission to minimize PEDV-risk. More research should be conducted to determine if contaminated surfaces can lead to PEDV infectivity and to determine the best feed mill PEDV-decontamination strategies.

Key words: PEDV, feed mill, decontamination, swabs, PCR

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## Introduction

Feed mills feeding Porcine Epidemic Diarrhea Virus (PEDV)-contaminated herds were found to have a greater likelihood for an environmental sample to test positive or suspect for PEDV particles (Greiner, 2014)<sup>5</sup>. Thus, there is a need for feed mill managers to strengthen biosecurity protocols and reduce the risk of transmission. However, the extent of environmental contamination that occurs after producing batches of feed that are PEDV-positive is unknown. Therefore, the objective for this study was to utilize PCR to evaluate the potential for environmental PEDV contamination in a feed mill after a PEDV-positive batch of feed is produced.

## Procedures

The 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.

### *Baseline Environmental Decontamination, Evaluation, and Containment*

Before 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)<sup>6</sup>. 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 140°F for at least 24 h and cooled to room temperature before baseline swabs (World Bioproducts, Mundelein, IL) were collected from designated surfaces (Figure 1). 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 (HEPA) filters preventing contaminated air from leaving the facility.

## Feed Manufacturing

The U.S. PEDV prototype strain cell culture isolate USA/IN/2013/19338 was divided into three 500 mL aliquots with one aliquot used in each replication. The virus was then mixed into 4.5 kg of feed, which was subsequently added to 45 kg of PEDV-free swine diet and mixed in a 4 ft<sup>3</sup> electric paddle mixer (H. C. Davis Sons Manufacturing, model# SS-L1; Bonner Springs, KS) for 5 min and then discharged, conveyed into a bucket elevator (Universal Industries, model# SC97278, Cedar Falls, IA) and collected into biohazard containers. Subsequent 50 kg batches of PEDV-free feed (Sequence 1 to Sequence 4) were similarly mixed, discharged, and conveyed. Also, feed from the

<sup>5</sup> Greiner, L. 2014. Evaluation of the risk of a feed mill being contaminated with PEDV or SdCV. <http://www.pork.org/wp-content/uploads/2014/05/greiner-14-165-main-020615.pdf> (Accessed 28 July 2015).

<sup>6</sup> Huss, A. R., R. A. Cochrane, A. Deliephan, C. R. Stark, and C. K. Jones. 2015. Evaluation of a biological pathogen decontamination protocol for animal feed mills. *J. Food Prot.* *In press.*

positive batch was processed using a pilot-scale single pass conditioner and pellet mill (Model CL5, CPM, Waterloo, IA).

### *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. Swabs were categorized by sampled material or type of environmental surface from specific locations (Figure 1). 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 completion of Sequence 4 and after pelleting the positive batch, the pellet mill was allowed to cool, disassembled, and swabs were collected at the metal interior of the 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, and the two steps of chemical cleaning, and heat-up.

The environmental swabbing sampling areas were outlined in heat-stable marker when possible to form five subsampled areas. Subsampled areas within a location were equal in size; however, the areas ranged from approximately 2.3 in<sup>2</sup> to 6.5 in<sup>2</sup> 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 two of chemical cleaning and heat-up, the entire surface of all five areas were swabbed to establish and confirm baseline levels.

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 placed back inside its plastic transport container that contained 2 mL of phosphate buffered saline (PBS) (pH 7.4; 1X; Life Technologies Corp. Grand Island, NY) until analyzed by PEDV PCR. After analysis, PBS from swabs were stored at -112°F.

### **Results and Discussion**

Unexpectedly, after the negative control was manufactured, 22.2% (2 of 9) and 33.3% (1 of 3) rubber surfaces had detectable PEDV RNA, with all positive samples occurring during the second replicate (Table 2). 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, the 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. After the positive control was manufactured, all feed samples and equipment swabs had detectable PEDV RNA, 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 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, 66.7% of mixer swabs, and 100% of bucket elevator swabs had detectable PEDV RNA. After Sequence 3 and 4, none of the feed samples had detectable PEDV RNA, yet 44.4% of the mixer swabs and 100% of the bucket elevator swabs had detectable 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.

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 detectable 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 detectable 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 detectable 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).

In conclusion, the results of the present study indicate that the equipment surfaces contained detectable viral RNA for several sequences which could lead to cross-contamination, thus making a PEDV-contaminated feed mill a potential route of PEDV infection into farms. Most concerning, once a feed mill is contaminated with PEDV, genetic material in dust continued to be detected on most surfaces until chemically cleaned. 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, because feed has been demonstrated to be a vehicle of PEDV transmission. Additional research is needed to determine if contaminated dust is infective and to further define the best ways to improve the biosecurity in our commercial feed mills to minimize the risk of biological pathogen contamination.

**Table 1. Effect of sequencing batches of feed on Porcine Epidemic Diarrhea Virus (PEDV) contamination of feed and equipment**

Item	Treatment <sup>1</sup>					
	After Negative	After Positive	After sequence 1	After sequence 2	After sequence 3	After sequence 4
Equipment swabs, %						
Mixer <sup>2</sup>	- <sup>3</sup> (0/9) <sup>4</sup>	100.0 (9/9)	100.0 (9/9)	66.7 (6/9)	44.4 (4/9)	44.4 (4/9)
Bucket elevator <sup>5</sup>	- (0/24)	100.0 (24/24)	100.0 (24/24)	100.0 (24/24)	100.0 (24/24)	100.0 (24/24)
Equipment swabs, Ct <sup>6</sup>						
Mixer	-	29.2	33.9	34.9	35.4	34.8
Bucket elevator	-	30.8	31.8	32.9	32.5	32.1

<sup>1</sup> 500 ml of tissue culture containing  $4.5 \times 10^6$  TCID<sub>50</sub>/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 the previous batch was discharged from the mixer, through the bucket elevator, and exited the spout. None of the equipment was cleaned between treatments.

<sup>2</sup> Values represent the mean of 3 swabs from inside the mixer per treatment  $\times$  3 repetitions.

<sup>3</sup> No detectable PEDV RNA (Ct > 45).

<sup>4</sup> 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.

<sup>5</sup> Values represent the mean of 8 swabs from inside the bucket elevator per treatment  $\times$  3 repetitions.

<sup>6</sup> Mean cycle threshold (Ct) value of samples with detectable PEDV RNA below 45.



**Table 2. Effect of sequencing batches of feed on Porcine Epidemic Diarrhea Virus (PEDV) contamination of environmental surfaces as determined by quantitative real-time PCR**

Item	Baseline <sup>2</sup>	Treatment <sup>1</sup>							
		After negative	After positive	After sequence 1	After sequence 2	After sequence 3	After sequence 4	After chemical cleaning with ammonium glutaraldehyde blend <sup>3</sup>	After chemical cleaning with sodium hypochlorite <sup>4</sup>
Swab location, %									
Metal <sup>5</sup>	- <sup>6</sup> (0/8) <sup>7</sup>	- (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 <sup>8</sup>	- (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 <sup>9</sup>	- (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 <sup>10</sup>	- (0/20)	NC <sup>11</sup>	100.0 (15/15)	NC	NC	NC	NC	33.3 (5/15)	13.3 (2/15)
Swab location, Ct <sup>12</sup>									
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

<sup>1</sup> 500 mL of tissue culture containing  $4.5 \times 10^6$  TCID<sub>50</sub>/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, which then exited the end spout before locations were sampled.

<sup>2</sup> Baseline represents the initial set of swabs to ensure there was no detectable PEDV RNA before each repetition and one set of swabs after completion of the study.

<sup>3</sup> Diluted to 1:256 with potable water.

<sup>4</sup> Diluted to 5-10% with potable water.

<sup>5</sup> Metal includes a sample from the garage door and stainless steel table ledge per treatment  $\times$  3 repetitions.

<sup>6</sup> No detectable PEDV RNA (Ct > 45).

<sup>7</sup> 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.

<sup>8</sup> Concrete includes a sample from the drain, low traffic and high traffic floor area per treatment  $\times$  3 repetitions.

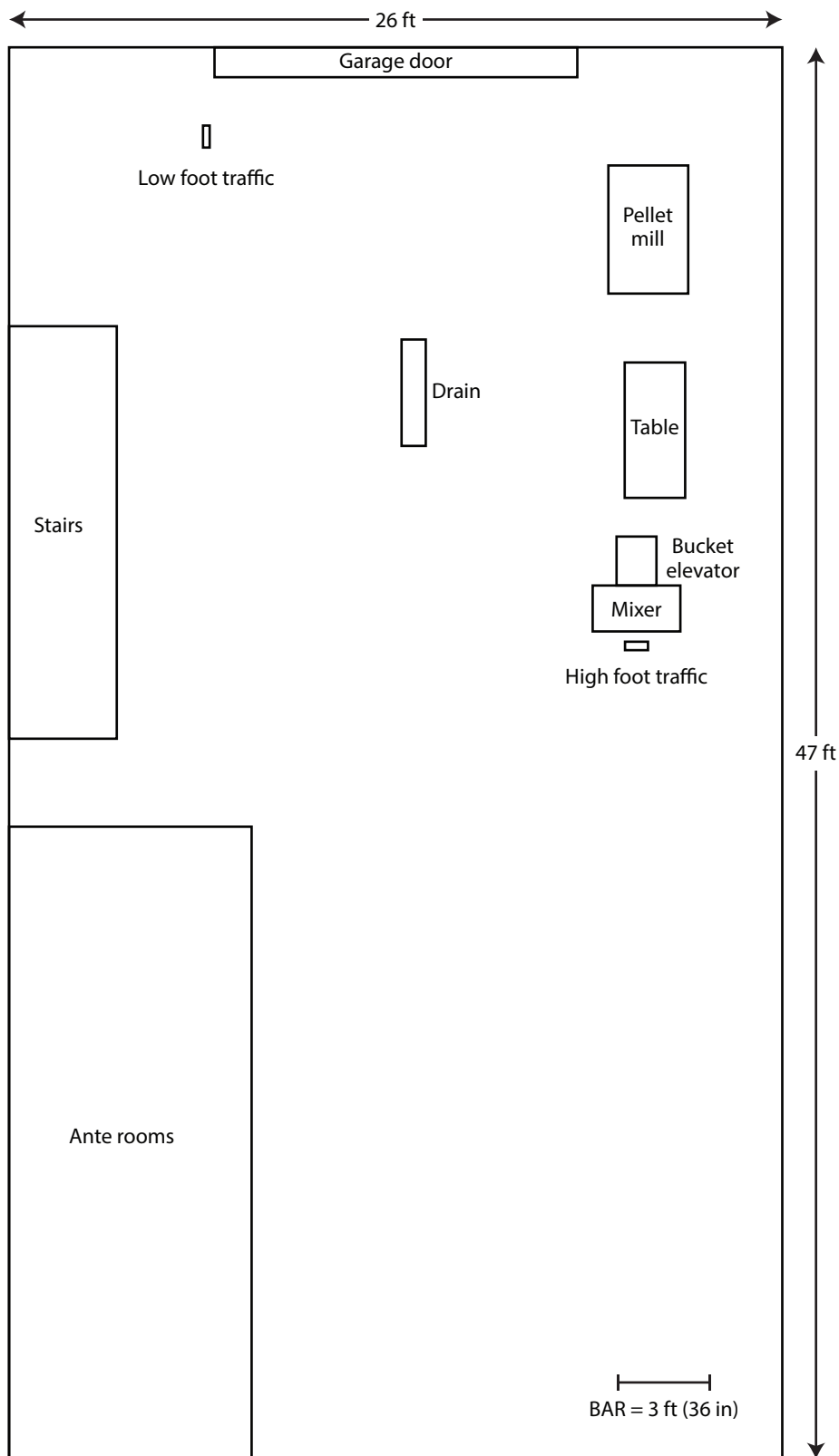
<sup>9</sup> Rubber includes a sample from the bottom of a pair of rubber boots per treatment  $\times$  3 repetitions.

<sup>10</sup> Only the positive treatment was processed. Five samples were taken from the pellet mill after processing the positive treatment  $\times$  3 repetitions.

<sup>11</sup> Sample not collected.

<sup>12</sup> Mean cycle threshold (Ct) value of samples with detectable 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 PCR analysis include high and low foot traffic areas, drain, garage door, pellet mill, table ledge, bucket elevator, and feed mixer as well as rubber boot bottoms.**