RESEARCH REPORT

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SWINE HEALTH

Title: Determining the impact of conditioning time and temperature in pelleted diets on porcine epidemic diarrhea virus (PEDv) survivability in complete swine diets – **NPB #14-159**

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INDUSTRY SUMMARY

Porcine Epidemic Diarrhea Virus (PEDv) has profoundly affected the U.S. swine industry since it emerged May 2013. Since late January 2014, it has been suspected that PEDv outbreaks have been associated with consumption of PEDv positive feed or feed ingredients. However, information is lacking which confirms the ability of feed to be a vector in PEDv transmission and no data is available which describes the minimum infectious dose of PEDv in a feed matrix. Additionally, it is believed that the normal temperature and retention times utilized by commercial pellet mills will be adequate to mitigate PEDv infectivity; however, no research has been conducted to test this hypothesis. Therefore, the purpose of this project was: 1) determine the minimum infectious dose of PEDv in a feed matrix and 2) determine if the retention time and temperatures used in commercial pellet mills will influence PEDv infectivity. Our results confirmed that feed can be a vehicle for PEDv transmission and that the minimum infectious dose of PEDv in a feed matrix is quite low. A PEDv dose that corresponded to a PCR Ct value of 37 was low enough to lead to infectivity. In layman's terms, this is theoretically equivalent to 1 g of infected pig feces being diluted in approximately 500 tons of feed. Our results also showed that the pelleting process utilized in many commercial mills can act as a point-in-time mitigation step in PEDv-associated risk prevention plans because none of the virus-inoculated and processed feed lead to infectivity in the pig bioassay model, even though the PCR analysis indicated that PEDv RNA was present in the processed feed. In contrast, the non-processed feed did lead to PEDv infectivity.

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SCIENTIFIC ABSTRACT

Feed has been confirmed as a potential vehicle for porcine epidemic diarrhea virus (PEDv) transfer (Dee et al., 2014a). In order to determine the overall magnitude of risk, a two-part study was conducted to identify the minimum infectious dose of PEDv in feed and evaluate the effectiveness of thermal processing in mitigating PEDv infectivity. The first part of the project involved determining the minimum dose of PEDv required to produce infection using a 10-d old pig bioassay model. The PEDv isolate (USA/IN/2013/19338 P7) was 10-fold serially diluted to produce 9 different PEDv doses with corresponding PCR cycle thresholds (Ct) of 14.0 to >45. Aliquots (500 ml) of the dilutions were uniformly mixed into 4.5 kg batches of complete swine diet using a laboratory scale mixer to ensure uniform viral distribution. The inoculated feed was then mixed with PBS overnight before extraction of the supernatant that was subsequently used for a 10-d old pig bioassay. The second portion of the project was designed to determine the effectiveness of thermal processing similar to that used in commercial pellet mills as a mitigation step in controlling PEDv transfer. This study had treatments arranged in a 2x3x3 factorial with two PEDv dosages (low and high), three condition times (45, 90, and 180s) and three temperatures (68.3, 79.4, 90.6°C). Based on the results of the previous bioassay from the minimum infectious dose study, an infectious titer of 1 x 10^2 TCID50/g (low dose) and infectious titer of 1 x10^4 TCID50/g (high dose) of feed was utilized from the same PEDv isolate to conduct the pelleting study. For each concentration, aliquots (500 ml) of the PEDv dilutions were mixed into 4.5 kg batches of complete swine diet. Each batch of inoculated feed was processed at 1 of the 9 potential temperature x time combinations using a pilot-scale single pass conditioner and pellet mill. The inoculated processed feed (100 g) was then mixed with 400 ml of PBS to form a supernatant, which was subsequently used for the bioassay. There was a loss of approximately 10 Ct values when PEDv cultured media was added to unprocessed feed in both studies. In the minimum infectious dose portion of the study, pigs showed clinical signs of disease when the four most concentrated doses of PEDv were added to feed; the least of which was at a concentration of 5.6X10^1 TCID50/g (Ct=27 in tissue culture media and Ct=37 in feed); thus establishing the minimum infectious dose of PEDv in a feed matrix. Additionally, this study confirmed a detectable PEDv Ct in feed can result in pig infectivity and the Ct related to the infective minimum dose can be above the positive/negative threshold used by some laboratories. Processing infected feed will raise the Ct value regardless of condition time or temperature suggesting there was less PEDv RNA detected after thermal processing. None of the low dose or high dose pigs that were challenged with thermally-processed, PEDv-inoculated feed resulted in evidence of PEDv infection; therefore thermal processing of feed appears to be an effective step to reduce or eliminate infectivity of PEDv contaminated feed.

INTRODUCTION

Within the past year, several porcine epidemic diarrhea virus (PEDv) outbreaks in the U.S. and Canada were suspected to be associated with consumption of PEDv PCR-positive feed or feed ingredients (Pasick et al., 2014). Since then, feed has been confirmed as a potential vehicle for PEDv transmission (Dee et al., 2014a) and has prompted investigations into reducing infectivity in contaminated diets or feed ingredients (Dee et al., 2014b; Lee et al., 2014). In a review by Nitikanchana (2014), a theoretical temperature and time relationship was proposed to reduce the infectivity of PEDv in complete feeds based on data extrapolated from PEDv environmental survival studies. Typical swine feed pelleting conditioning retention times and temperatures encompass the theoretical temperature and time relationship proposed. We are unaware of any available direct

research confirming this time and temperature relationship using conditioner and pelleting systems that are present in modern feed manufacturing facilities.

It is known that the minimum infectious dose of PEDv using clarified homogenates of intestinal mucosa from a PEDv-infected piglet is quite low. Clinical signs and PEDv viral replication were noted in pigs that had been inoculated with a 10^{-8} dilution of the intestinal homogenate (Goyal, 2014). However, we are unaware of any data defining a minimum infectious dose of PEDv in feed.

OBJECTIVES

1. Confirm the minimum infectious dose of PEDv in a feed matrix using a 10 d old pig bioassay (Exp. 1).
2. Determine the impact of conditioning time and temperature of pelleted complete feed at two PEDv dosages on PEDv inactivation as measured by RT-PCR and bioassay (Exp. 2).

MATERIAL AND METHODS

Virus Isolation, Propagation and Titration

Virus isolation, propagation, and titration were performed in Vero cells (ATCC CCL-81) as previously described (Chen et al. 2014). The United States (US) PEDv prototype strain cell culture isolate USA/IN/2013/19338, passage 8 (PEDv19338) was used to inoculate feed in these studies. The stock PEDv used in the experiments contained 5.6 x10^5 $TCID_{50}$/ml.

Feed

The feed used was a corn soybean meal-based swine diet (Table 1) and was manufactured at the Kansas State University O.H. Kruse feed mill (Manhattan, KS). A subsample of this feed was obtained prior to inoculation and confirmed negative by real-time PCR (RT-PCR) for the presence of PEDv RNA at the Kansas State University Veterinary Diagnostic Laboratory (Manhattan, KS).

PEDv Inoculum

In Exp. 1, PEDv was propagated and 8 serial 10-fold dilutions of the virus were made using tissue culture medium. A 500 ml aliquot of the original viral stock, each serial PEDv dilution and one virus-negative culture medium control were mixed into 4.5 kg batches of the swine diet to form 10 experimental treatments.

Feed and virus were mixed using a manual, bench-top stainless steel paddle mixer which had been validated for mixing efficiency testing using a standard protocol (McCoy, 1994). The optimal mixing time was determined as 2 minutes or greater to achieve uniform mixing. The 500 ml of media was added slowly to the mixer while operating the mixer. After the addition, the feed was mixed for 2.5 minutes. Batches of feed were mixed in order of lowest to highest virus concentration with a batch of non-inoculated feed (flush) mixed in-between each batch. Subsamples of each batch of feed and each of the flush batches were analyzed for presence of PEDv RNA by RT-PCR. After each inoculated batch and subsequent flush, the mixer was cleaned of any residual feed particulate matter before beginning the mixing process for the next batch.

Three subsamples (100 g/sample) of PEDv inoculated feed were obtained after mixing from each batch and were used to make a 20% suspension. Briefly, the 100 g sample of feed was added to 400 ml of cold phosphate buffered saline (PBS, pH 7.4) in 500 ml bottles, thoroughly mixed and stored at 4°C for approximately twelve hours. The feed suspension was evaluated using a PEDv N-gene based RT-PCR assay (Lowe et al, 2014). Also, aliquots were harvested and frozen at -80°C until use in the pig bioassay.

In Exp. 2, PEDv low dose ($1x10^2$ $TCID_{50}$/g) and high dose ($1x10^4$ $TCID_{50}$/g) concentrations in the feed were used based on the results obtained in Exp. 1 to inoculate the same swine diet used in Exp. 1 (Table 1). Within each dose the batches of inoculated feed were processed at 1 of 3 condition times (45, 90, 180 sec) with 1 of 3 processing temperatures (68.3, 79.4, and 90.6°C, corresponding to 155, 175, and 195°F). Thus, treatments were arranged in a 2 (PEDv dose) x 3 (conditioning time) x 3 (processing temperature) arrangement. Additionally, 3 batches of unprocessed (meal based) feed were evaluated. These batches consisted of feed inoculated with virus free tissue culture medium, feed inoculated with the low dose PEDv and feed inoculated with the high dose PEDv concentration. Conditioning times were chosen to represent a typical conditioning time, an extended time using a typical conditioner and an extended time that would require a modified conditioner. The temperature range reflects a typical nursery diet processing temperature on the low end and the maximum achievable temperature under typical commercial production of swine feed on the high end.

For each concentration, aliquots (500 ml) of the PEDv dilutions were uniformly blended into 4.5 kg batches of feed using the same equipment and procedures as used in Exp. 1. Feed from each of the conditioning time by temperature combinations (9 batches within each PEDv dose) was processed using a pilot-scale single pass conditioner and pellet mill (Model CL5, CPM, Waterloo, IA). Prior to processing the first batch, non-inoculated feed was processed until the exit temperature of the feed was stable at the target temperature. In between each inoculated batch, a minimum of 5 kg of virus free feed was processed. The objective of this was twofold. The first objective was to provide a flush of the equipment and prevent virus carry over. The second was to stabilize the processing temperature so the contaminated feed was processed under uniform temperature conditions. The low dose batches were processed prior to the high dose batches. Within dose, the highest temp and longest retention time was processed first and then temperature was reduced to achieve the medium and low temperature batch before moving to the middle retention time. The same progression was used between the medium and low retention time such that the final batch within each dose was the low temperature and shortest retention time treatment.

Similar to Exp. 1, three 100 g samples of each batch of feed were added to 400 ml of cold phosphate buffered saline (PBS, pH 7.4) in 500 ml bottles, thoroughly mixed and stored at 4°C for approximately twelve hours. The feed suspension supernatants were evaluated using a PEDv N-gene based real-time RT-PCR and aliquots were harvested and frozen at -80°C until use in the pig bioassay.

Pigs

Ninety-six crossbred, 10 day-old pigs (30 pigs for Exp. 1 and 66 pigs for Exp. 2) of mixed sex were sourced from a single commercial, crossbred farrow-to-wean herd with no prior exposure to PEDv. Upon arrival, piglets were ear tagged, weighed and administered a dose of cefitiofur (Excede, Zoetis, Florham Park, NJ). Also, upon arrival fecal swabs were obtained and confirmed negative for PEDv, porcine delta coronavirus (PDCoV) and transmissible gastroenteritis virus (TGEV) using a RT-PCR assay as previously described (Lowe et al., 2014). To further confirm PEDv negative status, serum was collected and confirmed negative for PEDv antibody by an indirect fluorescent antibody (IFA) assay (Madson et al., 2014) and TGEV antibody by ELISA

conducted at the Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL). Pigs were allowed 2 days of adjustment to the new pens before the bioassay began.

Bioassay

The Iowa State University Institutional Animal Care and Use Committee (protocol log #5-14-7793-S) reviewed and approved the pig bioassay protocol.

In Exp. 1, 30 pigs were randomly allocated by weight into 1 control and 9 challenge groups (3 pigs per treatment). The bioassay procedures were similar to and conducted in the same facilities as those previously described (Madsen et al., 2014; Thomas et al., 2014). Briefly pigs from each experimental treatment were housed in separate rooms that each had independent ventilation systems. Rooms had solid flooring that was minimally rinsed to reduce PEDv aerosols. Pig 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 PBS feed suspension supernatants by orogastric gavage using an 8 gauge French catheter (0 dpi).

In Exp. 2, 66 pigs were randomly allocated by weight into 2 unprocessed feed without virus control groups, 1 low dose unprocessed feed challenge group, 1 high dose unprocessed feed challenge group and 18 processed feed challenge groups. Pigs were offered the same milk replacer and pelleted feed *ad libitum* as previously described in Exp. 1. Each pig was administered the feed suspension fluid (10 ml/pig) using a similar procedure as Exp. 1.

Rectal swabs were collected on d 0, 2, 4, 6, and 7 dpi from all piglets and tested for PEDv RNA by quantitative RT-PCR. Fresh small intestine, cecum and colon were collected at necropsy at 7 dpi along with an aliquot of cecal content. One section of formalin-fixed proximal, middle, distal jejunum and ileum were collected as previously described for histopathology (Madson et al, 2014). Cecal content was evaluated for PEDv by qRT-PCR.

Histopathology

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 a total of six sections of intestine) were evaluated by a veterinary pathologist blind to the treatments. In each of the sections, one full-length villous and crypt were measured, based on tissue orientation, using a computerized image system as previously described (Madson, et al., 2014). Thus, one crypt and villi was measured per section of jejunum and ileum for a total of 6 values per pig. The three values per jejunum and ileum were averaged for calculating the villous/crypt ratio.

Porcine epidemic diarrhea virus immunohistochemistry slides were prepared on the sections of ileum as previously described (Madson et al., 2014). Antigen detection was scored based on the following criteria: No signal (0), mild (1-10% signals), moderate (11-25% signals), abundant (26-50% signals), and diffuse (>50-100%).

Statistical analysis

In Exp. 1 an analysis of variance was performed to evaluate the effect of PEDv dose on PEDv RNA in feed and on fecal shedding and fecal content for those doses where PEDv RNA was detected. The association

between the PEDv innocula Ct and feed after inoculation was evaluated using linear regression for those doses where PEDv RNA was detected in feed. An ANOVA was also performed for villus height, crypt depth, villous height to crypt depth ratio, and immunohistochemistry. For these response criteria a single degree of freedom polynomial contrast was used to compare PEDv doses where PEDv shedding was evident to those where it was not detectable. In Exp 2, ANOVA was performed to evaluate PEDv RNA feed Ct values, Villus height, crypt depth, villous height to crypt depth ratio, and immunohistochemistry.

RESULTS

Proximate analysis of the corn soybean meal-based gestation diet used in Exp. 1 and 2 was as expected within limits of analytical variation (Table 1).

Exp. 1. Serial dilutions of PEDv had corresponding PCR cycle thresholds (Ct) of 14.0 to greater than 45. When the aliquots of virus were added to feed, only the four highest doses had detectable PEDv RNA and an increase in Ct value (linear, $P < 0.01$, R^2 0.98) as the PEDv dose decreased (Table 2). Results indicated that every 1-log reduction in PEDv concentration resulted in an increase in 3.4±0.21 Ct in feed with detectable PEDv RNA as measured by RT-PCR. Furthermore, when the PEDv was added to the feed, those feed dilutions that were considered PEDv-positive had an increase of 9.6±0.4 Ct compared to the RT-PCR results of the tissue culture media that was utilized to inoculate the feed.

Additionally in Exp. 1, by using the flush, a model was created to determine if batch-to-batch transfer of PEDv would occur. When PEDv-negative feed was flushed after each serial dilution, the only detectable PEDv RNA was found in the sample collected after mixing the highest PEDv dose (5.6×10^4 $TCID_{50}$) and corresponded to a Ct value of 38.

Fecal shedding of PEDv (Table 2) was not detected in rectal swabs from negative control pigs for the duration of the study. Porcine epidemic diarrhea virus RT-PCR analysis of fecal swabs from pigs challenged with PEDv-inoculated feed supernatant indicated that fecal shedding and clinical disease was detected in all pigs challenged with 5.6×10^2 $TCID_{50}$/g to 5.6×10^4 $TCID_{50}$/g by 2 dpi and continued through 7 dpi. For the pigs challenged with 5.6×10^1 $TCID_{50}$/g, two of the 3 pigs had PEDv-positive fecal swabs at 2 dpi, but all three pigs had PEDv-positive fecal swabs at 4 dpi and continued to shed through the termination of the study. Pigs challenged with the PEDv treatments ranging from 5.6×10^0 $TCID_{50}$/g to 5.6×10^{-4} $TCID_{50}$/g had no PEDv-positive fecal swabs collected for the 7 dpi, nor was any of the cecum content at 7 dpi PEDv-positive. These findings suggest that the minimum infectious dose of PEDv in a feed matrix is 5.6×10^1 $TCID_{50}$/g, which corresponded to a Ct of 37 when the feed was analyzed by RT-PCR.

When comparing pigs fed doses that had fecal RNA shedding compared to those where RNA was not detected the villous height was shorter (371.8 ± 25.4 vs 470.8 ± 22.7 µm, P <0.01; Table 3), crypt depth tended to be greater (152.8 ± 9.3 vs 131.5 ± 8.3 µm, P =0.10), and villous height to crypt depth ratio was lower (2.6 ± 0.3 vs 3.7 ± 0.2 µm, P <0.01). Immunohistochemistry was positive for pigs challenged with any of the four highest doses of PEDv, which confirmed that infection was established.

Exp. 2. There was no PEDv RNA detected in the unprocessed PEDv free feed. When the low PEDv dose media (1×10^3 $TCID_{50}$/ml; Ct 20.0) was mixed with the feed, the resulting feed Ct value was 31.0 and when the high PEDv dose media (1×10^5 $TCID_{50}$/ml; Ct of 13.0) was mixed with feed the resulting Ct value was 24.0 (Table 4). The low dose unprocessed feed PEDv was 6.8 ± 1.8 Ct higher ($P < 0.01$) than the high dose unprocessed feed Ct value. Within the respective dose, regardless of time or temperature, feed processing increased ($P < 0.01$) the Ct compared to the unprocessed PEDv inoculated feed. Within PEDv dose there were no consistent differences among the conditioning time x temperature combinations

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

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The villous height for pigs challenged with the non-inoculated feed was higher ($P < 0.01$) compared to the height in pigs challenged with the low or high dose PEDv unprocessed feed. Porcine epidemic diarrhea virus IHC immnoreactivity 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.

DISCUSSION

The results of this study clearly demonstrated the minimum infectious dose of PEDv in feed is 5.6 X 10^1 TCID50/g. PEDv infection was confirmed thru visible clinical signs characteristic of PEDv disease along with typical histopathologic changes, positive PEDv IHC, and RT-PCR positive fecal samples present in pigs that received inoculated feed supernatant at the minimum infectious dose and greater. Additionally, the minimum infectious dose in feed had a corresponding PCR Ct value of 37, which may be above reported diagnostic values as previously mentioned (Table 5). Therefore, a detectable Ct in feed can result in infectivity, however definitive interpretation of feed PEDv PCR results should be made cautiously especially when determining clinical relevance. Interestingly, there was a consistent Ct difference of approximately 10 (3 logs) when PEDv was blended into feed which equates to a 1000-fold difference in the amount of PEDv RNA that was detected. The authors hypothesize that the loss in Ct count could be due to either the degradation of RNA when added to the feed or binding of the viral particles or viral RNA to feed particles. The second hypothesis is intriguing because this would indicate a lower sensitivity of RNA detection in a feed matrix. More studies should be conducted to investigate possible viral reversible binding and possible infectivity of released virus in a bioassay model.

None of the pigs in the time and temperature pellet study that were inoculated with feed processed by a conditioner/pellet mill similar to that used in commercial production showed clinical signs of disease or pathologic lesions of PEDv regardless of viral dosage, condition time or pellet temperature. Therefore, it appears thermal processing conducted in conditions typical for a full-scale feed mill is an effective point-in-time step to decrease risk of PEDv transmission in feed.

In Experiment 1, we demonstrated that PEDv can be transferred from one batch to the next via the contamination of the mixing equipment. However, carry over was observed only after a high dose of PEDv contamination was present. This suggests that a sequencing protocol could be used to minimize PEDv carryover prior to making feed for high-risk growth phases (i.e. feed offered to sows or early nursery pigs). Additional research should be conducted to determine the effectiveness of feed sequencing through the feed manufacturing process as a possible PEDv mitigation step.

In conclusion, an effective and repeatable model for virus-inoculation of feed was demonstrated. Supernatant from inoculated feed not thermally processed and orally dosed to 10 d old pigs induced clinical disease and histologic changes at a minimum infectious dose of 5.6 X 10^1 TCID/g, which corresponded to a feed Ct of 37. Additionally, infected feed processed at common pellet mill temperatures and conditioner retention times effectively inactivated the virus at a low and high concentration and did not cause detectible clinical or microscopic disease in 10 d old pigs. The viral feed model undoubtedly demonstrates a novel vector

for PEDv transmission and raises concern about feed mill biosecurity, a topic unheard of two or three years ago. Additional research about PEDv viral properties in feed and the impact of mitigation steps that can be utilized in the feed mill (i.e. feed sequencing, use of specialty feed ingredients or other treatments, feed processing, etc.) should be further investigated.

LITERATURE CITED

Chen, Q. et al. 2014. Isolation and Characterization of Porcine Epidemic Diarrhea Viruses Associated with the 2013 Disease Outbreak among Swine in the United States. Journal of Clinical Microbiology 52:234-243.

Dee, S. et al. 2014a. An evaluation of contaminated complete feed as a vehicle for porcine epidemic diarrhea virus infection of naive pigs following consumption via natural feeding behavior: proof of concept. BMC veterinary research 10:176.

Dee, S., C. Neill, T. Clement, J. Christopher-Hennings, and E. Nelson. 2014b. An evaluation of a liquid antimicrobial (Sal CURB(R)) for reducing the risk of porcine epidemic diarrhea virus infection of naive pigs during consumption of contaminated feed. BMC veterinary research 10:220.

Goyal, S. 2014. Environmental stability of PED (porcine epidemic diarrhea virus). http://www.pork.org/wp-content/uploads/2014/05/goyal-13-215-main.pdf (Accessed 15 December 2014.)

Lee, J. H. et al. 2014. Porcine epidemic diarrhea virus infection: Inhibition by polysaccharide from Ginkgo biloba exocarp and mode of its action. Virus research 195:148-152.

Madson, D. M. et al. 2014. Pathogenesis of porcine epidemic diarrhea virus isolate (US/Iowa/18984/2013) in 3-week-old weaned pigs. Veterinary microbiology 174:60-68.

McCoy, R. A. 1994. Mixer Testing. In: R. R. McElhiney, editor, Feed Manufacturing Technology IV. American Feed Industry Association, Arlington, VA. p. 548-550.

Nitikanchana, S. 2014. Potential Alternatives to Reduce Porcine Epidemic Diarrhea Virus (PEDV) Contamination in Feed Ingredients. http://www.asi.k-state.edu/species/swine/research-and-extension/PEDV%20contamination%20in%20feed%20ingredients_Feb%2026.pdf (Accessed 31 December 2014.)

Pasick, J. et al. 2014. Investigation into the role of potentially contaminated feed as a source of the first-detected outbreaks of porcine epidemic diarrhea in Canada. Transboundary and emerging diseases 61:397-410.

Thomas, P., L. A. Karriker, A. Ramirez, J. Zhang, J. S. Ellingson, D. J. Holtkamp. 2014. Methods for inactivating PEDV in hog trailers. http://www.pork.org/wp-content/uploads/2014/05/pages-from-2014swinebooklet_final10-30-14-2-3.pdf (Accessed 12 January 2015.)

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
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 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}.

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

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

Table 2. Influence of porcine epidemic diarrhea virus (PEDv) inoculated feed on N-gene RT-PCR cycle threshold (Ct) of feed, fecal swabs and fecal contents of pigs[1]

PEDv Concentration in the feed, TCID50/g[2]	Tissue Culture Media, Ct	Feed, Ct	Fecal swabs, Ct					
			0 dpi[3]	2 dpi	4 dpi	6 dpi	7 dpi	7 dpi Cecum content, Ct
Virus-free feed	–[4]	–	–	–	–	–	–	–
5.6×10^{-4}	–	–	–	–	–	–	–	–
5.6×10^{-3}	38.0	–	–	–	–	–	–	–
5.6×10^{-2}	34.3	–	–	–	–	–	–	–
5.6×10^{-1}	30.6	–	–	–	–	–	–	–
5.6×10^{0}	27.4	–	–	–	–	–	–	–
5.6×10^{1}	24.3	37.1	–	33.2[5]	20.7	19.8	25.3	23.1
5.6×10^{2}	20.7	33.6	–	27.3	22.2	21.3	24.2	26.5
5.6×10^{3}	16.6	29.5	–	30.7	22.4	21.2	25.2	24.0
5.6×10^{4}	14.0	27.0	–	27.4	21.0	21.9	25.2	25.4
SEM		0.3	NA	1.9	1.9	2.1	2.8	2.4

[1]An initial tissue culture containing 5.6×10^5 $TCID_{50}$/ml of PEDv was serially diluted using tissue culture media. These dilutions were then 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 one time via oral gavage on d 0 to each of three pigs per feed dilution (10 ml per pig). Thus each value represents the mean of three replicates. Pigs were initially 10 d old and 3.6 kg BW.

[2] Titer was estimated by assuming that mixing of 500 ml of PEDV at 5.6×10^5 $TCID_{50}$/ml with 4.5 kg feed gave rise to a titer of 5.6×10^4 $TCID_{50}$/g feed.

[3]Day post inoculation.

[4]Cycle threshold (Ct) was established at >45 as negative.

[5]There was one pig that was negative; Ct value of 45 was used to account for this pig.

Table 3. Morphologic and immunohistochemistry evaluation of small intestine from pigs that were challenged with porcine epidemic diarrhea virus (PEDv) inoculated feed[1]

	Morphology[2]			
PEDv Concentration in the feed, TCID50/g[3]	Villus height, µm	Crypt depth, µm	Villus height to crypt depth ratio	Immunohistochemistry (IHC)[4]
Virus-free feed	485.8	132.8	3.7	0
5.6×10^{-4}	527.7	136.3	4.3	0
5.6×10^{-3}	464.3	120.7	3.9	0
5.6×10^{-2}	491.3	116.3	4.3	0
5.6×10^{-1}	436.0[5]	136.3	3.2	0
5.6×10^{0}	434.7	147.7	3.0	0
5.6×10^{1}	390.0	191.0	2.3	0.7
5.6×10^{2}	302.0	151.7	2.1	0.3
5.6×10^{3}	365.3	141.3	2.6	0.7
5.6×10^{4}	429.7	127.3	3.5	1
SEM	50.7	18.6	0.5	0.3

[1]An initial tissue culture containing 5.6×10^5 $TCID_{50}$/ml of PEDv was serially diluted using tissue culture media. These dilutions were then 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 one time via oral gavage on d 0 to each of three pigs per feed dilution (10 ml per pig). Thus, each value represents the mean of three pigs per dilution and 3 villi measured per pig. Pigs were initially 10 d old and 3.6 kg BW.

[2]Intestinal cross-sections were fixed in formalin and stained with hematoxylin and eosin for evaluation.

[3]Inoculated feed with calculated titers. Titer was estimated by assuming that mixing of 500 ml of PEDV at 5.6×10^5 $TCID_{50}$/ml with 4.5 kg feed gave rise to a titer of 5.6×10^4 $TCID_{50}$/g feed.

[4]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 treatment, thus the mean of 9 values

[5]There was one pig that was negative; Ct value of 45 was used to account for this pig.

Table 4. 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. 2[1]

Item		PEDv N-gene Real Time-PCR, cycle threshold (Ct)							
				Fecal swabs					Cecum contents
		Tissue culture	Feed	0[2] dpi	2 dpi	4 dpi	6 dpi	7 dpi	7dpi
Unprocessed virus-free feed		–[3]	–	–	–	–	–	–	–
Low dose inoculated feed[4]		20.0	31.0	–	22.4	18.2	18.8	24.1	26.7
Temp[5]	Time[6]								
68.3°C	45s		42.6	–	–	–	–	–	–
	90s		39.5	–	–	–	–	–	–
	180s		45.0	–	–	–	–	–	–
79.4°C	45s		36.7	–	–	–	–	–	–
	90s		39.7	–	–	–	–	–	–
	180s		42.3	–	–	–	–	–	–
90.6°C	45s		39.7	–	–	–	–	–	–
	90s		37.4	–	–	–	–	–	–
	180s		35.9	–	–	–	–	–	–
High dose inoculated feed[7]		13.0	24.0	–	23.0	15.3	20.4	24.3	24.0
Temp	Time								
68.3°C	45s		30.2	–	–	–	–	–	–
	90s		29.7	–	–	–	–	–	–
	180s		30.2	–	–	–	–	–	–
79.4°C	45s		30.1						
	90s		29.5	–	–	–	–	–	–
	180s		30.2	–	–	–	–	–	–
90.6°C	45s		30.1						
	90s		30.6	–	–	–	–	–	–
	180s		30.0	–	–	–	–	–	–

[1]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 one time via oral gavage on d 0 to each of three pigs per treatment (10 ml per pig). Thus, each value represents the mean of three pigs per treatment. Pigs were initially 10 d old and 3.6 kg BW.

[2]Day post inoculation.

[3]A cycle threshold (Ct) of >45 was considered negative for presence of PEDv RNA.

[4]For low dose feed, PEDv (1×10^3 $TCID_{50}$/ml) was diluted into feed to provide a dose of 1×10^2 $TCID_{50}$/g of feed.
[5]Temperature of feed exiting the conditioner.

[6]Retention time. The amount of time required for feed to pass through the conditioner.

[7]For high dose feed, PEDv (1×10^5 $TCID_{50}$/ml) was diluted into feed to provide a dose of 1×10^4 $TCID_{50}$/g of feed.

Table 5. Effects of porcine epidemic diarrhea virus (PEDv) dose, pelleting temperature, and conditioning retention time on morphologic and immunohistochemistry evaluation of small intestine from pigs, Exp. 2[1]

Item		Morphology[2]			
		Villus height, µm	Crypt death, µm	Villus height to crypt depth ratio	Immunohistochemistry (IHC)[3]
Unprocessed virus-free feed		495.7	101.7	4.9	0
Low dose inoculated feed[4]		414.3	91.0	4.6	0.3
Temp[5]	Time[6]				
68.3°C	45s	481.6	101.3	4.8	0
	90s	489.3	108.1	4.6	0
	180s	504.4	115.6	4.4	0
79.4°C	45s	508.6	108.9	4.7	0
	90s	476.4	103.6	4.6	0
	180s	441.8	103.6	4.3	0
90.6°C	45s	443.2	97.8	4.6	0
	90s	495.7	101.7	4.9	0
	180s	441.8	103.6	4.3	0
High dose inoculated feed[7]		309.3	112.6	3.1	1.7
Temp	Time				
68.3°C	45s	423.1	105.3	4.0	0
	90s	429.4	118.3	3.7	0
	180s	389.2	100.0	4.0	0
79.4°C	45s	432.8	117.3	3.7	0
	90s	390.6	102.5	3.7	0
	180s	448.7	104.5	4.3	0
90.6°C	45s	383.1	119.5	3.2	0
	90s	446.4	102.8	4.4	0
	180s	408.9	105.7	3.9	0
	SEM	29	10.1	0.4	0.2

[1]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 one time via oral gavage on d 0 to each of three pigs per treatment (10 ml per pig). Thus, each value represents the mean of three pigs per treatment necropsied at 7 days post infection. Pigs were initially 10 d old and 3.6 kg BW.

[2]Intestinal cross-sections were fixed in formalin and stained with hematoxylin and eosin 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 treatment, thus the mean of 9 values.

[4]For low dose feed, PEDv (1 x 10^3 $TCID_{50}$/ml) was diluted into feed to provide a dose of 1 x 10^2 $TCID_{50}$/g of feed.

[5]Temperature of feed exiting the conditioner.

[6]Retention time. The time feed was inside the conditioner.

[7]For high dose feed, PEDv (1 x 10^5 $TCID_{50}$/ml) was diluted into feed to provide a dose of 1 x 10^4 $TCID_{50}$/g of feed.

Table 5. Comparison of reported Ct values for PEDv RT-PCR assay across four diagnostic laboratories.

	Veterinary Diagnostic Laboratory			
	Kansas State University	**Iowa State University**	**University of Minnesota**	**South Dakota State University**
Ct values	**≤36 = positive** **37-39= weak positive** **≥39 = negative**	**<35 = positive** **>35 = negative**	**≤35 = positive** **36-39= suspect** **>39 = negative**	**≤ 37 = positive** **>37 = not detected**