

# Effect of pelleting on survival of porcine epidemic diarrhea virus–contaminated feed<sup>1</sup>

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**ABSTRACT:** Porcine epidemic diarrhea virus (PEDV) is a heat-sensitive virus that has devastated the U.S. swine industry. Because of its heat sensitivity, we hypothesized that a steam conditioner and pellet mill mimicking traditional commercial thermal processing may mitigate PEDV infectivity. Pelleting, a common feed processing method, includes the use of steam and shear forces, resulting in increased temperature of the processed feed. Two thermal processing experiments were designed to determine if different pellet mill conditioner retention times and temperatures would impact PEDV quantity and infectivity by analysis of quantitative reverse transcription PCR and bioassay. In Exp. 1, a 3 × 3 × 2 factorial design was used with 3 pelleting temperatures (68.3, 79.4, and 90.6°C), 3 conditioning times (45, 90, or 180 s), and 2 doses of viral inoculation (low, 1 × 10<sup>2</sup> tissue culture infectious dose<sub>50</sub> (the concentration used to see cytopathic effect in 50% of the cells)/g, or high, 1 × 10<sup>4</sup> tissue culture infectious dose<sub>50</sub>/g). Noninoculated and PEDV-inoculated unprocessed mash were used as controls. The low-dose PEDV–infected mash had 6.8 ± 1.8 cycle threshold (Ct) greater ( $P < 0.05$ ) PEDV than the high-dose mash. Regardless of time or temperature, pelleting reduced ( $P < 0.05$ ) the quantity of detectable

viral PEDV RNA compared with the PEDV-inoculated unprocessed mash. Fecal swabs from pigs inoculated with the PEDV-positive unprocessed mash, regardless of dose, were clinically PEDV positive from 2 to 7 d (end of the trial) after inoculation. However, if either PEDV dose of inoculated feed was pelleted at any of the 9 tested conditioning time × temperature combinations, no PEDV RNA was detected in fecal swabs or cecum content. Based on Exp. 1 results, a second experiment was developed to determine the impact of lower processing temperatures on PEDV quantity and infectivity. In Exp. 2, PEDV-inoculated feed was pelleted at 1 of 5 conditioning temperatures (37.8, 46.1, 54.4, 62.8, and 71.1°C) for 30 s. The 5 increasing processing temperatures led to feed with respective mean Ct values of 32.5, 34.6, 37.0, 36.5, and 36.7, respectively. All samples had detectable PEDV RNA. However, infectivity was detected by bioassay only in pigs from the 37.8 and 46.1°C conditioning temperatures. Experiment 2 results suggest conditioning and pelleting temperatures above 54.4°C could be effective in reducing the quantity and infectivity of PEDV in swine feed. However, additional research is needed to prevent subsequent recontamination after pelleting as it is a point-in-time mitigation step.

**Key words:** feed, pelleting, porcine epidemic diarrhea virus, swine

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## INTRODUCTION

Recent research has confirmed that porcine epidemic diarrhea virus (PEDV) can be transmitted by swine feed and ingredients (Dee et al., 2014, 2015; Schumacher et al., 2015). In a review by Nitikanchana (2014), a theoretical temperature ×

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time relationship was proposed to reduce the infectivity of PEDV in complete feeds based on data extrapolated from PEDV environmental survival studies (Pospischil et al., 2002; Thomas et al., 2015). Typical swine feed pellet mill conditioner retention times and temperatures encompass the theoretical temperature  $\times$  time relationship proposed. Although Goyal (2013) corroborated this relationship using benchtop measures, there is no research confirming this time  $\times$  temperature relationship using a conditioner and pellet mill that are present in modern feed manufacturing facilities. Although it would be uncommon to set target pellet mill conditioning temperatures below 68°C, it is possible that feed may be produced below these limits during start up of the pellet mill or during a pellet mill plug. If feed begins to plug in the pellet mill die, the initial attempt to resolve the plug is to turn off steam to the conditioner. This can lead to significant quantities of pelleted feed not reaching the target conditioning temperature. If PEDV particles were in the feed, the resulting feed conditioned at a lower temperature may still have infectious PEDV particles that may potentially contaminate the pellet cooler, after-pellet feed handling equipment, trucks, feed lines, and feeders at the farm. Therefore, we hypothesize that PEDV dose and pelleting parameters will reduce PEDV quantity and infectivity.

## MATERIALS AND METHODS

All procedures involving pigs were approved by the Iowa State University Institutional Animal Care and Use Committee. A corn–soybean meal–based mash swine diet (Table 1) was manufactured at the Kansas State University O.H. Kruse Feed Technology Innovation Center in Manhattan, KS, and used in all experiments. A subsample of this feed was obtained prior to inoculation and confirmed negative by quantitative reverse transcription PCR (qRT-PCR) for the presence of PEDV RNA at the Kansas State University Research Park Molecular Diagnostics Development Laboratory in Manhattan, KS.

### Porcine Epidemic Diarrhea Virus Isolate

The mash swine feed was inoculated with the U.S. PEDV prototype strain cell culture isolate USA/IN/2013/19338, passage 8 (PEDV19338). Virus isolation, propagation, and titration were performed in Vero cells (ATCC CCL-81; American type culture collection, Manassas, VA) as described by Chen et al. (2014). The stock virus titer contained  $4.5 \times 10^6$  tissue culture infectious dose (the concentration used to see cytopathic effect in 50% of the cells; TCID<sub>50</sub>/mL) and was divided

**Table 1.** Diet composition used in Exp. 1 and 2

Item	Negative control
Ingredient, %	
Corn	79.30
Soybean meal, 46.5 CP	15.70
Choice white grease	1.00
Monocalcium phosphate	1.40
Limestone, ground	1.15
Salt	0.50
L-Threonine	0.03
Trace mineral premix <sup>1</sup>	0.15
Sow add pack <sup>2</sup>	0.50
Vitamin premix <sup>3</sup>	0.25
Phytase <sup>4</sup>	0.02
Total	100.00
Formulated analysis, %	
DM	91.4
CP	17.1
Crude fiber	3.7
Ether extract	3.5
Ca	0.78
P	0.52

<sup>1</sup>Each kilogram contains 26.4 g Mn, 110 g Fe, 110 g Zn, 11 g Cu, 198 mg I, and 198 mg Se.

<sup>2</sup>Each kilogram contains 220,000 mg choline, 88 mg biotin, 660 mg folic acid, and 1,980 mg pyridoxine.

<sup>3</sup>Each kilogram contains 4,400,000 IU vitamin A, 660,000 IU vitamin D<sub>3</sub>, 17,600 IU vitamin E, 1,760 mg menadione, 3,300 mg riboflavin, 11,000 mg pantothenic acid, 19,800 mg niacin, and 15.4 mg vitamin B<sub>12</sub>.

<sup>4</sup>High Phos 2700 GT (DSM Nutritional Products, Parsippany, NJ).

into 500-mL aliquots that were stored at  $-80^{\circ}\text{C}$  prior to use, with 1 aliquot used in each replication.

### Experiment 1

Experiment 1 evaluated the role of PEDV dose and varying time  $\times$  temperature combinations during pelleting on PEDV quantity and infectivity. Treatments were arranged in a  $3 \times 3 \times 2 + 1 + 2$  factorial arrangement with 3 pellet mill conditioning temperatures (68.3, 79.4, and 90.6°C), 3 conditioning times (45, 90, and 180 s), and 2 PEDV doses (low dose,  $1 \times 10^2$  TCID<sub>50</sub>/g and 20 cycle time [Ct], and high dose,  $1 \times 10^4$  TCID<sub>50</sub>/g and 13 Ct). Two types of controls included a single noninoculated meal–based negative control and 2 PEDV-inoculated meal–based positive controls that were not thermally processed (1 at each dose). The PEDV dosages were selected based on the known minimum infectious dose of PEDV in swine feed determined by Schumacher et al. (2015). Pellet mill conditioning temperatures and times were selected based on traditional industry parameters for pellet quality and extreme parameters for hygienic pelleting to mitigate other biological hazards, such as *Salmonella* spp. (Cochrane et al., 2015).

### ***Porcine Epidemic Diarrhea Virus Inoculum.***

After mixing the basal diet, negative control mash samples were collected. Next, a feed inoculum was created by mixing a 500-mL aliquot of the stock virus into a 4.5-kg batch of feed. The feed and virus were mixed using a benchtop laboratory scale stainless steel paddle mixer (Cabela's Inc., Sidney, NE) for a total of 2.5 min with 1 rotation of the paddles per second.

The above procedures were then repeated for the high-dose inoculation. Samples of each mash diet were aseptically collected as the positive low-dose and high-dose feed controls prior to pelleting.

***Thermal Processing.*** The low- and high-dose inoculated feeds were thermally processed using a pilot-scale single-pass conditioner and pellet mill (model CL5; CPM, Waterloo, IA). Prior to pelleting the first treatment, noninoculated feed was processed until the exit temperature of the feed was stable at the target temperature. To help reduce cross-contamination, the low-dose batches were pelleted prior to the high-dose batches. During thermal processing, within each dose, the treatment with the lowest temperature and longest retention time was pelleted first. The temperature was then held constant until the other 2 retention times were achieved, and samples were collected. This process was then repeated at the 2 higher temperatures. The temperature for each treatment was measured at the discharge from the conditioner to the pellet die feeder screw. Once the low-dose treatments were completed, the same procedure was used to pellet the high-dose treatments. For each temperature, time, and dose combinations, 3 pelleted samples were collected for qRT-PCR and bioassay analysis. In addition, between each inoculated batch, a minimum of 5 kg of virus-free feed was processed through the pellet mill. This was done to prevent virus carryover between treatments and to stabilize the conditioning temperature to ensure the contaminated feed was processed under uniform temperature conditions.

***Samples Preparation and Storage.*** Three 100-g samples of each batch of feed were added to 400 mL of cold PBS (Life Technologies, Grand Island, New York; pH 7.4) in 500-mL bottles (square Nalgene bottles; Thermo Scientific, Waltham, MA) and thoroughly mixed and stored at 4°C for approximately 12 h. A 2-mL aliquot of feed suspension was then evaluated using a PEDV N gene-based qRT-PCR at Kansas State University (Manhattan, KS). A 20-mL aliquot was also harvested and frozen at -80°C for use in the bioassay.

***Bioassay.*** The Iowa State University Institutional Animal Care and Use Committee reviewed and approved the pig bioassay protocol. A total of 63 pigs of mixed sex were sourced from a single commercial, crossbred farrow-to-wean herd with no prior exposure to PEDV. Additionally, all pigs were confirmed nega-

tive for PEDV, porcine delta coronavirus, and transmissible gastroenteritis virus based on fecal swab. To further confirm PEDV-negative status, collected blood serum was analyzed for PEDV antibodies by an indirect fluorescent antibody assay and for transmissible gastroenteritis virus antibodies by ELISA, both conducted at the Iowa State University Veterinary Diagnostic Laboratory (Ames, IA). Pigs were allowed 2 d of adjustment to the new pens before the bioassay began.

A total of 21 rooms (63 pigs, 3 per room) were assigned to treatment groups with 1 negative control room, 2 positive control rooms (low and high dose), and 18 rooms representing treatment diets (3 conditioning temperatures × 3 conditioning times × 2 PEDV dose levels). Each of the 3 pigs in each room received a separate feed sample of the experimental diet via oral gavage. For example, feed samples inoculated with the low dose of PEDV ( $1 \times 10^2$  TCID<sub>50</sub>/g and 20 Ct) and conditioned at 68.3°C for 45 s were fed to 1 room. Within that room, each pig was inoculated with a different feed sample from the experimental treatment. This resulted in 3 pigs per time × temperature × dose treatment.

During the bioassay, rectal swabs were collected on 0, 2, 4, 6, and 7 d after inoculation (dpi) from all pigs and tested for PEDV RNA qRT-PCR. Following humane euthanasia at 7 dpi, small intestine, cecum, and colon samples were collected at necropsy along with an aliquot of cecal contents. One section of formalin-fixed proximal, middle, and distal jejunum and ileum was collected per pig for histopathology and immunohistochemistry (IHC; Chen et al., 2014).

## ***Experiment 2***

Based on the results of Exp. 1, a second study was designed to evaluate the effect of increasing pellet mill temperatures on PEDV inactivation. The experiment was designed to mimic a real-life situation that could occur in the feed mill, which is a pellet mill plug. In this situation, the feed would become lodged in the pellet mill die and the steam would be turned off to free the material within the die. This situation leads to feed not reaching the target pellet temperature. Therefore, the purpose of Exp. 2 was to evaluate 5 increasing conditioner temperatures ranging from 37 to 71°C on PEDV inactivation if a plug were to occur within the pellet mill.

***Porcine Epidemic Diarrhea Virus Inoculum.*** Experiment 2 was performed in the Cargill Feed Safety Research Center at Kansas State University (Manhattan, KS). To accomplish the objective for Exp. 2, PEDV-free feed was first collected, used as the PEDV-negative control, and used to make the PEDV inoculum. Only the high-dose PEDV stock virus ( $1 \times 10^4$  TCID<sub>50</sub>/g and 16 Ct) was used for Exp. 2. An inoculum premix was

created by mixing a 500-mL aliquot of the stock virus into a 4.5-kg batch of feed of swine feed using procedures established in Exp. 1. The feed and inoculum were mixed using a benchtop laboratory scale stainless steel paddle mixer (Cabela's Inc.) for a total of 2.5 min with 1 rotation of the paddles per second.

The PEDV feed inoculum (4.5 kg of feed + 500 mL of stock virus) was then added to 45 kg of PEDV-free swine diet to form the positive control in a 0.11 m<sup>3</sup> electric paddle mixer (model number SS-L1; H.C. Davis Sons Manufacturing Co., Inc., Bonner Springs, KS). The entire batch was then mixed for 5 min, creating the PEDV-positive control, discharged for 10 min into biohazard containers, and finally held at -2°C for approximately 1 h, until thermal processing.

**Thermal Processing.** Porcine epidemic diarrhea virus-free feed was pelleted using the same pilot-scale single-pass conditioner and pellet mill (model CL5; CPM) used in Exp. 1. To mimic a plug within the pelleting system, the pellet mill was heated by pelleting PEDV-free feed with a conditioning temperature of 71°C, which would represent normal production temperature. The pellet mill was heated to 71°C for 60 min to have the entire pellet mill reach optimum temperature. The temperature was again measured by a thermometer at the discharge from the conditioner to the pellet die feeder screw. At 60 min, the steam valve was then turned off until the conditioning temperature dropped below 37°C to mimic procedures commonly used to resolve a plug in the conditioner or pellet die. Next, PEDV-inoculated feed was placed into the pellet mill hopper. Once PEDV-inoculated feed started passing through the pellet mill, steam was slowly added, and 5 pelleted samples were collected at targeted hot mash conditioner temperatures of 37, 46, 54, 62, and 71°C ( $\pm 1.2^\circ\text{C}$ ) using a 30-s conditioning time. These conditioning temperatures were selected based on a previously determined prediction equation for the specific pellet mill.

Experiment 2 was performed 3 separate times (days) within the feed safety research center with complete decontamination of the facility between each run to create 3 true replications.

**Samples Preparation and Storage.** Three 100-g samples of each batch of feed were added to 400 mL of cold PBS (Life Technologies; pH, 7.4) in 500-mL bottles (square Nalgene bottles; Thermo Scientific) and thoroughly mixed and stored at 4°C for approximately 12 h. A 2-mL aliquot of feed suspension was then evaluated using a PEDV N gene-based qRT-PCR at Kansas State University. A 20-mL aliquot was also harvested and frozen at -80°C for use in the bioassay.

**Bioassay.** A total of 48 pigs of mixed sex were sourced from a single commercial, crossbred farrow-to-wean herd with no prior exposure to PEDV.

Procedures and evaluation for the bioassay were performed as described in the Exp. 1 bioassay.

A total of 16 rooms (48 pigs, 3 per room) were assigned to treatment groups with 1 negative control room and 15 rooms representing treatment diets (5 conditioning temperatures  $\times$  3 replicates/temperature). Each of the 3 pigs in each room received a separate feed sample representing 1 of 3 processing days of the experimental diet via oral gavage. For example, feed samples conditioned at 37°C were fed to 3 different rooms. Within each room, Pig 1 received the sample manufactured on Thermal Processing Day 1, Pig 2 received the sample manufactured on Thermal Processing Day 2, and Pig 3 received the sample manufactured on Processing Day 3. This resulted in 9 pigs per conditioning temperature.

Each pig from the negative control room was given a 10-mL aliquot of inoculum created from the 0.11 m<sup>3</sup> electric paddle mixer. Different from the negative control room, each pig in each challenge room was given an aliquot of inoculum from the replicate, temperature and processing day, resulting in 3 samples of the same temperature treatment and different processing days. One room was representative of a treatment with 3 rooms per treatment. Rectal swabs and intestinal sections were evaluated in the same fashion as the Exp. 1 bioassay.

### Statistical Analysis

Statistical analysis was performed using SAS version 9.4 (SAS Inst. Inc., Cary, NC). In Exp. 1, PROC MIXED of SAS was used to evaluate PEDV RNA feed Ct values, villus height, crypt depth, villous height-to-crypt depth ratio, and IHC. Fixed effects included temperature, time, dose, and their combination. In Exp. 2, data of the effects of conditioner temperature on feed Ct values, villus height, crypt depth, and villus height-to-crypt depth ratio were analyzed as a completely randomized design using PROC GLIMMIX in SAS with pig as the experimental unit by a pairwise comparison. Treatment was the fixed effect. Results for treatment criteria were considered significant at  $P \leq 0.05$  and marginally significant from  $P > 0.05$  to  $P \leq 0.10$ .

## RESULTS

### Experiment 1

There was no PEDV RNA detected in the unprocessed PEDV-free feed. When the low-dose PEDV (Ct 20) was mixed with the feed, the resulting feed Ct value was 31, and when the high PEDV dose media (Ct 13) was mixed with feed, the resulting Ct value was 24 (Table 2). The low dose processed treatments

**Table 2.** Effects of porcine epidemic diarrhea virus (PEDV) dose, pelleting temperature, and conditioning retention time on PEDV detection from feed, pig fecal swabs, and cecum contents, Exp. 1<sup>1</sup>

PEDV dose, temperature, <sup>2</sup> and time <sup>3</sup>	PEDV N gene quantitative reverse transcription PCR, cycle threshold (Ct) <sup>4</sup>							
	Tissue culture	Fecal swabs					Cecum contents	
		Feed	0 dpi <sup>5</sup>	2 dpi	4 dpi	6 dpi	7 dpi	7 dpi
Unprocessed virus-free feed <sup>6</sup>		45.0	–	–	–	–	–	–
Low-dose inoculated feed <sup>7</sup>	20.0	30.7 <sup>e</sup>	–	22.4	18.2	18.8	24.1	26.7
68.3°C 45 s		42.6 <sup>ab</sup>	–	–	–	–	–	–
90 s		39.5 <sup>bcd</sup>	–	–	–	–	–	–
180 s		45.0 <sup>a</sup>	–	–	–	–	–	–
79.4°C 45 s		36.7 <sup>cd</sup>	–	–	–	–	–	–
90 s		39.7 <sup>bc</sup>	–	–	–	–	–	–
180 s		42.3 <sup>ab</sup>	–	–	–	–	–	–
90.6°C 45 s		39.7 <sup>bc</sup>	–	–	–	–	–	–
90 s		37.4 <sup>cd</sup>	–	–	–	–	–	–
180 s		35.9 <sup>d</sup>	–	–	–	–	–	–
High-dose inoculated feed <sup>8</sup>	13.0	23.9 <sup>f</sup>	–	23.0	15.3	20.4	24.3	24.0
68.3°C 45 s		30.2 <sup>e</sup>	–	–	–	–	–	–
90 s		29.7 <sup>e</sup>	–	–	–	–	–	–
180 s		30.2 <sup>e</sup>	–	–	–	–	–	–
79.4°C 45 s		30.1 <sup>e</sup>	–	–	–	–	–	–
90 s		29.5 <sup>e</sup>	–	–	–	–	–	–
180 s		30.2 <sup>e</sup>	–	–	–	–	–	–
90.6°C 45 s		30.1 <sup>e</sup>	–	–	–	–	–	–
90 s		30.6 <sup>e</sup>	–	–	–	–	–	–
180 s		30.0 <sup>e</sup>	–	–	–	–	–	–
SEM		1.27						

<sup>a–f</sup>Means within a column with different superscripts differ ( $P < 0.05$ ).

<sup>1</sup>An initial tissue culture containing a low dose and high dose of PEDV was used to inoculate batches of feed. Three feed samples per batch were collected and diluted in PBS. The supernatant from each sample was then collected for pig bioassay. The supernatant was administered 1 time via oral gavage on d 0 to each of 3 pigs per treatment (10 mL per pig). Therefore, each value represents the mean of 3 pigs per treatment. Pigs were initially 10 d old and 3.6 kg BW.

<sup>2</sup>Temperature of feed exiting the conditioner.

<sup>3</sup>Retention time: the amount of time required for feed to pass through the conditioner.

<sup>4</sup>In each instance a (–) signals a negative pig in the bioassay and a (+) represents a positive fecal swab in the bioassay. Each day after inoculation within each treatment has 3 symbols with each row and column, which represents 1 of the 3 pigs in each treatment.

<sup>5</sup>dpi = days after inoculation.

<sup>6</sup>A Ct of >45 was considered negative for the presence of PEDV RNA.

<sup>7</sup>For low-dose feed, PEDV ( $1 \times 10^3$  tissue culture infectious dose [TCID]<sub>50</sub>(the concentration used to see cytopathic effect in 50% of the cells)/mL) was diluted into feed to provide a dose of  $1 \times 10^2$  TCID<sub>50</sub>/g of feed.

<sup>8</sup>For high-dose feed, PEDV ( $1 \times 10^5$  TCID<sub>50</sub>/mL) was diluted into feed to provide a dose of  $1 \times 10^4$  TCID<sub>50</sub>/g of feed.

ranged from 36 to 45 Ct compared with the high-dose processed treatments of either 30 or 31 Ct (Table 2).

As expected, fecal shedding of PEDV was not detected in rectal swabs from negative control pigs for the duration of the study (Table 2). Fecal swabs from pigs fed the low- and high-PEDV dose positive control treatment (inoculated but nonprocessed feed) were PEDV positive from 2 dpi through the end of the study at 7 dpi. Cecum contents at 7 dpi and IHC determined at 7 dpi were also positive for the positive control pigs (Tables 2 and 3). However, if either the low- or high-dose PEDV feed was processed at any of the 9 possible conditioning time  $\times$  temperature combinations, no PEDV RNA was detected in fecal swabs or cecum contents at 7 dpi.

The villous height for pigs challenged with the noninoculated feed was higher ( $P < 0.05$ ) compared with the villous height in pigs challenged with the high-dose PEDV unprocessed feed (Table 3). Porcine epidemic diarrhea virus IHC immunoreactivity was not visible in the cytoplasm of villous enterocytes of low- or high-dose challenged pigs from any of the time and temperature pellet treatment combination for the duration of the study. However, immunoreactivity was detected within the low- and high-PEDV dose positive control treatment (inoculated but nonprocessed feed; Table 3).

**Table 3.** Effects of porcine epidemic diarrhea virus (PEDV) dose, pelleting temperature, and conditioning retention time on morphologic and immunohistochemistry (IHC) evaluation of the small intestine from pigs, Exp. 1<sup>1</sup>

Item	Morphology <sup>2</sup>			IHC <sup>3</sup>	
	Villus height, $\mu\text{m}$	Crypt depth, $\mu\text{m}$	Villus height-to-crypt depth ratio		
Unprocessed virus-free feed	463.4 <sup>abcd</sup>	112.5	4.2 <sup>abcde</sup>	0 <sup>b</sup>	
Low-dose inoculated feed <sup>4</sup>	414.3 <sup>de</sup>	91.0	4.6 <sup>abcd</sup>	0.3 <sup>b</sup>	
68.3°C <sup>5</sup> 45 s <sup>6</sup>	481.6 <sup>abcd</sup>	101.3	4.8 <sup>abc</sup>	0 <sup>b</sup>	
90 s	489.3 <sup>abcd</sup>	108.1	4.6 <sup>abcd</sup>	0 <sup>b</sup>	
180 s	504.4 <sup>abc</sup>	115.6	4.4 <sup>abcd</sup>	0 <sup>b</sup>	
79.4°C	45 s	508.6 <sup>ab</sup>	108.9	4.7 <sup>abcd</sup>	0 <sup>b</sup>
90 s	476.4 <sup>abcde</sup>	103.6	4.6 <sup>abcd</sup>	0 <sup>b</sup>	
180 s	460.6 <sup>abcde</sup>	93.1	4.9 <sup>ab</sup>	0 <sup>b</sup>	
90.6°C	45 s	443.2 <sup>abcde</sup>	97.8	4.6 <sup>abcd</sup>	0 <sup>b</sup>
90 s	514.5 <sup>a</sup>	103.3	5.0 <sup>a</sup>	0 <sup>b</sup>	
180 s	441.8 <sup>abcde</sup>	103.6	4.3 <sup>abcde</sup>	0 <sup>b</sup>	
High-dose inoculated feed <sup>7</sup>	309.3 <sup>f</sup>	112.6	3.1 <sup>f</sup>	1.7 <sup>a</sup>	
68.3°C	45 s	423.1 <sup>cde</sup>	105.3	4.0 <sup>abcdef</sup>	0 <sup>b</sup>
90 s	429.4 <sup>bcde</sup>	118.3	3.7 <sup>def</sup>	0 <sup>b</sup>	
180 s	389.2 <sup>ef</sup>	100.0	4.0 <sup>bcdef</sup>	0 <sup>b</sup>	
79.4°C	45 s	432.8 <sup>abcde</sup>	117.3	3.7 <sup>cdef</sup>	0 <sup>b</sup>
90 s	390.6 <sup>ef</sup>	102.5	3.7 <sup>cdef</sup>	0 <sup>b</sup>	
180 s	448.7 <sup>abcde</sup>	104.5	4.3 <sup>abcd</sup>	0 <sup>b</sup>	
90.6°C	45 s	383.1 <sup>ef</sup>	119.5	3.2 <sup>ef</sup>	0 <sup>b</sup>
90 s	446.4 <sup>abcde</sup>	102.8	4.4 <sup>abcd</sup>	0 <sup>b</sup>	
180 s	408.9 <sup>de</sup>	105.7	3.9 <sup>bcdef</sup>	0 <sup>b</sup>	
SEM	29	10.2	0.4	0.2	

<sup>a-f</sup>Means within a column with different superscripts differ ( $P < 0.05$ ).

<sup>1</sup>An initial tissue culture containing a low dose or high dose of PEDV was used to inoculate batches of feed. Three feed samples per batch were collected and diluted in PBS. The supernatant from each sample was then collected for pig bioassay. The supernatant was administered 1 time via oral gavage on d 0 to each of 3 pigs per treatment (10 mL per pig). Therefore, each value represents the mean of 3 pigs per treatment necropsied at 7 d after infection. Pigs were initially 10 d old and 73.6 kg BW.

<sup>2</sup>Intestinal cross-sections were fixed in formalin and stained with hematoxylin and eosin for evaluation.

<sup>3</sup>Three sections of ileum were evaluated and averaged into 1 categorical value per pig. Categorical values were assigned for each pig (0 = no signal, 1 = mild, 2 = moderate, 3 = abundant, and 4 = diffuse) and reported as the mean from 3 pigs per treatment and, thus, the mean of 9 values.

<sup>4</sup>For low-dose feed, PEDV ( $1 \times 10^3$  tissue culture infectious dose [TCID]<sub>50</sub> (the concentration used to see cytopathic effect in 50% of the cells)/mL) was diluted into feed to provide a dose of  $1 \times 10^2$  TCID<sub>50</sub>/g of feed.

<sup>5</sup>Temperature of feed exiting the conditioner.

<sup>6</sup>Retention time: the time feed was inside the conditioner.

<sup>7</sup>For high-dose feed, PEDV ( $1 \times 10^5$  TCID<sub>50</sub>/mL) was diluted into feed to provide a dose of  $1 \times 10^4$  TCID<sub>50</sub>/g of feed.

## Experiment 2

When PEDV-inoculated feed was processed at 5 different conditioning temperatures (37, 46, 54, 62, and 71°C), the respective mean cycle threshold (Ct) values as detected by qRT-PCR were 32.5, 34.6, 37.0, 36.5, and 36.7, respectively (Table 4). All 9 of the feed samples conditioned at 37, 46, or 54°C had detectable PEDV RNA, whereas 8 of the 9 feed samples processed at 62 and 71°C had detectable PEDV RNA. It was observed that Ct value increased as conditioning temperature increased to 54°C, with little change thereafter.

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 4). Of the 9 total pigs gavaged with aliquots from the PEDV-positive diet conditioned at 37°C,

a fecal swab from 1 pig (Room 7, Replicate 2) yielded detectable PEDV RNA at 2 dpi, and all 3 pigs in Room 7 had fecal swabs and cecum contents with detectable viral particles by 4 through 7 dpi. In addition, 3 pigs gavaged with aliquots from the treatment conditioned at 46°C had detectable 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 detectable 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 temperature treatment (Table 5). Furthermore, pigs challenged with feed conditioned at 46 or 71°C had different ( $P < 0.05$ ) crypt depths when compared with one another but were statis-

**Table 4.** Influence of processed porcine epidemic diarrhea virus (PEDV)–inoculated feed on quantitative reverse transcription PCR cycle threshold (Ct) of feed, fecal swabs, and cecum contents of pigs, Exp. 2<sup>1</sup>

Item	KSU <sup>2</sup> feed inoculum,	Fecal swabs, Ct					
	Ct	0 dpi <sup>3</sup>	2 dpi	4 dpi	6 dpi	7 dpi	7 dpi cecum content, Ct
Processed feed, % positive <sup>4</sup>							
Negative	– <sup>5</sup>	–	–	–	–	–	–
37.8°C	100.0 (9/9)	–	(1/9)	(3/9)	(3/9)	(3/9)	(3/9)
46.1°C	100.0 (9/9)	–	(3/9)	(3/9)	(3/9)	(3/9)	(3/9)
54.4°C	100.0 (9/9)	–	–	–	–	–	–
62.8°C	88.9 (8/9)	–	–	–	–	–	–
71.1°C	88.9 (8/9)	–	–	–	–	–	–
Processed feed, Ct values <sup>6</sup>							
Negative	–	–	–	–	–	–	–
37.8°C	32.5 <sup>a</sup>	–	15.8	27.5	16.6	17.8	16.9
46.1°C	34.6 <sup>b</sup>	–	24.5	15.2	15.4	17.9	18.8
54.4°C	37.0 <sup>c</sup>	–	–	–	–	–	–
62.8°C	36.5 <sup>c</sup>	–	–	–	–	–	–
71.1°C	36.7 <sup>c</sup>	–	–	–	–	–	–

<sup>a-c</sup>Means within a column with different superscripts differ ( $P < 0.05$ ).

<sup>1</sup>500 mL of tissue culture containing  $4.5 \times 10^6$  tissue culture infectious dose [TCID]<sub>50</sub> (the concentration used to see cytopathic effect in 50% of the cells)/mL of PEDV was inoculated into a 4.5 kg batch of feed and then added to 45 kg of PEDV-negative feed to form the positive treatment. One feed sample per temperature per replicate  $\times$  3 replicates was collected, divided into 3 aliquots, and diluted in PBS to form supernatants. Therefore, each feed supernatant value per treatment represents the mean of 3 replicates  $\times$  3 repetitions.

<sup>2</sup>KSU = Kansas State University (Manhattan, KS).

<sup>3</sup>dpi = days after inoculation.

<sup>4</sup>Means represent the percent of samples that had detectible RNA by PEDV quantitative reverse transcription PCR analysis ( $<45$  Ct).

<sup>5</sup>No detectible PEDV RNA (Ct  $>45$ ).

<sup>6</sup>Mean Ct value of samples with detectible PEDV RNA below 45.

tically similar to all other treatments (Table 5). This led to pigs challenged with feed conditioned at 37°C having the lowest villus height:crypt depth ratio followed by increasing ratios as temperature increased (Table 5). Porcine epidemic diarrhea virus IHC immunoreactivity was visible in the cytoplasm of villous enterocytes of pigs challenged with the 2 lowest processed temperatures, 37 and 46°C, when harvested at 7 dpi.

## DISCUSSION

Temperature and time studies have been performed on PEDV in feces, feed, and ingredients. However, some of those temperatures are not applicable to the feed and ingredient industries. Fecal material has been tested at 71°C for 10 min, 63°C for 10 min, 54°C for 10 min, 38°C for 12 h, 20°C for 24 h, and 20°C for 7 d on metal surfaces (Thomas et al., 2015). From these time  $\times$  temperature combinations, only the 71°C for 10 min and 20°C for 7 d led to bioassay-negative pigs (Thomas

**Table 5.** Morphologic and immunohistochemistry (IHC) evaluation of the small intestine from pigs that were challenged with porcine epidemic diarrhea virus (PEDV)–inoculated feed processed at increasing temperatures, Exp. 2<sup>1</sup>

Item	Morphology <sup>2</sup>			IHC <sup>3</sup>
	Villus height, $\mu$ m	Crypt depth, $\mu$ m	Villus height-to-crypt depth ratio	
Processed feed				
Negative	365.1 $\pm$ 34.9 <sup>ab</sup>	190.7 $\pm$ 17.3 <sup>ab</sup>	1.9 $\pm$ 0.2 <sup>abc</sup>	0
37.8°C	299.9 $\pm$ 20.1 <sup>b</sup>	192.4 $\pm$ 10.0 <sup>ab</sup>	1.6 $\pm$ 0.1 <sup>ac</sup>	0.7
46.1°C	394.2 $\pm$ 20.1 <sup>a</sup>	230. $\pm$ 10.0 <sup>a</sup>	1.7 $\pm$ 0.1 <sup>bc</sup>	0.6
54.4°C	393.9 $\pm$ 20.1 <sup>a</sup>	200.0 $\pm$ 10.0 <sup>ab</sup>	2.0 $\pm$ 0.1 <sup>ab</sup>	0
62.8°C	405.1 $\pm$ 20.1 <sup>a</sup>	206.8 $\pm$ 10.0 <sup>ab</sup>	2.0 $\pm$ 0.1 <sup>ab</sup>	0
71.1°C	420.9 $\pm$ 20.1 <sup>a</sup>	184.1 $\pm$ 10.0 <sup>b</sup>	2.3 $\pm$ 0.1 <sup>a</sup>	0

<sup>a-c</sup>Means within a column with different superscripts differ ( $P < 0.05$ ).

<sup>1</sup>500 mL of tissue culture containing  $4.5 \times 10^6$  tissue culture infectious dose [TCID]<sub>50</sub> (the concentration used to see cytopathic effect in 50% of the cells)/mL of PEDV was inoculated into a 4.5 kg batch of feed and then added to 45 kg of PEDV-negative feed to form the positive treatment. One feed sample per temperature per replicate  $\times$  3 replicates was collected, divided into 3 aliquots, and diluted in PBS to form supernatants. Therefore, each feed supernatant value per treatment represents the mean of 3 replicates  $\times$  3 repetitions.

<sup>2</sup>Intestinal cross-sections were fixed in formalin and stained with hematoxylin and eosin for evaluation.

<sup>3</sup>Three sections of ileum were evaluated and averaged into 1 categorical value per pig. Categorical values were assigned for each pig (0 = no signal, 1 = mild, 2 = moderate, 3 = abundant, and 4 = diffuse) and reported as the mean from 3 pigs per treatment and, thus, the mean of 9 values.

et al., 2015). However, all of the other treatments led to infection in pigs (Thomas et al., 2015). When comparing the temperatures used to those in the present study, the first issue that arises is that the 54°C treatments differed in the bioassay results. In this study, all pigs were bioassay negative when feed was treated at 54°C. This could be due to the nature of the pellet mill as steam is directly added to the feed and then the feed is forced through a pellet die. As the feed is forced through the die, it could also undergo frictional heat from being pushed through the small diameter holes. However, in both trials in which 71°C was reached, it led to a PEDV-negative bioassay regardless of the treatment time.

Porcine epidemic diarrhea virus has also been shown to survive up to 7 d in fresh feces at 30, 50, and 70% relative humidity in combination with 40, 50, and 60°C and at room temperature for 14 d when placed into a slurry (Goyal, 2013). The major difference between the data from the fecal material and the present feed study is that the use of a pellet mill inactivates PEDV at a much lower temperature (54.4°C) compared with an incubator (71°C).

Ingredients and complete feed have been evaluated at 60, 70, 80, or 90°C for 0, 5, 10, 15, or 30 min (Trudeau et al., 2015). In each instance, there was no difference between the ingredients that were heated for

30 min (Trudeau et al., 2015). There was also no detectable virus after 30 min of heating at 90°C (Trudeau et al., 2015). However, these samples were placed in Vero-81 cells and not directly into pigs. In a separate study, time, temperature, and relative humidity were evaluated. In that study, 99.99% of PEDV was inactivated by heating at 90°C and 70% relative humidity for 10 min (Goyal, 2014). A temperature of 90°C in both studies and in the current study all resulted in a negative result.

More extreme temperatures have also been evaluated in which 145°C inactivated PEDV after 10 min (Trudeau et al., 2016). When comparing the previous studies to the present study, the use of a pellet mill inactivated PEDV at a faster rate (30 s) and much lower temperature (54.4°C). This could be due to the heat source and the frictional heat that occurs as the pellet exits the pellet die.

Another commercial process that is used for some ingredients in the feed industry, including plasma, is the use of spray drying. This process uses high temperatures throughout the manufacturing process, which makes it an option to test against PEDV. In a study by Gerber et al. (2014), inlet temperatures of 166°C and outlet temperature of 80°C were used and these conditions led to the inactivation of infectious PEDV (Goyal, 2014). In a separate study, bovine plasma was subjected to the spray drying process in which the inlet temperature was 200°C and either 70 or 80°C throughout the outlet (Pujols and Segalés, 2014). The bovine plasma subjected to spray drying was not infectious after the process (Pujols and Segalés, 2014). However, after the spray drying process, the plasma was reinoculated with PEDV and subjected to storage times of 7, 14, or 21 d and temperatures of 4, 12, or 22°C (Pujols and Segalés, 2014). The virus was not infectious at any of the time points at 22°C but was infectious in 1 out of 5 samples in 12°C at 7 d and 4 out of 5 samples in 4°C at 7 d (Pujols and Segalés, 2014). The initial high temperatures, which were greater than those used in this study, led to the inactivation of the virus; however, the low storage temperatures of 4 and 12°C did not inactivate the virus. This is extremely important to note because manufacturing equipment requires start up time and in some instances, as shown in the present study, the heat source is turned off to clear lodged material. This, in turn, could potentially lead to contamination of material later in processing and transportation. From this study, it was deemed that a temperature of 54.4°C or higher is required inactivate PEDV in complete feed when using a pellet mill and placing the feed into a swine bioassay. It is important to point out a few limitations of the 2 studies. The first is that the studies were performed on a pilot-size pellet mill. Every pellet mill will operate differently depending on the size, steam addition, time, and material

flowing through the process. The second issue is the heating and cooling time of pellets. For this study, pellets were immediately placed on ice to cool them. If a pellet mill cooler would have been used, the cooling time would have been longer, in which case the pellets would have maintained their internal heat for a longer time period. The third limitation is the use of a bioassay. Currently, a bioassay is the best diagnostic method to measure infectivity. However, as explained by Davies (2015), the risk of an individual pig becoming infected is very low but carries a high collective risk. In the bioassay, pigs received only 10 mL of a sample compared with their normal feed consumptions. If larger amounts of the pelleted feed were given to the pigs, then the likelihood of infection would have increased in the study. However, research generated using the same feed and pig bioassay model has led to infection within the animals (Schumacher et al., 2015; Cochrane et al., 2016). Because of the issues associated with this study, further research needs to be performed on a larger scale looking at the use of different pellet mills and pellet cooling times.

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