EVALUATION OF PORCINE EPIDEMIC DIARRHEA VIRUS IN FEED MANUFACTURING

by

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Abstract

Biological hazards in animal feed are a growing concern for the feed industry. Porcine epidemic diarrhea virus (PEDV) is the first viral pathogen confirmed to be transmissible in swine feed and feed ingredients. This led to investigations identifying the magnitude of transmissible risk PEDV imposes and strategies to mitigate infectivity in contaminated diets. The objective of the first experiment was to evaluate the minimum infectious dose of PEDV in virus-inoculated feed. Pigs became infected when PEDV concentrations at or above 5.6×10^1 50% tissue culture infectious dose/g ($TCID_{50}$/g); corresponding feed cycle threshold (Ct) of 37 or below was utilized. Evaluation of a mitigation strategy for PEDV contaminated diets is also important since cross-contamination during feed manufacturing is possible. Therefore, the objective of the second experiment was to determine the effectiveness of feed batch sequencing as a method to minimize the risk of PEDV cross-contamination. This method was effective to reduce but not eliminate infectious PEDV carryover risk. Furthermore, feed that lacked detectible PEDV RNA as analyzed by quantitative real-time reverse transcription PCR assay (qPCR) was infectious. The third study was an observational study complementary to the previous experiment where the magnitude of virus contamination in the feed manufacturing facility was characterized during feed batch sequencing. Widespread contamination of the facility occurred and surfaces remained contaminated until chemically cleaned. The final experiment was conducted to assess PEDV RNA detection in feed and spray dried porcine plasma (SDPP) when analyzed by qPCR across 5 diagnostic laboratories. Overall, it appears qPCR PEDV RNA detection in feed and SDPP was precise as quantified by low coefficient of variation across laboratories, with the exception of one %CV from SDPP inoculated with low virus load from one laboratory. Although the magnitude of the Ct value difference was large in only 1 of 5 laboratories, comparisons of Ct

values across laboratories should be interpreted cautiously. Finally, qPCR can be a useful surveillance tool for detection of PEDV RNA in non-clinical samples such as feed and SDPP.

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Dedication

I would like to dedicate this thesis in honor of my parents, Dean and Carol Schumacher.

Chapter 1 - Evaluation of the Minimum Infectious Dose of Porcine Epidemic Diarrhea Virus in Virus-Inoculated Feed

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Abstract

Objective

To determine the minimum infectious dose of porcine epidemic diarrhea virus (PEDV) in virus-inoculated feed

Animals

30 crossbred 10-day-old pigs.

Procedures

Tissue culture PEDV was diluted to form 8 serial 10-fold dilutions. An aliquot of stock virus (5.6×10^5 $TCID_{50}$/mL) and each serial PEDV dilution were mixed into 4.5-kg batches of feed to create 9 PEDV-inoculated feed doses; 1 virus-negative dose of culture medium in feed was also created. Pigs were challenge exposed via oral administration of PEDV-inoculated feed, and fecal swab specimens were collected. All pigs were euthanized 7 days after challenge exposure; fresh tissues were collected and used for PCR assay, histology, and immunohistochemistry.

Results

The PCR cycle threshold (Ct) decreased by approximately 10 when PEDV was added to feed, compared with results for equivalent PEDV diluted in tissue culture medium. Pigs became infected with PEDV when challenge exposed with 4 highest concentrations (lowest concentration to cause infection, 5.6×10^1 $TCID_{50}$/g; Ct = 27 in tissue culture medium and 37 in feed.

Conclusions and clinical relevance

In this study, PEDV in feed with detectable Ct values of 27 to 37 was infective. The Ct was 37 for the lowest infective PEDV dose in feed, which may be above limit of detection

established for PEDV PCR assays used by some diagnostic laboratories. Overall, results indicated 5.6×10^1 $TCID_{50}$/g was the minimum PEDV dose in feed that can lead to infection in 10-day-old pigs under the conditions of this study. (Am J Vet Res 2016;77:xxx–xxx)

Abbreviations

Ct	Cycle threshold
IHC	Immunohistochemistry
PEDV	Porcine epidemic diarrhea virus
qPCR	Real-time quantitative PCR

Introduction

Porcine epidemic diarrhea virus suddenly and profoundly affected the United States swine industry in its emergence in May 2013 [1]. Although the direct route of transmission is fecal-oral transmission, little is known about other possible routes of transmission and risk factors for spread among swine populations, including the role of transport vehicles and aerosolized virus [2-4]. Recently, several PEDV outbreaks were suspected to be associated with the consumption of PEDV-containing feed or feed ingredients [5]. Since those outbreaks were reported, it has been confirmed that feed is a potential vehicle for PEDV transmission, and this has prompted investigations into reducing infectivity risk attributable to contaminated diets or feed ingredients [6-9]. Additionally, PEDV is highly transmissible in the United States; however, little is known about the overall magnitude of transmissible risk that PEDV-infected feed constitutes. Furthermore, the authors are aware of no data that define the minimum infectious dose of PEDV detected in feed. Therefore, the objective of the study reported here was to determine the infectious dose of PEDV in feed by use of a 10-day-old pig bioassay.

Materials and Methods

Animals

Thirty crossbred 10-day-old pigs of both sexes were obtained from a commercial crossbred farrow-to-wean herd that had no prior exposure to PEDV. Immediately after pigs arrived at the research facility, pigs received identification ear tags; pigs were then weighed and administered a dose of cefitiofur.[a] Fecal swab specimens were obtained and confirmed negative for PEDV, porcine delta coronavirus, and transmissible gastroenteritis virus by use of virus-specific qPCR assays conducted at the Iowa State University Veterinary Diagnostic Laboratory. To further confirm the pigs were not infected with PEDV, serum samples were obtained and

confirmed to have negative results for antibodies against PEDV by use of an indirect fluorescent antibody assay and antibodies against transmissible gastroenteritis virus by use of an ELISA, both of which were conducted at the Iowa State University Veterinary Diagnostic Laboratory. Pigs were allowed 2 days to acclimate to their surroundings before the study began. All procedures involving pigs were approved by the Iowa State University Institutional Animal Care and Use Committee.

Virus isolation, propagation, and titration

Virus isolation, propagation, and titration were performed in Vero cells[b] as described elsewhere [10]. The US PEDV prototype (strain cell culture isolate USA/IN19338/2013) was used to inoculate feed study. The stock solution of PEDV contained 5.6×10^5 $TCID_{50}$/mL.

Feed

The feed used in the study was manufactured at the Kansas State University O.H. Kruse Feed Technology Innovation Center in Manhattan, Kansas. The feed was based on corn and soybean meal and included vitamin and trace mineral premixes as well as a source of phytase[c] (Appendix 1). Chemical analysis of the feed revealed that it contained 91.40% dry matter, 17.10% crude protein, 3.70% crude fiber, 0.78% calcium, 0.52% phosphorous, and 3.50% fat. A subsample of feed was obtained prior to inoculation and confirmed to have negative results for PEDV RNA by use of a qPCR assay performed at the Kansas State University Veterinary Diagnostic Laboratory.

PEDV inoculum

A stock solution of PEDV cell passage 8 with a titer of 5.6×10^5 $TCID_{50}$/mL (which corresponded to a PCR Ct value of 14) was used to create serial 10-fold dilutions (diluted with tissue culture medium) and generate 8 dilutions with virus titers ranging from 5.6×10^4

$TCID_{50}$/mL to 5.6×10^{-3} $TCID_{50}$/mL. A 500-mL aliquot of the viral stock solution, 500 mL of each serial dilution, and 500 mL of virus-negative culture medium were each mixed into 4.5-kg batches of feed to provide 10 experimental treatments (9 PEDV-inoculated treatments and 1 virus-negative control treatment). Feed and solutions were mixed with a manual, bench-top stainless steel paddle mixer[d] that had been validated for mixing efficiency by use of a standard testing protocol [11]. The 500 mL of solution was added slowly to the feed during mixing. After the solution was added, the feed was mixed for 2.5 minutes. A batch of noninoculated feed was mixed between each batch of PEDV-inoculated feed to act as a flush. After each PEDV-inoculated batch and subsequent flush was mixed, the mixer was cleaned of residual feed before beginning the mixing process for the next batch. Batches of feed were mixed in order of lowest virus concentration to highest virus concentration. Subsamples of each batch of feed and each of the flush batches were analyzed for presence of PEDV RNA by use of a qPCR assay.

Three subsamples (100 g/subsample) of PEDV-inoculated feed were obtained 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 (4°C) PBS solution (pH, 7.4) in 500-mL bottles; contents were thoroughly mixed, and the bottles were stored at 4°C for approximately 12 hours. The feed suspension was evaluated by use of a PEDV N-gene–based qPCR assay [10, 12]. Also, aliquots of the feed suspension were harvested and frozen at –80°C until used in the pig bioassay.

Inoculation of PEDV-containing feed

The 30 pigs were randomly allocated to treatment using a spread sheet based random number generator to 1 control and 9 challenge-exposure groups (3 pigs/group). Bioassay procedures were similar to those previously described [13] and were conducted in the same

facilities as previously described [13]. Briefly, pigs of each experimental group were housed in separate rooms that each had separate ventilation systems. Each rooms had a solid floor that was minimally rinsed to reduce PEDV aerosols. Pigs were fed liquid milk replacer twice daily and provided a commercial pelleted diet[e] ad libitum; pigs also had ad libitum access to water. Each pig was administered 10 mL of the feed suspension (PBS solution) supernatants by orogastric gavage with an 8F catheter (day 0). Fecal swab specimens were collected from the rectum of each pig on days 0, 2, 4, 6, and 7 and tested for PEDV RNA by use of a qPCR assay. At the completion of the study (day 7), pigs were euthanized by IV administration of an overdose of pentobarbital sodium solution.[f] Samples of fresh small intestine, cecum, and colon and an aliquot of cecal content were collected during necropsy. One section of formalin-fixed tissues from the proximal, middle, and distal aspects of the jejunum and 1 from the ileum were collected for histologic examination of which only ileum samples were evaluated, as previously described [14]. Cecal content was evaluated for PEDV by use of a qPCR assay.

RNA extraction and qPCR assay

Nucleic acids were extracted from aliquots of the virus dilutions (50 μL), feed suspensions (100 μL), and fecal swab specimens (100 μL) by use of an RNA-DNA kit[g] and a magnetic particle processor was used for DNA/RNA extraction[h] use in accordance with the manufacturer's instructions. Nucleic acids were eluted into 90 μL of elution buffer. Five microliters of RNA template (total reaction volume, 25 μL) was used for the qPCR assay kit,[i] as previously described [4, 12, 13].

Histologic examination

Tissues were processed in a routine manner, fixed in neutral-buffered formalin, embedded in paraffin, sectioned, and stained with H&E stain. Three serial sections from a piece

of ileum were evaluated by a veterinary pathologist (LLS), who was unaware of the treatment administered to each pig. For each of the 3 sections, 1 full-length villous and 1 crypt were measured by use of a computerized image system.[j] Mean villous length and crypt depth for each intestinal segment were used for statistical analysis. Mean values were determined and used to calculate the villous height-to-crypt depth ratio for each pig. Slides for IHC analysis of PEDV were prepared by use of the sections of ileum, as previously described [14]. Antigen detection was scored by use of the following criteria: 0 = no stain (0% stained tissue), 1 = mild (1% to 10% stained tissue), 2 = moderate (11% to 25% stained tissue), 3 = abundant (26% to 50% stained tissue), and 4 = diffuse (> 50% to 100% stained tissue).

Statistical analysis

A statistical analysis program[k] was used to perform an ANOVA to evaluate the effect of PEDV dose on PEDV RNA in feed, fecal shedding, and fecal content for those doses in which PEDV RNA was detected. The association between the Ct for the PEDV inoculum and the Ct for the feed after inoculation was evaluated by use of linear regression analysis for those doses in which PEDV RNA was detected in feed. One pig had a negative result for the qPCR assay, and a Ct value of 45 was used to account for this pig. An ANOVA was also performed for villus height, crypt depth, villous height-to-crypt depth ratio, and results of IHC analysis. For these response criteria, a single degree of freedom polynomial contrast was used to compare PEDV doses in which PEDV shedding was evident with those in which PEDV was not detected.

Results

qPCR assay of PEDV inoculum

Serial dilutions of PEDV in tissue culture medium with theoretical titers of 5.6×10^4 $TCID_{50}$/mL to 5.6×10^{-3} $TCID_{50}$/mL had corresponding qPRC assay Ct values of 16.6 to > 45

(Table 1.1). When aliquots of virus were added to feed, only the 4 highest concentrations had detectable PEDV RNA with a linear increase in Ct value (R^2, 0.98; $P = 0.01$) as the PEDV dose decreased. Results indicated that every reduction of 1 (log10) in PEDV concentration resulted in a mean ± SD increase in Ct value of 3.4 ± 0.21 for feed with detectable PEDV RNA, as measured by use of the qPCR assay. Furthermore, when PEDV was added to feed, those feed dilutions that had detectable PEDV RNA had a mean increase in Ct value of 9.6 ± 0.4, compared with results for the equivalent virus dilutions in tissue culture medium. Additionally, use of the non–PEDV-inoculated feed to flush between mixing of treatments resulted in a method that could be used to determine whether batch-to-batch transfer of PEDV would occur. Use of PEDV-negative feed to flush the mixer between each serial dilution resulted in detectable PEDV RNA only in the flush sample collected after mixing the highest PEDV concentration (5.6×10^4 $TCID_{50}$/g), which corresponded to a Ct value of 38.

qPCR assay of pig bioassay samples

Fecal shedding of PEDV was not detected in fecal swab specimens collected from negative control pigs for the duration of the study (Table 1.2). The qPCR analysis of fecal swab specimens obtained from pigs challenge exposed with PEDV-inoculated feed revealed fecal shedding and clinical disease in all pigs challenge exposed with 5.6×10^2 $TCID_{50}$/g to 5.6×10^4 $TCID_{50}$/g by day 2, which continued through day 7. Two of the 3 pigs challenge exposed with 5.6×10^1 $TCID_{50}$/g had PEDV-positive fecal swab specimens at day 2, but all 3 of these pigs had PEDV positive fecal swab specimens at days 4 through 7. Pigs challenge exposed with PEDV-inoculated feed ranging from 5.6×10^0 $TCID_{50}$/g to 5.6×10^{-4} $TCID_{50}$/g had no PEDV-positive fecal swab specimens throughout the 7 days of the study, nor did any of the cecal contents collected on day 7 have positive results when tested for PEDV. These findings suggested that the

minimum infectious dose whereby infection was detected in feed was 5.6×10^1 $TCID_{50}$/g, which corresponded to a Ct of 37 when PEDV was analyzed by use of the qPCR assay.

Histologic examination and IHC analysis

Pigs that had fecal shedding of RNA, compared with those in which RNA was not detected in fecal swab specimens, had a significantly ($P = 0.01$) shorter mean ± SD villous height (347.7 ± 25.8 μm vs 470.8 ± 23.0 μm, respectively), greater crypt depth (166.9 ± 8.7 μm vs 131.5 ± 7.8 μm, respectively), and smaller villous height-to-crypt depth ratio (2.2 ± 0.3 vs 3.7 ± 0.2, respectively; Table 1.3). Positive results for IHC staining were observed in enterocytes of pigs challenge exposed with any of the 4 highest concentrations of PEDV; this confirmed that infection was established.

Discussion

In the study reported here, the lowest detectable infectious dose of PEDV in feed was 5.6×10^1 $TCID_{50}$/g, as characterized by results of the pig bioassay. Infection with PEDV after challenge exposure with the minimum infectious dose and greater was confirmed by use of various assays. Results for qPCR assay of fecal samples of pigs challenge exposed with PEDV-inoculated feed indicated the presence of detectable RNA. Shortened villi in infected pigs was a typical histopathologic finding consistent with PEDV infection. Finally, enterocytes had positive results for IHC staining, which confirmed the presence of viral antigen.

Surprisingly, the lowest infectious dose detected in feed had a corresponding qPCR assay Ct value of 37, which may be considered higher than the cutoff Ct when the sample is reported to have a negative result at some veterinary diagnostic laboratories [14]. Infectivity above the Ct detection limit of the qPCR assay has been reported in other studies [13, 15] of the PEDV infectious dose that involved the use of intestinal scrapings or tissue culture fluid. Investigators

of 1 study [13] reported that tissue culture inoculum with a theoretical titer of 0.0056 $TCID_{50}$/mL had a corresponding Ct value of > 45, which is a value considered as genetic material that is not detectable. Interestingly, the inoculum was found to be infectious in 1 of 4 neonatal pigs [13]. Additionally, the response was age dependent, with a much lower minimum infectious dose in neonatal pigs than in weaned pigs when challenge exposed at dilutions ranging from 10^{-3} $TCID_{50}$/ml to 10^{-8} $TCID_{50}$/ml. Similarly, investigators of another study [15] used clarified intestinal homogenates of PEDV-infected pigs to generate serial dilutions used for challenge exposure of 10-day-old pigs. Viral dilutions > 10^{-8} $TCID_{50}$/ml had no detectable genetic material, yet challenge exposure result in diarrhea and detectable RNA from mucosal samples with a Ct value as low as 16. Results of those studies and the study reported here indicated that PEDV is highly infectious in neonatal pigs and infectivity is at the higher end of qPCR assay detection limits. This suggests that the PEDV minimum infectious dose is quite low in young pigs.

The cell culture virus isolate used in 1 of the aforementioned studies [13] was also used in the study reported here. Cell passage 8 is quite low for cell culture and the isolate that caused severe disease in neonatal pigs. Moreover, an established cell culture isolate is known to be purer than is the isolate obtained from a clinical sample, and it is also easier to quantify and generate a homologous titer with more consistent virulence during bioassay.

Young pigs reportedly excrete feces containing ≥ 10^9 PEDV genomic equivalents/mL [16]. On the basis of the lowest infective dose for the present study, it can be estimated that 1 g of this fecal matter could potentially contaminate up to 450,000 kg of feed. Also, large amounts of PEDV are present in the environment of infected farms, and given the fact that feed deliveries need to occur on a regular basis, it is theoretically possible that infectious material is transferred

from an infected farm through a feed mill to another farm. Thus, feed delivery personnel and transport vehicles may potentially be a substantial risk factor for PEDV transmission. Prior to the introduction of PEDV into the United States, there were few reports that implicated feed as a source for viral transmission. Other researchers have investigated PEDV survivability in feed and feed ingredients and examined chemical methods to mitigate transmission risk [17, 18]. Survivability may be dependent on the feed, and viability appears to be different for individual ingredients than for complete diets [18]. Although the magnitude of transmission risk via feed is unknown, education of feed mill operators and delivery personnel about biosecurity is warranted. For example, the importance of biosecurity in regard to minimizing the risk of virus transmission via PEDV contamination of feed mills has been reported [19].

Interestingly, there was a consistent difference of approximately 10 in the Ct value between PEDV diluted in tissue culture medium and PEDV blended into feed, which equates to a 1,000-fold (3 [log10]) difference in the amount of PEDV RNA, assuming that a reduction of 1 (log10) in virus concentration corresponds to an increase in Ct value of 3.3. However, it must be mentioned that the process of diluting virus in culture medium and diluting virus in feed differs. First, dilution of PEDV in culture medium was a liquid-to-liquid dilution, whereas adding virus to feed was a liquid-to-solid dilution, although both were considered 10-fold dilutions. Second, virus diluted in culture medium was directly used for RNA extraction and testing by use of a qPCR assay. However, an additional processing step for feed (resuspend feed in PBS solution to create a 20% suspension) was performed before RNA extraction and testing by use of a qPCR assay. This step further diluted the virus concentration and could have accounted for a difference in Ct value of approximately 2 to 3. Differences in the procedures used to detect PEDV in feed versus the liquid dilution in culture medium could possibly have contributed to the observed

differences in Ct values. Prior to the study reported here, we valuated several elution and extraction protocols that did not result in appreciable differences in the Ct value. Thus, the exact reason that the Ct value of detected virus added to feed differed from that for virus in culture medium is unknown, and other factors may have contributed to the differences. We hypothesize that the increase in Ct value also could have been attributable to degradation of RNA when virus was added to the feed or binding of the virus or viral RNA to feed particles. For example, a strain of food-borne Norovirus adhered to plant cell wall material via carbohydrate moieties, which is a method that may enhance viral persistence and thwart decontamination efforts [20]. Perhaps there was a similar binding mechanism for PEDV in feed with unknown consequences on resulting infectivity. This hypothesis is intriguing because it would indicate a lower sensitivity of RNA detection when conducted with a feed matrix. Additional studies should be conducted to elucidate the reason that there was an approximate increase in Ct value of 10 when the virus was placed in feed and determine whether this effect will influence infectivity.

In the present study, PEDV could be transferred from one batch of feed to the next via contamination of the mixing equipment. However, detectable transmission was observed only after a high dose of PEDV was used. This suggested that a sequential flush protocol could be used to minimize PEDV transmission when mixing feed for high-risk pigs, such as sows or young nursery-age pigs. Additional studies should be conducted to determine the effectiveness of the sequence of the feed manufacturing process as a possible means of mitigating transmission of PEDV.

For the study reported here, an effective and repeatable method for virus inoculation of feed was used. All supernatants from inoculated feed dilutions with detectible Ct values were infectious to 10-day-old pigs. Furthermore, the lowest dose for which PEDV infection was

detected corresponded to a Ct value of 37 for the PEDV-inoculated feed. This Ct value may be above the PCR assay detection threshold of some diagnostic laboratories, which would thus render false-negative results. Overall, results indicated that 5.6×10^1 $TCID_{50}$/g was the minimum PEDV dose for which we detected infectivity for PEDV-inoculated feed.

Sources and manufacturers

a. Excede, Zoetis, Florham Park, NJ.

b. ATCC CCL-81, American Type Culture Collection, Rockville, MD.

c. High Phos 2700 GT, DSM Nutritional Products, Parsippany, NJ.

d. Stainless steel meat mixer, Cabela's Inc, Sidney, Neb.

e. All Natural Starter 2, Heartland Co-Op, Alleman, IA.

f. Fatal-Plus, Vortech Pharmaceuticals Ltd, Dearborn, Mich.

g. MagMAX pathogen RNA/DNA kit, Thermo Fisher Scientific, Waltham, Mass.

h. Kingfisher-96, Thermo Fisher Scientific, Waltham, Mass.

i. Path-ID Multiplex One-Step RT-PCR kit, Thermo Fisher Scientific, Waltham Mass.

j. Nikon Eclipse TI-U microscope, Nikon Instruments Inc, Melville, NY.

k. SAS, version 9.3, SAS Institute Inc, Cary, NC.

References

1. Huang Y-W, Dickerman AW, Piñeyro P, Li L, Fang L, Kiehne R, et al. Origin, evolution, and genotyping of emergent porcine epidemic diarrhea virus strains in the United States. MBio. 2013;4(5):e00737-13. doi: 10.1128/mBio.00737-13
2. Saif L, M. B. Pensaert, K. Sestak, S.G. Yeo, K. Jung., editor. Coronaviruses. 10th ed. Chichester, UK: Wiley-Blackwell; 2012.
3. Alonso C, Goede DP, Morrison RB, Davies PR, Rovira A, Marthaler DG, et al. Evidence of infectivity of airborne porcine epidemic diarrhea virus and detection of airborne viral RNA at long distances from infected herds. Vet Res. 2014;45(1):73. doi: 10.1186/s13567-014-0073-z
4. Lowe J, Gauger P, Harmon K, Zhang J, Connor J, Yeske P, et al. Role of transportation in spread of porcine epidemic diarrhea virus infection, United States. Emerg Infec Dis. 2014;20(5):872-4. doi: 10.3201/eid2005.131628
5. Pasick J, Berhane Y, Ojkic D, Maxie G, Embury-Hyatt C, Swekla K, et al. Investigation into the role of potentially contaminated feed as a source of the first-detected outbreaks of porcine epidemic diarrhea in Canada. Transbound Emerg Dis. 2014;61(5):397-410. doi: 10.1111/tbed.12269
6. Jones CK. Post-processing contamination chemical mitigation strategies to control PEDV in feed and feed ingredients2014. Available from: http://research.pork.org/FileLibrary/ResearchDocuments/14-158-JONES-KSt.pdf. Accessed 14 March 2015.
7. Dee S, Clement T, Schelkopf A, Nerem J, Knudsen D, Christopher-Hennings J, et al. 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 Vet Res. 2014;10(1):176. doi: 10.1186/s12917-014-0176-9

8. Lee JH, Park JS, Lee SW, Hwang SY, Young BE, Choi HJ. Porcine epidemic diarrhea virus infection: Inhibition by polysaccharide from Ginkgo biloba exocarp and mode of its action. Virus Res. 2014;195:148-52. doi: 10.1016/j.virusres.2014.09.013

9. Dee S, Neill C, Clement T, Christopher-Hennings J, Nelson E. An evaluation of a liquid antimicrobial (Sal Curb®) for reducing the risk of porcine epidemic diarrhea virus infection of naive pigs during consumption of contaminated feed. BMC Vet Res. 2014;10(1):220. doi: 10.1186/s12917-014-0220-9

10. Chen Q, Li G, Stasko J, Thomas JT, Stensland WR, Pillatzki AE. Isolation and characterization of porcine epidemic diarrhea viruses associated with the 2013 disease outbreak among swine in the United States. J Clin Microbiol. 2014;52. doi: 10.1128/jcm.02820-13

11. McCoy RA, editor. Feed Manufacturing Technology IV. Arlington, VA: American Feed Industry Association; 1994.

12. Madson DM, Magstadt DR, Arruda PH, Hoang H, Sun D, Bower LP, et al. Pathogenesis of porcine epidemic diarrhea virus isolate (US/Iowa/18984/2013) in 3-week-old weaned pigs. Vet Microbiol. 2014;174:60-8. doi: 10.1016/j.vetmic.2014.09.002

13. Thomas JT, Chen Q, Gauger PC, Gimenez-Lirola LG, Sinha A, Harmon KM, et al. Effect of porcine epidemic diarrhea virus infectious doses on infection outcomes in naive conventional neonatal and weaned pigs. PLoS ONE. 2015;10(10):e0139266. doi: 10.1371/journal.pone.0139266

14. Schumacher LL, Dritz SS. Feed diagnostics: What does it really mean—or does it even matter? 46th annual meeting of the American Association of Swine Veterinarians; February 2015; Orlando, Florida.

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

16. Jung K, Annamalai T, Lu Z, Saif LJ. Comparative pathogenesis of US porcine epidemic diarrhea virus (PEDV) strain PC21A in conventional 9-day-old nursing piglets vs. 26-day-old weaned pigs. Vet Microbiol. 2015;178(1-2):31-40. doi: 10.1016/j.vetmic.2015.04.022

17. Dee S, Neill C, Clement T, Singrey A, Christopher-Hennings J, Nelson E. An evaluation of porcine epidemic diarrhea virus survival in individual feed ingredients in the presence or absence of a liquid antimicrobial. Porc Health Manage. 2015;1(1):1-10. doi: 10.1186/s40813-015-0003-0

18. Cochrane RA, Woodworth JW, Dritz SS, Huss AR. Evaluating chemical mitigation of porcine epidemic diarrhea virus in swine feed and ingredients. Jt Meet Abstr Am Dairy Sci Assoc; 2015; Des Moines, IA.

19. Greiner LL. Evaluation of the likelihood of detection of porcine depidemic diarrhea viurs or porcine delta coronavirus ribonucleic acid in areas within feed mills. J Swine Health and Prod. 2016;24(4):198-204

20. Esseili MA, Wang Q, Saif LJ. Binding of human GII.4 norovirus virus-like particles to carbohydrates of romaine lettuce leaf cell wall materials. Appl Environ Microbiol. 2012;78(3):786-94. doi: 10.1128/aem.07081-11

Tables

Table 1.1 Composition of feed inoculated with PEDV and used for challenge exposure of 10-day-old pigs

Ingredient	%
Corn	79.30
Soybean meal*	15.70
Choice white grease	1.00
Calcium phosphate (monocalcium)	1.40
Limestone	1.15
Salt	0.50
L-Threonine	0.03
Trace mineral premix†	0.15
Additional additive premix‡	0.50
Vitamin premix§	0.25
Phytase[c]	0.02
Total	**100.00**

*Contained 46.5% crude protein. †Each kilogram contained 26.4 g of Mn, 110 g of Fe, 110 g of Zn, 11 g of Cu, 198 mg of I, and 198 mg of Se. ‡Each kilogram contained 4,409 U of vitamin E, 44 mg of biotin, 992 mg of pyridoxine, 331 mg of folic acid, and 110,229 mg of choline. §Each kilogram contained 4,400,000U of vitamin A, 551,146 U of vitamin D_3, 17,637 U of vitamin E, 1,764 mg of menadione, 3,300 mg of riboflavin, 11,023 mg of pantothenic acid, 19,841 mg of niacin, and 15 mg of vitamin B_{12}.

Table 1.2 The Ct values for a qPCR assay to detect PEDV in feed fed to and fecal swab specimens and cecal contents obtained from 10-day-old pigs (3 pigs/treatment).

PEDV in feed ($TCID_{50}$/g)*	Tissue culture medium	Feed	Fecal swab specimens					
			Day 0	Day 2	Day 4	Day 6	Day	Cecal contents†
Virus-free feed	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
5.6×10^{-4}	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
5.6×10^{-3}	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
5.6×10^{-2}	38.0	Neg	Neg	Neg	Neg	Neg	Neg	Neg
5.6×10^{-1}	34.3	Neg	Neg	Neg	Neg	Neg	Neg	Neg
5.6×10^{0}	30.6	Neg	Neg	Neg	Neg	Neg	Neg	Neg
5.6×10^{1}	27.4	37.1	Neg	33.2	20.7	19.8	25.3	23.1
5.6×10^{2}	24.3	33.6	Neg	27.3	22.2	21.3	24.2	26.5
5.6×10^{3}	20.7	29.5	Neg	30.7	22.4	21.2	25.2	24.0
5.6×10^{4}	16.6	27.0	Neg	27.4	21.0	21.9	25.2	25.4
SEM	ND	0.3	NA	1.9	1.9	2.1	2.8	2.4

An initial stock solution of PEDV containing 5.6×10^5 $TCID_{50}$/mL with a Ct value of 14 was serially diluted with tissue culture medium; these dilutions were then used to inoculate batches of feed. Then, 3 feed samples/batch were collected and diluted in PBS solution, and 10 mL of supernatant from each sample was administered via oral gavage (day 0) to each of the 3 pigs for that treatment group. Thus, each value represents the mean of 3 replicates.

*The titer was estimated by assuming that mixing PEDV (500 mL; 5.6×10^5 $TCID_{50}$/mL) with 4.5 kg of feed would provide a titer of 5.6×10^4 $TCID_{50}$/g of feed. †Pigs were euthanized on day 7; cecal contents were collected during necropsy.

NA = Not applicable. Neg = Negative result because a Ct value > 45 was established as the cutoff for a negative result.

Table 1.3 Results of histologic examination and IHC evaluation of samples of the ileum obtained from 10-day-old pigs challenge exposed with PEDV-inoculated feed (3 pigs/treatment group).

	Histologic findings			
PEDV in feed ($TCID_{50}$/g)*	**Villus height (μm)**	**Crypt depth (μm)**	**Villus height-to-crypt depth ratio**	**IHC score†**
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	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}	333.6	183.5	1.8	1.0
SEM	51.5	17.4	0.5	0.3

†Three serial sections of ileum were evaluated for each pig. Antigen detection was scored as follows: 0 = no signal (0% stained tissue), 1 = mild (1% to 10% stained tissue), 2 = moderate (11% to 25% stained tissue), 3 = abundant (26% to 50% stained tissue), and 4 = diffuse (> 50% to 100% stained tissue). The mean was calculated (3 samples/pig × 3 pigs/treatment) for each treatment. ***See*** Table 1.2 for remainder of key.

Chapter 2 - Utilizing Feed Batch Sequencing to Decrease the Risk of Porcine Epidemic Diarrhea Virus (PEDV) Cross-Contamination During Feed Manufacturing

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Abstract

Feed has been identified as a vector of transmission for porcine epidemic diarrhea virus (PEDV). The objective of this study was to determine the effects of feed batch sequencing on PEDV cross-contamination. Porcine epidemic diarrhea virus-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. Feed was then analyzed for PEDV RNA by qPCR and for infectivity by bioassay. Sequence 1 feed had higher ($P < 0.05$) qPCR Ct values than the positive treatment and sequence 2 feed had higher ($P < 0.05$) Ct values than sequence 1, regardless of sampled location. Mixer feed from sequence 2, 3, and 4 was qPCR negative whereas conveyor feed was qPCR negative from sequence 3 and 4. Bioassay for negative, positive, sequence 1 and 2 mixer treatments was conducted in group 1 and consisted of 30 mixed sex (3.92 ± 0.88 kg BW) pigs confirmed negative for PEDV allocated to 1 of 10 treatment rooms. Bioassay for sequence 3 and 4 mixer treatments and all conveyor treatments was conducted in group 2 and consisted of 36 mixed sex (3.18 ± 0.79 kg BW) pigs confirmed negative for PEDV allocated to 1 of 12 treatment rooms. Group 2 was performed 11 mo after group 1; pigs were initially 10 d old. Control pigs remained PEDV negative for the study. All pigs from the mixer positive treatment (9/9) and

conveyor positive treatment (3/3) were qPCR positive on fecal swabs by the end of the study. One replicate of pigs from mixer sequence 1 were qPCR positive (3/3) by 7dpi. One replicate of mixer pigs from sequence 2 were qPCR positive (3/3) by 7dpi although no detectable PEDV RNA was found it the feed. The results demonstrate sequenced batches had reduced quantities of PEDV RNA although sequenced feed without detectible PEDV RNA by qPCR can be infectious. Therefore, a sequencing protocol can reduce but not eliminate the risk of producing infectious PEDV carryover from the first sequenced batch of feed.

Introduction

Porcine epidemic diarrhea virus (PEDV) profoundly affected the United States swine industry since its emergence in May 2013 [1]. A few reports of PEDV outbreaks in the U.S. and Canada were suspected to be caused by consumption of PEDV-contaminated feed or feed ingredients [2]. Feed has since been confirmed as one of the many routes of PEDV-transmission, which has led to investigations into identifying ways to mitigate infectivity of contaminated diets or feed ingredients [3-5]. Preliminary work from our previous studies suggested PEDV cross-contamination of feed can occur during feed manufacturing [6]. However, infectivity of this batch-to-batch contamination was not established. Due to the lack of additional data detailing PEDV cross-contamination during feed manufacturing, it is hypothesized that strategically sequencing batches during feed production may reduce the risk of PEDV cross-contamination. Therefore, the objective of this experiment was to determine the efficacy of feed batch sequencing methods to minimize the risk of PEDV cross-contamination as measured by real-time reverse transcription PCR (qPCR) and pig bioassay.

Materials and Methods

The feed manufacturing portion of the experiments was approved by the Kansas State University Institutional Biosafety Committee and 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 RNA in the feed, equipment, and environment as measured by qPCR. The pig bioassay portion of the 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. All animal work was conducted at the Iowa State University Veterinary Medical Research Institute (Ames, IA).

Virus and Virus Aliquot Transportation and Handling

PEDV virus isolation, propagation, and titration were performed as described elsewhere [7]. The US PEDV prototype (strain cell culture isolate USA/IN19338/2013 cell passage 8) was used to inoculate feed in this study. The stock solution of PEDV contained 4.5×10^6 50% tissue culture infectious dose/mL ($TCID_{50}$/mL). This isolate has been previously shown to be pathogenic in young pigs [8]. The virus was divided into three, 500 mL aliquots and stored at -80°C. One aliquot was used in each replication. In 1 of 3 replicates, a 500 mL aliquot was shipped frozen on dry ice from Iowa State University Veterinary Diagnostic Laboratory (ISU VDL) to the FSRC. In 2 of 3 replicates, a frozen 500 mL aliquot was retrieved from ISU VDL by currier and began to slowly thaw at room temperature in a cooler without ice until arrival at the FSRC. In all replicates, the 500 mL aliquots were allowed to thaw overnight at 4°C in the FSRC until used the following day for the experiment.

Swine Diet

A corn soybean meal-based diet was manufactured at the Kansas State University O.H. Kruse Feed Technology Innovation Center (Manhattan, KS) (Table 2.1). A subsample of the feed was obtained prior to inoculation for each repetition and was confirmed PEDV negative by qPCR.

Negative Feed Treatment

Fifty kg of swine diet was mixed in a 0.113 m^3 electric paddle mixer (H. C. Davis Sons Manufacturing model# SS-L1; Bonner Springs, KS) that was previously validated to mix a 50 kg batch of feed with CV less than 10%, as per standard mixing efficiency protocol [9]. The feed

was mixed for 5 min before aseptically sampled. Clean disposable gloves were worn while using a disposable plastic cup to subsample five equally spaced locations within the mixer. The subsampled feed formed a 400 to 500 g sample which was placed in a closeable plastic specimen bag. Feed was then discharged at a rate of approximately 4.5 kg/min into the conveyor (Universal Industries, Cedar Falls, IA) which had a boot pit depth of 2.54 cm from the edge of the cup to the boot bottom and contained 74 buckets (each 114 cm^3). Feed carried by the buckets then exited the conveyor through a downspout where an additional 400 to 500 g sample was collected directly into a plastic specimen bag once the feed stream began. Mixer and conveyor specimen bags were set on ice in a cooler until transported the same day for qPCR analysis. Bagged feed samples were then temporarily stored at -20°C until discarded when no longer needed.

PEDV Inoculum and Positive Feed Treatment

The PEDV inoculum premix was established by mixing a 500 mL aliquot of stock virus into a 4.5 kg batch of the swine diet using procedures established in a prior experiment [4]. The PEDV inoculum premix (4.5 kg of feed + 500 mL of stock virus) was then added to 45 kg of swine diet to form the positive experimental treatment and was mixed, discharged, sampled, and handled as described above.

Sequenced Feed Treatments

Following the positive feed treatment, four subsequent 50 kg batches of PEDV-free swine diet were each mixed, discharged, and sampled as described previously to form sequence feed treatment 1, 2, 3, and 4 and was mixed, discharged, sampled, and handled as described before. The equipment was not cleaned between any feed treatments until completion of the study to mimic commercial feed manufacturing conditions.

Feed Sample Processing Procedures and Storage

Each mixer and conveyor sample was divided into 3 subsamples (100 g/sample) and then used to make a 20% suspension. Briefly, the 100 g sample was added to 400 mL of PBS (Life Technologies; pH, 7.4) in 500 mL bottles (Nalgene square bottles; Thermo Scientific, Waltham, MA); contents were thoroughly mixed and allowed to settle at 4ºC overnight. Aliquots were then collected without remixing the supernatant by using sterile serologic pipettes and pipette controller (Pipetboy; Integra Biosciences, Hudson, NH). A 4 mL aliquot of the feed suspension was evaluated by Kansas State University (KSU) using a PEDV spiked gene-based qPCR assay as described below. Twenty mL aliquots for bioassay and an additional 30 mL saved/backup aliquots were harvested from all treatment samples and placed in sterile conical polypropylene centrifuge tubes (Tornado tubes; MidSci, St. Louis, MO). Group 1 bioassay aliquots are from mixer negative, mixer positive and mixer sequence 1 and 2 feed treatments and were stored frozen at −80ºC until challenged in pigs within 1 mo of sample collection. Group 2 bioassay aliquots were from mixer sequence 3 and 4 and from all conveyor feed treatments (negative, positive, and sequence 1 to sequence 4) and were stored frozen at −80ºC until challenged in pigs 11 mo later.

RNA extraction and PEDV qPCR

All feed samples were analyzed at Kansas State University Molecular Diagnostics Development Laboratory (Manhattan, KS) for the presence of PEDV RNA by qPCR. Nucleic acids were extracted from a 50 µL sample of feed supernatant. Automated extraction was carried out on a KingFisher magnetic particle processor (Thermo Scientific, Waltham, MA) using a MagMAX-96 Viral RNA Isolation Kit (Life Technologies, Grand Island, NY). All manufacturer's instructions were followed, with the exception of a final elution volume of 60 µl.

Each 96-well extraction run included an extraction positive control (PEDV stock virus) and an extraction negative control (1× PBS). An in-house-developed duplex qPCR assay targeting the spike gene (S) of PEDV and host 18S rRNA (internal control) was used for the detection and quantification of PEDV. The 20 µl reaction mixture comprised 1× Path-ID Multiplex RT-PCR buffer, 2 µl Path-ID Multiplex Enzyme Mix (Thermo Scientific, Waltham, MA), 500 nM of each of three PEDV primers and two 18S primers and 62.5 nM of each probe (PEDV and 18S), and 4 µl of the extracted nucleic acid. Amplification was performed on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The thermal cycling parameters were: 10 min reverse transcription at 48°C, 10 min of reverse transcriptase inactivation/initial denaturation at 95°C followed by 45 cycles of 10 sec at 95°C and 40 sec at 60°C.

All animal samples and stock virus were analyzed by Iowa State University Veterinary Diagnostic Laboratory (ISU VDL). Nucleic acids were extracted from initial stock virus (50 µl), bioassay inoculum (100 µl), and rectal swabs (100 µl) and eluted into 90 µl of elution buffer using a RNA/DNA kit (MagMAX Pathogen RNA/DNA Kit; Thermo Scientific, Waltham, MA) and a Kingfisher-96 magnetic particle processor following the manufacturer's instructions. Samples were analyzed for PEDV using a previously described PEDV nucleocapsid (N) gene-based qPCR assay [8]. Five µl of RNA template was used in the qPCR setup in a 25 µl reaction using a Path-ID Multiplex One-Step RT-PCR Kit (Thermo Scientific, Waltham, MA) and amplification reactions were conducted on an ABI 7500 Fast instrument (Thermo Scientific Waltham, MA) following previously described procedures [8].

Animals

To assess infectivity of feed treatments, 2 pig studies were performed following a previously established protocol [8]. Bioassay for negative, positive, sequence 1 and 2 mixer

treatments was conducted in group 1. Bioassay for sequence 3 and 4 mixer treatments and all conveyor treatments was conducted in group 2. Group 2 was performed 11 mo after group 1. A total of sixty-six, 10 d old pigs were purchased from a conventional breeding farm and delivered to the Iowa State University Laboratory Animal Resource facilities. All pigs were injected with a dose of ceftiofur (Exede; Zoetis, Florham Park, NJ) i.m. upon arrival. All pigs were confirmed negative for PEDV, porcine delta coronavirus (PDCoV), transmissible gastroenteritis virus (TGEV) and porcine rotaviruses (groups A, B, and C) by virus specific qPCR on rectal swabs and were serologically negative for PEDV. Pigs were blocked by weight and then randomly divided into groups of 3 per room. Rooms had independent ventilation systems and solid flooring that was minimally rinsed to reduce PEDV aerosols. Pigs were fed liquid milk replacer (Esbilac; PetAg, Hampshire, IL) and commercially pelleted diet (All Natural Starter 2; Heartland Co-op, Alleman, IA). Pigs had ad libitum access to feed and water at all times. After 2 days of acclimation, each pig was administered PBS feed suspension inoculum (as described above) by orogastric gavage using an 8–gauge French catheter (0 dpi, day post inoculation). Rectal swabs were collected daily but analyzed on -2, 0, 2, 4, 6, and 7 dpi (with remaining swabs saved if additional analysis was required) from all pigs at ISU VDL for PEDV RNA by qPCR. All pigs were euthanized at 7 dpi for necropsy by i.v. overdose of pentobarbital sodium solution (Fatal-Plus; Vortech Pharmaceuticals Ltd, Dearborn, MI). One section of formalin-fixed proximal, middle, distal jejunum and ileum was submitted for histopathology along with an aliquot of fresh cecal contents for PEDV qPCR to the Iowa State University Veterinary Diagnostic Laboratory (Ames, IA).

Mixer Feed Treatment Pig Study Design

A total of 16 rooms (3 pigs per room) were assigned to the mixer feed treatment groups (1 negative control room and 15 challenge rooms). Each pig from the mixer negative control room was orogavaged with a 10 mL aliquot of inoculum created from the negative control feed collected from the mixer during each of replicate 1, 2, and 3, thus each negative pig represented of 1 of 3 replicates. Different from the mixer negative control room, each pig from the mixer challenge rooms (positive, sequence 1 to sequence 4) was given a 10 mL aliquot of inoculum from the same replicate, thus one room represented one replication per treatment.

Conveyor Feed Treatment Pig Study Design

A total of 6 rooms (3 pigs per room) were assigned to the conveyor feed treatment groups negative, positive, sequence 1 to sequence 4 (1 negative control and 5 challenge rooms). Pigs were given a 30 mL aliquot that combined three, 10 mL aliquots derived from 1 feed treatment from 1 replicate. Thus each pig represented 1 of 3 replicates per treatment and one room represented each treatment.

Histopathology and Immunohistochemistry

Microscopic evaluation on formalin-fixed tissues were performed following a previously established protocol [8, 10]. Briefly, three serial sections of ileum were microscopically evaluated by a veterinary pathologist blinded to the individual animal identifications and treatments. In each of the sections, 1 full-length villus and crypt were measured, based on tissue orientation, using a computerized image system (Nikon Eclipse TI-U microscope with NIS-Elements imaging software, basic research version 3.3, Nikon Instruments Inc., Melville, NY). Thus, one crypt and villi was measured per section of ileum for a total of 3 values per pig. The 3

values per ileum were averaged into 1 value per pig for calculating villus height, crypt depth, and villus-to-crypt-depth ratio.

Porcine epidemic diarrhea virus immunohistochemistry (IHC) slides were prepared on sections of ileum as previously described [10]. Antigen detection was scored based on the following criteria: 0 = no signal (no tissue stained), 1 = mild (1-10% tissue stained), 2 = moderate (11-25% tissue stained), 3 = abundant (26-50% tissue stained), and 4 = diffuse (>50-100% tissue stained).

Statistical Analysis

Data were analyzed using the GLIMMIX procedure in SAS (SAS Inst. Inc., Cary, NC) as a completely randomized design to determine the main effects of treatment, location (mixer vs. conveyor), and their interaction on PEDV Ct values with feed sample as the experimental unit. For villus height, crypt depth, and villus-height-to-crypt-depth ratio, pig was the experimental unit. These data were analyzed using PROC GLIMMIX to determine differences in morphology using an overall model F- test to determine model utility and the LSMEANS procedure to compare differences between bioassay controls and treatment selections by pairwise comparisons. Samples considered negative by qPCR were evaluated as a value of 45 (thermocycler parameter was set at 45 cycles to minimize false negatives) in the statistical model. SEM were calculated and reported as pooled SEM values due to uneven sample size in the mixer bioassay. Results for treatment criteria were considered significant at $P \leq 0.05$.

Results

Detection of PEDV RNA in Feed

As expected, no PEDV RNA was detected by qPCR when the negative control treatment was sampled from the mixer or conveyer (Table 2.2). After the positive feed treatment was

manufactured, all samples from the mixer and conveyer had detectible PEDV RNA (mean Ct = 31.7 and 30.9, respectively). From the mixer and after sequence 1, fewer samples (7/9) had detectible PEDV RNA and the mean Ct increased (Ct = 39.6; $P < 0.05$) compared to the positive feed treatment; however, no samples produced detectible PEDV RNA after sequence 2, 3, or 4. For the samples collected from the conveyor, after sequence 1 there were fewer samples with detectible PEDV genetic material (7/9) and the samples resulted in an increase in the mean feed Ct (Ct = 39.4; $P < 0.05$) as compared to the positive treatment. Unlike mixer feed from sequence 2, PEDV genetic material was detected in 2 of 9 conveyor feed samples from sequence 2 and again the mean Ct increased (Ct = 43.7; $P < 0.05$) as compared to sequence 1. Like the mixer, no conveyor feed samples had detectible RNA after sequence 3 and 4. The main effect of treatment ($P = 0.001$) had an effect on feed Ct values whereas the main effect of location and interaction of location by treatment was unaffected ($P = 0.18$ and $P = 0.72$, respectively).

PEDV Bioassay of Feed Obtained from the Mixer and Conveyor

Fecal virus shedding from pigs challenged with feed treatments is summarized in Table 2.3. All pigs used in the mixer bioassay were qPCR negative on rectal swabs collected before inoculation and at 0 dpi. Additionally, all pigs from the mixer negative control feed treatment remained qPCR negative on rectal swabs throughout the study and in cecum contents at 7 dpi. All pigs from the mixer positive feed treatment were qPCR positive on rectal swabs at 2 dpi and continued to shed virus to the end of the study (7 dpi). One pig from a sequence 1 treatment room was PEDV qPCR positive at 2 dpi; by 4 dpi, 3 of 3 pigs in this room had detectible PEDV RNA on fecal swabs and continued to shed virus to the end of the study. Although none of the feed from mixer sequence 2 had detectible PEDV RNA, one pig was qPCR positive on rectal swab at 2 dpi and by 4 dpi, 3 of 3 pigs from this treatment room were PEDV qPCR positive on

fecal swabs and remained positive to termination of the study. 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.

As expected, 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 from this room had qPCR positive rectal swabs and shed virus to the end of the study and in 7 dpi cecum contents. Although most of the feed (7/9) from conveyor sequence 1 was qPCR positive, none of the pigs shed PEDV during the study. Additionally, some of the feed (2/9) from conveyor sequence 2 was qPCR positive and again none of the pigs had detectible PEDV RNA on fecal swabs during the study. None of the pigs from conveyor feed sequence 3 and 4 shed PEDV on fecal swabs nor had detectible PEDV in cecum contents at 7 dpi.

Histologic Examination and IHC Analysis

Villus height and crypt depth were measured, villus-height-to-crypt-depth ratios were calculated and the magnitude of IHC staining was scored and summarized in Table 2.4. There was no statistical difference in villus height, villus/crypt ratio or IHC scores from mixer feed treatments ($P = 0.60$, $P = 0.88$, and $P = 0.34$, respectively). Immunohistochemistry staining was negative on pigs from the positive mixer treatment. In contrast, 2 pigs each from mixer sequence 1 and mixer sequence 2 had positive IHC staining.

Discussion

The potential for PEDV contamination in feed manufacturing facilities is a concern since research has confirmed feed and feed ingredients as vectors for PEDV transmission [2, 3]. Since little is known about viral cross-contamination during animal feed production, the objective of

this experiment was to determine the efficacy of feed batch sequencing as a method of reducing PEDV cross-contamination when manufacturing feed using a pilot scale mixer and conveying equipment. The U.S. PEDV prototype strain cell culture isolate USA/IN/19338/2013 was used to inoculate a single batch of feed to create the potential for cross-contamination. The cell culture virus isolate as used previously [8] and in this experiment had a cell passage of 8 which is still quite low for cell culture. Moreover, the isolate was pathogenic and demonstrative of causing disease in neonatal pigs [8]. Again, virulence of this isolate was demonstrated in this study where all pigs challenged with positive feed in the mixer and conveyor bioassay exhibited signs of infectivity by 7 dpi.

The results clearly demonstrate that cross-contamination in the feed manufacturing process is possible as indicated by feed collected from the first sequence that was infective. Although cross-contamination occurred, feed batch sequencing did reduce the amount of detectible PEDV RNA in feed after sequencing 2 batches of swine diet following the positive feed treatment. Thus, sequencing virally contaminated feed appears to be similar to sequencing medicated feed in effort to reduce carryover. Sequencing a batch of medicated-free feed has been shown to reduce significant drug carryover in medicated feed manufacturing [11, 12] and has been adopted by the Food and Drug Administration (FDA) as an approved cleanout procedure for manufacturing medicated feed [13]. Similar to medicated feed, the amount of PEDV detectible RNA in knowingly contaminated feed from our study decreased after sequencing and therefore appears to potentially mitigate cross-contamination during feed manufacturing.

Another finding from this study is that cross-contamination seems to occur at different locations during feed manufacturing. Manufacturing equipment was not cleaned nor disinfected between treatments during feed mixing and conveying to mimic the feed manufacturing process

in a commercial mill. Even after 2 sequences, detectible PEDV RNA persisted in feed from the conveyor whereas no PEDV RNA was detected in feed from the mixer. This observation could be due to cross-contamination that occurred within the boot of the conveyor. Buckets within the conveyor are designed to pick up their load from the boot—which is dead space or pit area—that fills with feed at the bottom foot pulley. This space is filled with previously discharged product, thus charging the boot. In commercial settings, manual clean-out of the boot is not done on a regular basis [14] due to time constraints, difficult accessibility, messiness, and was seemingly unnecessary prior to this research. Therefore, the boot can create a potential source of cross-contamination as batches of feed are manufactured. Another source for persistent PEDV detection in conveyed feed could have originated from contaminated equipment surfaces following PEDV positive feed production. In a complementary study to the current experiment, we monitored the rapid widespread contamination that ensued after production of a PEDV-contaminated batch of feed and demonstrated swabs collected from the plastic conveyor buckets and rubber belt remained qPCR positive during feed sequencing [15]. An additional source of cross-contamination is possibly from the mixer. Although the mixer was empty and clean between discharges by commercial feed manufacturing standards, some feed (approximately 1.4 kg via preliminary data) always remained at the bottom of the tank. Therefore, it is possible for cross-contamination of feed to occur at multiple sites during feed manufacturing.

Concerns of PEDV cross-contamination raises questions about how to eliminate the pathogen from contaminated feed production facilities. Undesired microorganisms are quite difficult to remove once introduced, therefore enhanced protocols for feed mill housekeeping could be critical to prevent cross-contamination [16]. However, housekeeping in general can be difficult for some systems for a variety of reasons such as additional labor, constant

accumulation of dust and debris, and lack of downtime to perform cleaning protocols. Additionally, wet disinfection is not ideal for feed mills since mainly dry ingredients are used and because most of the equipment has limited accessibility needed for chemical cleaning [17]. Instead, feed production facilities must rely on physical cleaning and good manufacturing procedures to prevent spread of microorganisms, however these methods have been proven to increase contamination [17]. Even with chemical disinfectant, PEDV genetic material has proven difficult to eliminate in the FSRC [18]. In other studies and in our own preliminary data, RNA can still be detectable by qPCR following disinfection treatment [19]. Therefore, perhaps more enhanced measures are needed for cleaning and decontamination if a feed mill becomes contaminated with PEDV.

In the current study, infectivity of feed was assessed by bioassay. The concerning results are the demonstration that qPCR negative samples from sequence 2 were infectious in a swine bioassay. These results parallel previous studies where infected tissue homogenates titrated beyond detection limits of qPCR (i.e. qPCR negative) were positive by bioassay [20] and a serial dilution of PEDV cell culture fluid titrated beyond detection limits of qPCR was infective in neonatal pigs [8]. This demonstrates that in some situations, bioassay is more sensitive at detecting PEDV relative to qPCR. Additionally, another bioassay result from this study demonstrated that feed batch sequencing appears to reduce the magnitude of infectivity. For example, in the mixer bioassay, only one pig from sequence 1 and one pig from sequence 2 became infected by 2 dpi in contrast to the mixer positive control where 9 of 9 pigs were infected by 2 dpi. This is likely due to the lower amount of virus in sequenced batches that can induce an infection. Similarly, in another study that used serial PEDV tissue culture dilutions to determine the minimum infectious dose, only one neonatal pig became infected at the lowest serial titration

as compared to all pigs that became infected when challenged with higher PEDV concentrations [8]. Therefore, it seems sequencing decreased the magnitude of infectivity similarly seen with minimum infectious dose studies [4, 8].

Since the sequencing protocol did not eliminate the risk of producing infectious feed after the first sequence, this suggests that other strategies in addition to sequencing may be needed in the feed processing chain. Such strategies may include thermal or chemical mitigation to further decrease the risk of PEDV transmission [5, 6]. This may be especially true considering the stage of pig production sequenced feed is fed. For example, high health herds from nucleus farms, boar studs, or breeding stock multiplication units are critical to swine production. Porcine epidemic diarrhea infection in these herds would disrupt swine production and have devastating economic impact. Thus, sequencing alone may not provide enough hazard mitigation for these swine herds since the magnitude of the risk of infection increases when feeding larger populations [21]. Further research by our group is underway investigating additional PEDV mitigation methods during feed manufacturing.

Although the feed from the positive control conveyor feed was infective in bioassay, the supernatant from conveyor sequence 1 and conveyor sequence 2 was bioassay negative even though the feed was qPCR positive. One factor that might influence this lack of infectivity in the presence of qPCR positive samples is extended storage time. The conveyor samples were retained at -80°C until challenged 11 mo later due to limitations of bioassay facility availability. Bioassay experiments challenging pigs with stored PEDV samples have been previously performed [22, 23], however the duration was not specified. Therefore, it would be reasonable inference that storage duration was less than in the current study. Although most samples containing any type of virus are routinely stored frozen at low temperatures to maintain

infectivity [24], 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 [25]. Therefore, it is possible that sequenced conveyor feed treatments did not contain enough viable PEDV that survived during storage. Although none of the sequenced feed treatments from the conveyor demonstrated infectivity, it is possible there was a storage duration effect, however this hypothesis is untested and remains to be proven.

Interestingly, immunohistochemistry (IHC) staining was negative on pigs from the positive mixer treatment. Immunohistochemistry is a point-in-time method for detecting and visualizing the distribution of virus replication in paraffin embedded tissues. Immunoreactivity is therefore dependent on stage of infection and quantity of detectible antigen expression within tissues [26]. Thus, clinical interpretation of any IHC positive or negative staining should be done in combination with other diagnostic methods such qPCR [26]. In the present study, pigs from the positive control were infected early as determined by qPCR. It appears that by the end of the study, the immune system had time to clear virus replication in enterocytes resulting in no tissue staining at the time of harvest. In contrast, pigs from mixer sequence 1 and mixer sequence 2 that had positive immunostaining became infected later in the study and had active virus replication detectible in tissue sections at time of harvest. These results agree with others that the stage of infection can influence IHC results. For example, fecal swabs with PEDV qPCR Ct value of 30 and above (indicative of an ongoing infection as compared to an acute infection) have been correlated with virus detection without evidence of lesions or immunoreactivity [27]. Furthermore, it has been demonstrated that PEDV inoculated pigs necropsied at 4 dpi had positive IHC and qPCR results consistent with an acute infection in comparison to inoculated

pigs harvested at 28 dpi were no IHC staining or an active infection was observed [8]. Therefore, the stage of infection is important for IHC detectability in PEDV studies.

It is noteworthy in the current study and similar to our previous studies [6], lateral transmission of PEDV to pigs housed in the same treatment rooms occurred, usually within 48 hrs. Once an infected pig sheds PEDV, the environmental contamination is likely high which facilitates fecal-oral exposure to the other pigs [28]. Porcine epidemic diarrhea virus is highly infectious in naive young pigs as demonstrated during the North American outbreak in 2013, and these results further highlight the high pig-to-pig transmissibility of the virus as reported by others [28].

In conclusion, we confirmed the hypothesis that batch-to-batch carryover of PEDV-infected feed can result in subsequent cross-contamination of infectious PEDV in feed. The results of the present study suggest that a sequencing protocol can be used as a risk-reduction but not risk-elimination procedure for infectious PEDV carryover from the first sequenced batch. Manufacturing feed with a high possibility of contamination prior to manufacturing diets for at risk animal populations (i.e. early nursery pigs or lactating sows) should be avoided to reduce exposure to infectious PEDV carryover. Concerning findings from this study revealed that sequenced qPCR negative feed was infectious. Additional research is needed to define ways to further minimize the risk of viral pathogen contamination during feed manufacturing.

References

1. Huang Y-W, Dickerman AW, Piñeyro P, Li L, Fang L, Kiehne R, et al. Origin, evolution, and genotyping of emergent porcine epidemic diarrhea virus strains in the United States. MBio. 2013;4(5):e00737-13. doi: 10.1128/mBio.00737-13
2. Pasick J, Berhane Y, Ojkic D, Maxie G, Embury-Hyatt C, Swekla K, et al. Investigation into the role of potentially contaminated feed as a source of the first-detected outbreaks of porcine epidemic diarrhea in Canada. Transbound Emerg Dis. 2014;61(5):397-410. doi: 10.1111/tbed.12269
3. Dee S, Clement T, Schelkopf A, Nerem J, Knudsen D, Christopher-Hennings J, et al. 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 Vet Res. 2014;10(1):176. doi: 10.1186/s12917-014-0176-9
4. Schumacher LL, Woodworth JC, Jones CK, Chen Q, Zhang J, Gauger PC, et al. Evaluation of the minimum infectious dose of porcine epidemic diarrhea virus in a virus-inoculated feed. Am J of Vet Res (In press). 2016
5. Cochrane RA. Chemical mitigation of microbial pathogens in animal feed and ingredients. MS Thesis, Kansas State Univ. 2015. Available: http://krex.k-state.edu/dspace/handle/2097/20105.
6. Woodworth JC. Determining the impact of conditioning time and temperature in pelleted diets on porcine epidemic diarrhea virus (PEDV) survivability in complete swine diets. National Pork Board. 2015. Available from:

http://research.pork.org/FileLibrary/ResearchDocuments/14-159-WOODWORTH-KSt.pdf. Accessed 14 March 2016.

7. Chen Q, Li G, Stasko J, Thomas JT, Stensland WR, Pillatzki AE. Isolation and characterization of porcine epidemic diarrhea viruses associated with the 2013 disease outbreak among swine in the United States. J Clin Microbiol. 2014;52. doi: 10.1128/jcm.02820-13
8. Thomas JT, Chen Q, Gauger PC, Gimenez-Lirola LG, Sinha A, Harmon KM, et al. Effect of porcine epidemic diarrhea virus infectious doses on infection outcomes in naive conventional neonatal and weaned pigs. PLoS ONE. 2015;10(10):e0139266. doi: 10.1371/journal.pone.0139266
9. McCoy RA. Mixer Testing. In: Schofield EK, editor. Feed manufacturing technology V. Arlington, VA: American Feed Industry Association; 2005. pp. 620-2.
10. Madson DM, Magstadt DR, Arruda PH, Hoang H, Sun D, Bower LP, et al. Pathogenesis of porcine epidemic diarrhea virus isolate (US/Iowa/18984/2013) in 3-week-old weaned pigs. Vet Microbiol. 2014;174:60-8. doi: 10.1016/j.vetmic.2014.09.002
11. Martinez-Kawas A. Evaluating flushing procedures to prevent drug carryover during medicated feed manufacturing MS Thesis, Kansas State Univ. 2008. Available:
12. Herrman T, Behnke K, Loughin T. Mixing and clean-out properties of sulfamethazine and carbadox in swine feed. J Swine Health and Prod. 1995;3(5):127-31
13. Current good manufacturing practice for medicated feeds, 21 C.F.R. part 225.65 (1976).
14. Tilley DR. Incidence and spread of insects from bucket elevator leg boots. PhD Dissertation, Kansas State Univ. 2013. Available: https://krex.k-state.edu/dspace/bitstream/handle/2097/15572/DennisTilley2013.pdf?sequence=1

15. Schumacher LL, Cochrane RA, Evans CE, Kalivoda JR, Woodworth JC, Huss AR, et al. Evaluating the effect of manufacturing porcine epidemic diarrhea virus (PEDV)-contaminated feed on subsequent feed mill environmental surface contamination [abstract]. J. Anim. Sci. 2016;94 Suppl 2. doi: 10.2527/msasas2016-164

16. Cochrane RA, Dritz SS, Woodworth JC, Stark CR, Huss AR, Cano JP, et al. Feed mill biosecurity plans: A systematic approach to prevent biological pathogens in swine feed. J Swine Health Prod. 2016;24(3):154-64

17. Huss AR, Cochrane RA, Deliephan A, Stark CR, Jones CK. Evaluation of a biological pathogen decontamination protocol for animal feed mills. J Food Prot. 2015;78(9):1682-8. doi: 10.4315/0362-028X.JFP-15-052

18. Huss AR, Schumacher LL, Cochrane RA, Poulsen E, Bai JF, Woodworth JC, et al. Proof-of-concept method to sanitize a feed mill contaminated with porcine epidemic diarrhea virus [abstract]. J. Anim. Sci. 2016;94 2. doi: 10.2527/msasas2016-217

19. Bowman AS, Nolting JM, Nelson SW, Bliss N, Stull JW, Wang Q. Effects of disinfection on the molecular detection of porcine epidemic diarrhea virus. Vet Microbiol. 2015;179. doi: 10.1016/j.vetmic.2015.05.027

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

21. Davies P, Sampedro F, Snider T, Bueno I, Bergeron J, Urriola P. Risk assessment of feed ingredients of porcine origin as vehicles for transmission of porcine epidemic diarrhea virus (PEDV). University of Minnesota. 2015. Available from:

http://research.pork.org/FileLibrary/ResearchDocuments/14-164-DAVIES-UofMN.pdf. Accessed 12 Feb 2015.

22. Alonso C, Goede DP, Morrison RB, Davies PR, Rovira A, Marthaler DG, et al. Evidence of infectivity of airborne porcine epidemic diarrhea virus and detection of airborne viral RNA at long distances from infected herds. Vet Res. 2014;45(1):73. doi: 10.1186/s13567-014-0073-z

23. Schneider PT, Zhang J, Ramirez A, Wang C, Holtkamp DJ. Evaluation of disinfection protocols to reduce virus transmission via livestock transport vehicles using model trailers and experimenal conditions. J Swine Health Prod. 2015;23(6):306-16

24. Block J, Joret J, Morlot M, Foliguet J. Persistence of poliovirus 1 during the freezing-thawing of hydroxide sludges from a water treatment plant under laboratory conditions. European journal of applied microbiology and biotechnology. 1978;5(4):331-40

25. Gould EA. Methods for long-term virus preservation. Mol Biotechnol. 1999;13(1):57-66. doi: 10.1385/mb:13:1:57

26. Ramos-Vara JA. Technical Aspects of Immunohistochemistry. Vet Pathol. 2005;42(4):405-26. doi: 10.1354/vp.42-4-405

27. Taplett A, Burrough E. Correlation of semi-quantitative reverse transcription of polymerase chain reaction assay results for porcine epidemic diarrhea virus and the presence of positive immunohistochemistry. 46th Amer Assoc of Swine Vet Ann Meet; 2015 Feb 28-Mar 3; Orlando, FL.

28. Crawford K, Lager K, Miller L, Opriessnig T, Gerber P, Hesse R. Evaluation of porcine epidemic diarrhea virus transmission and the immune response in growing pigs. Vet Res. 2015;46:49. doi: 10.1186/s13567-015-0180-5

Tables

Table 2.1 Diet composition, as fed basis

Ingredient, %	Composition
Corn	79.30
Soybean meal, 46.5% CP	15.70
Choice white grease	1.00
Calcium phosphate (monocalcium)	1.40
Limestone	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, %[5]	
DM	91.4
CP	17.1
Crude fiber	3.7
Ca	0.78
P	0.52
Fat	3.5

[1]Each kilogram of premix contains 73 g Fe, 73 g Zn, 22 g Mn, 11g Cu, 0.198 mg I, and 0.198 mg Se.

[2]Each kilogram of premix contains 4,409 IU vitamin E, 44 mg biotin, 992 mg pyridoxine, 331 mg folic acid, 110,229 mg choline, 40 mg chromium, 9,920 mg L-carnitine.

[3]Each kilogram of premix contains 4,409,171 IU vitamin A, 551,146 IU vitamin D_3, 17,637 IU vitamin E, 1,764 mg menadione, 3,300 mg riboflavin, 11,023 mg d-pantothenic acid, 19,841 mg niacin, 15 mg vitamin B_{12}.

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

[5]One sample was analyzed by Ward Laboratories Inc., Kearney, NE.

Table 2.2 Effect of batch sequencing feed on porcine epidemic diarrhea virus (PEDV) cross-contamination[1]

Item	Negative	Positive	Sequence 1	Sequence 2	Sequence 3	Sequence 4
Feed, Detectable RNA/Total[2]						
Mixer	0/9	9/9	7/9	0/9	0/9	0/9
Conveyor	0/9	9/9	7/9	2/9	0/9	0/9
Feed, Ct[3]						
Mixer	45.0[a]	31.7[c]	39.6[b]	45.0[a]	45.0[a]	45.0[a]
Conveyor	45.0[a]	30.9[c]	39.4[b]	43.7[a]	45.0[a]	45.0[a]

[a,b,c]Means with different superscripts differ (P <0.05).

[1]Tissue culture fluid containing 4.5×10^6 $TCID_{50}$/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 and analyzed by PEDV qPCR.

[2]Count of samples with detectible PEDV RNA (Ct <45)/number of samples analyzed.

[3]Mean cycle threshold (Ct) value of samples. A value of 45.0 was used for samples with no detectible PEDV RNA. For feed Ct analysis: Main effect of location $P = 0.18$. Main effect of treatment $P = 0.001$. Location × Treatment $P = 0.72$ and pooled SEM = 0.52.

Table 2.3 Pig bioassay results from manufactured and batch sequenced porcine epidemic diarrhea virus (PEDV) inoculated feed collected from mixing and conveying equipment[1]

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

[1]Tissue culture fluid containing 4.5×10^6 $TCID_{50}$/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 3.92 kg BW for group 1 and 3.2 kg BW for group 2. Feed from the mixer was inoculated in to pigs in 3 rooms with 3 pigs per room for the positive feed and sequences. One room with 3 pigs were inoculated with negative feed. Each pig in the negative control room was inoculated from each replicate. This same process was used for the feed from the conveyor so there was 1 room with each pig inoculated with feed from each replicate. Bioassay for negative, positive and sequence 1 and 2 was conducted in group 1. Bioassay for sequence 3 and 4 and conveyor samples was conducted in group 2. Group 2 was performed 11 mo after group 1.

[2]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 when cecum contents and tissues were collected.

[3]Count of pigs shedding detectible PEDV RNA/number of pigs analyzed.

Table 2.4 Pig morphologic and immunohistochemistry evaluation of ileum after manufactured and batch sequenced porcine epidemic diarrhea virus (PEDV) inoculated feed collected from mixer equipment[1]

	Morphology[2]			
Item	Villus height, μm	Crypt depth, μm	Villus height-to-crypt depth ratio, μm	Immunohistochemistry score (IHC)[3]
Feed from mixer[4]				
Negative[5]	375.0	166.0[ab]	2.3	0
SEM[6]	24.04	9.81	0.20	0.00
Positive	354.0	170.7[ab]	2.1	0.0
Sequence 1	366.2	165.3[ab]	2.2	0.6
Sequence 2	365.8	157.0[b]	2.3	0.8
Sequence 3	402.7	186.1[a]	2.2	0.0
Sequence 4	395.9	185.5[a]	2.2	0.0
SEM	20.37	8.31	0.13	0.10

[a,b]Means with different superscripts differ ($P < 0.05$).

[1]Each number is the mean of 6 pigs from the negative treatment and of 9 pigs per positive, sequence 1 to sequence 4 treatments. Only the crypt depth response criteria had statistical differences between feed treatments.

[2]Three serial cross-sections of ileum per pig were fixed in formalin and stained with hematoxylin and eosin (H&E) for evaluation.

[3]Three serial sections of ileum were evaluated and averaged into one categorical value per pig. Categorical values were assigned for each pig (0 = no signal (0% stained tissue), 1 = mild (1% to 10% stained tissue), 2 = moderate (11% to 25% stained tissue), 3 = abundant (26% to 50% stained tissue), 4 = diffuse (>50% to 100% stained tissue).

[4]Bioassay for negative, positive and sequence 1 and 2 was conducted in group 1. Bioassay for sequence 3 and 4 was conducted in group 2. Group 2 was performed 11 mo after group 1.

[5]Three pigs per negative treatment were in group 1 and 3 pigs per negative treatment were in group 2 for a total of 6 pigs averaged into one value.

[6]SEM are the pooled SEM values due to different sample variances: N = 6 pigs for negative treatment and N = 9 pigs for remaining treatments.

Chapter 3 - Characterizing the Rapid Spread of Porcine Epidemic Diarrhea Virus (PEDV) Through an Animal Food Manufacturing Facility

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Abstract

New regulatory and consumer demands highlight the importance of animal feed as a part of our national food safety system. Porcine epidemic diarrhea virus (PEDV) is the first viral pathogen confirmed widely transmissible in animal food. Because the potential for viral contamination in animal food is not well characterized, the objectives of this study were to 1) observe the magnitude of virus contamination in an animal food manufacturing facility, and 2) investigate a proposed method, feed sequencing, to decrease virus decontamination on animal food-contact surfaces. A U.S. virulent PEDV isolate was used to inoculate 50 kg swine feed, which was mixed, conveyed, and discharged into bags using pilot-scale feed manufacturing equipment. Surfaces were swabbed and analyzed for the presence of PEDV RNA by quantitative real-time polymerase chain reaction (qPCR). Environmental swabs indicated complete contamination of animal food-contact surfaces (0/40 vs. 48/48, positive baseline samples/total baseline samples, positive subsequent samples/total subsequent samples, respectively; $P < 0.05$) and near complete contamination of non-animal food-contact surfaces (0/24 vs. 16/18, positive baseline samples/total baseline samples, positive subsequent samples/total subsequent samples, respectively; $P < 0.05$). Flushing animal food-contact surfaces with a low-risk feed is commonly used to reduce cross-contamination in animal feed manufacturing. Thus, four subsequent 50 kg batches of virus-free swine feed was manufactured using the same system to test its impact on decontaminating animal food-contact surfaces. Even after 4 subsequent sequences, animal food-contact surfaces retained viral RNA (28/33 positive samples/total samples), with the conveying system being more contaminated than the mixer. A bioassay to test infectivity of dust from animal food-contact surfaces failed to produce infectivity. This study demonstrates the potential widespread viral contamination of surfaces in an animal food manufacturing facility and the

difficulty of removing contamination using conventional feed sequencing, which underscores the importance for preventing viruses from entering and contaminating such facilities.

Introduction

Federal regulations recognize animal feed as food and an important part of our national food supply. Recent changes in legislation through the Food Safety Modernization Act, along with evolving consumer demands, are placing greater emphasis on the role of animal food in the farm-to-fork food safety system [1]. Recently, porcine epidemic diarrhea virus (PEDV), a swine pathogen present in other parts of the world, was identified for the first time in the United States [2, 3]. The introduction of PEDV into U.S. herds was remarkable because of the sheer magnitude of infectivity and impact on animal health and welfare [4, 5]. Nonetheless, it was also significant because PEDV is one of the first viral pathogens confirmed transmissible in animal food. In one proof-of-concept study, suspected particulates of animal food and dust was found infectious [6]. Potential routes of viral introduction into the animal food manufacturing process have been identified [7]. Therefore, there is potential for viral contamination of animal food manufacturing facilities [8]. However, there is no available data describing the transmission of viruses in either animal or human food manufacturing facilities, nor are there established procedures to reduce or eliminate viral contamination on food-contact surfaces. This is particularly concerning because a proof-of-concept procedure proved elimination of PEDV RNA in an animal food manufacturing facility was challenging, and extreme decontamination measures including chemical disinfectants and heat were necessary [8]. More knowledge is needed to understand how a food-transmitted virus interacts with a manufacturing environment in order to ensure both animal and human health. Therefore, the objective of this study was to 1) characterize the extent of viral contamination in an animal food manufacturing facility and 2) test a proposed control method,

feed sequencing, to decrease viral decontamination on animal food-contact surfaces as measured by quantitative real-time PCR (qPCR) and infectivity by pig bioassay.

Materials and Methods

The animal food manufacturing portion of the experiments was conducted at the Kansas State University Cargill Food Safety Research Center (FSRC; Manhattan, KS), a 3-story biosafety level 2 biocontainment laboratory and animal food manufacturing facility containing pilot scale animal food manufacturing equipment. Procedures were approved by the Kansas State University Institutional Biosafety Committee (Approval No. 929.3). All manufacturing procedures were replicated three times. Decontamination occurred before and after each replicate to establish baseline and confirmed negative by the absence of PEDV RNA on animal food-contact and non-food contact surfaces as measured by qPCR as previously described [8].

The portion of the experiment evaluating infectivity in animals was conducted at Iowa State University. Procedures were approved by the Iowa State University Institutional Animal Care and Use Committee (Approval No. 1-16-8168-S).

Preparation of Inoculum

Virus isolation, propagation, and titration were performed in Vero cells (ATCC CCL-81) as previously described [9]. The U.S. PEDV prototype strain cell culture isolate USA/IN19338/2013 cell passage 8 was used to inoculate food in this study. The stock virus titer contained 4.5 x 10^6 $TCID_{50}$/ml, with a corresponding qPCR cycle threshold (Ct) value of 11. The virus was divided into three 500 ml aliquots that were stored at -80ºC, with one aliquot used per replication. For each replication, an aliquot was thawed overnight at 4°C, added to 4.5 kg of animal food using mixing procedures previously established [10] to form the animal food inoculum.

Animal Food Manufacturing

A corn-soybean meal-based diet with a composition typically fed to adult swine was manufactured at the Kansas State University O.H. Kruse Food Technology Innovation Center (Manhattan, KS) (Table 3.1). A subsample of the animal food was obtained prior to inoculation for each replication and confirmed PEDV negative by qPCR. Prior to inoculation, 50 kg of the animal food was mixed in a 0.113 m^3 electric paddle mixer (H. C. Davis Sons Manufacturing model# SS-L1; Bonner Springs, KS) that was previously validated to mix a 50 kg batch of animal food with CV less than 10%, as per standard mixing efficiency protocol [11]. The animal food was mixed for 5 min, then discharged at a rate of approximately 4.5 kg/min into the conveyor (Universal Industries, Cedar Falls, IA) that carried 74 buckets (each 114 cm^3) of animal food. The animal food was conveyed and exited through a downspout into biohazard bags.

Inoculation of Diet and Animal Food Manufacturing

The previously-prepared 5 kg of inoculum was added to 45 kg of virus-free animal food in a 0.113 m^3 electric paddle mixer (H. C. Davis Sons Manufacturing; Model SS-L1; Bonner Springs, KS) to form the positive control, and was mixed and discharged as described above. Four sequenced 50 kg batches (Sequence 1 to 4) of virus-free animal food were mixed and discharged following the positive control without any cleaning or decontamination between batches to mimic commercial animal food production conditions.

Environmental Observation

Prior to and after each batch of feed being manufactured, environmental surfaces were swabbed using large foam-tipped disposable swabs (World Bio-Products LLC, Woodinville, WA) that were pre-wetted with 2 ml of phosphate buffered saline. To collect samples, a clean

pair of disposable gloves was worn, each swab opened aseptically, and rubbed across the desired surface. Swabs were then capped and placed in a cooler with ice until analyzed.

Designated locations were sampled as illustrated in Figure 3.1. At each location, surfaces were outlined in heat-stable marker to form 5 equal-sized subsample areas. One randomly selected area was swabbed at each location before manufacturing (baseline), and after each manufactured batch of animal food. Designated surfaces included the drain, floor with high foot traffic, floor with low foot traffic, garage door, table ledge, mixer paddle, mixer interior lid and mixer interior of bottom, boots worn during the experiment, the interior of 4 plastic conveyer buckets (one swab each) and 4 rubber belt areas (one swab each) adjacent to the chosen buckets. Swabs were categorized by surface (metal, concrete, plastic vs. rubber) within zone (animal food-contact vs. non-animal food contact). Immediately after completion of the study, supernatant from swabs were transferred to 96-well plates and plates were stored frozen at -80°C until initiation of the bioassay. The plates were then thawed at room temperature, supernatant was pooled according to replicate and treatment for each pig and were then stored at 4°C overnight until used for bioassay the next day (0 DPI).

Pig Study

Eighteen pigs were purchased from a conventional breeding farm and delivered to the Iowa State University Laboratory Animal Resource (LAR) facilities. All pigs were administered an intramuscular dose of ceftiofur (Exede; Zoetis, Florham Park, NJ) per label instructions upon arrival and confirmed negative for PEDV, porcine delta coronavirus (PDCoV), transmissible gastroenteritis virus (TGEV) and porcine rotaviruses (groups A, B, and C) by virus specific qPCR on rectal swabs. In addition, pigs were confirmed PEDV antibody negative by fluorescent

foci neutralization serologic analysis performed at South Dakota State University Veterinary Diagnostic Laboratory (SDSU VDL).

Bioassay was conducted 11 months after animal food preparation and sample collection. A total of 6 rooms (3 pigs per room) were assigned to swabbed dust samples collected from the conveyer after production of each animal food treatment (1 negative control room and 5 challenge rooms). Pigs were blocked by weight, then randomly divided into groups of 3 per room. Rooms had independent ventilation systems and solid flooring that was minimally rinsed to reduce PEDV aerosols. Pig were fed liquid milk replacer (Esbilac; PetAg, Hampshire, IL) and commercially pelleted diet (All Natural Starter 2; Heartland Co-op, Alleman, IA). Pigs had ad libitum access to food and water at all times.

After 2 days of acclimation, each pig was administered the dust suspension from swabbed surfaces by orogastric gavage using an 8–gauge French catheter and 60 ml syringe (8 ml/pig), which marked day 0 post inoculation (0 DPI). The 8 ml aliquot combined eight 1-ml dust suspensions sampled from 4 buckets and 4 adjacent belt areas after manufacturing each food treatment from one replicate. Thus, each pig represented 1 of 3 replicates per treatment and each room represented each treatment.

Rectal swabs were analyzed from all pigs on -2, 0, 2, 4, 6, and 7 DPI. Swabs were submerged into 1 ml phosphate buffered saline (PBS, 1 × pH 7.4) immediately after collection and submitted to Iowa State University Veterinary Diagnostic Laboratory (ISU VDL) for PEDV RNA by qPCR. All pigs were euthanized at 7 DPI for necropsy by intravenous overdose of pentobarbital sodium solution as per label instructions (Fatal-Plus; Vortech Pharmaceuticals Ltd, Dearborn, MI). At necropsy, an aliquot of fresh cecal contents was submitted for PEDV qPCR to ISU VDL.

RNA extraction and quantitative PEDV RT-PCR (qPCR)

Dust samples from swabs were tested at Kansas State University Molecular Diagnostics Development Laboratory (Manhattan, KS) for PEDV using a PEDV spike (S) gene-based qPCR. Nucleic acids were extracted from a 50 µL sample of supernatant. Automated extraction was carried out on a KingFisher magnetic particle processor (Thermo Scientific, Waltham, MA) using a MagMAX-96 Viral RNA Isolation Kit (Life Technologies, Grand Island, NY). All manufacturer's instructions were followed, with the exception of a final elution volume of 60 µl. Each 96-well extraction run included an extraction positive control (PEDV stock virus) and an extraction negative control (1x PBS). Four µl of RNA template was used in qPCR setup in a 20 µl reaction using a real time RT-PCR kit (Path-ID Multiplex One-Step RT-PCR Kit; Thermo Scientific, Waltham, MA). Amplification reactions were conducted on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The thermal cycling parameters were: 10 min reverse transcription at 48°C, 10 min of reverse transcriptase inactivation/initial denaturation at 95°C followed by 45 cycles of 10 sec at 95°C and 40 sec at 60°C.

Animal samples and samples for bioassay were tested for PEDV using a previously described PEDV nucleocapsid (N) gene-based qPCR [12]. Nucleic acids were extracted from the stock virus (50 µl), bioassay inoculum (100 µl), and rectal swabs (100 µl), and eluted into 90 µl of elution buffer using an RNA/DNA kit (MagMAX Pathogen RNA/DNA Kit; Thermo Scientific, Waltham, MA) and a Kingfisher-96 magnetic particle processor (Thermo Scientific, Waltham, MA) following the manufacturer's instructions. Five µl of RNA template was used in qPCR setup in a 25 µl reaction using a real time RT-PCR kit (Path-ID Multiplex One-Step RT-PCR Kit; Thermo Scientific, Waltham, MA). Amplification reactions were conducted on an ABI

7500 Fast instrument (Thermo Scientific, Waltham, MA) following previously described procedures [12].

Statistical Analysis

Swabs were categorized as animal food-contact and non-animal food-contact surfaces. Within animal food-contact surface, Ct analysis of the metal mixer, plastic conveyer buckets, and rubber conveyer belt were performed using PROC GLIMMIX (SAS Institute, Inc., Cary, NC). Within animal food-contact surface, the statistical model evaluated the effect of treatment (negative, positive, sequence 1, sequence 2, sequence 3 and sequence 4) and surface (metal mixer, plastic conveyer buckets, and rubber conveyer belt) and the associated interaction. Each swab was classified from treatment and surface type. The LSMEANS procedure compared surface type among treatments within animal food-contact surfaces by pairwise comparison. The non-animal food-contact surfaces were reported in the results text using descriptive statistics; non-animal food-contact swabs were organized by surface type (metal garage, metal tabletop, concrete floor, and rubber boot bottoms worn during the experiment) among treatments. Samples considered negative by qPCR were evaluated as a value of 45 in the statistical model. Results were considered significant at $P \leq 0.05$.

Results

As expected, all animal food-contact negative control swabs were qPCR negative (Table 3.2). After the positive treatment was manufactured, the count of qPCR positive swabs increased to 100%. After sequence 1, 100% of swabs remained qPCR positive, and the mean Ct of samples from the metal mixer were higher ($P < 0.05$) than plastic conveyer buckets or rubber belt. After sequence 2, 67% of metal mixer swabs were qPCR positive, whereas 100% of plastic conveyer buckets and rubber belt swabs were qPCR positive. After sequence 3 and four, 44% of

metal mixer swabs were qPCR positive and 100% of plastic conveyer buckets and rubber belt were again qPCR positive. For mean Ct values, there was an animal-food contact surface × treatment interaction ($P < 0.05$). Following manufacturing of the positive batch of animal food, the mean Ct value of the metal mixer increased through sequence 3, however there was no significant Ct or further improvement after sequence 4. Unlike the metal mixer, the mean Ct value of surfaces from the conveyor rubber belt did not change after sequencing animal food following manufacturing of the positive animal food treatment. For the plastic conveyer buckets, following sequence 1 there was a Ct increase ($P < 0.05$) followed by another increase after sequence 2, however sequence 2 and 3 did not differ. Additionally, after sequence 4, Ct values did not differ after sequence 3, however was lower ($P < 0.05$) than Ct values after sequence 2.

All non-animal food-contact surface baseline swabs were qPCR negative. Non-animal food-contact swabs were analyzed by surface type (metal garage, metal tabletop, concrete floor, and rubber boot bottoms worn during the experiment). Unexpectedly, in 1 of 3 repetitions, 1.7% of non-animal food-contact surface swabs were qPCR positive after the negative treatment was manufactured, although the animal food was qPCR negative. For all repetitions, after the positive treatment and after sequence 1, 89% of non-food-contact surface swabs were qPCR positive. After sequence 2, 94% of non-food-contact surface swabs were qPCR positive. After sequence 3, 89% of non-food-contact surface swabs were positive that again increased to 94% after sequence 4. The percentage of positive swabs from non-animal food-contact metal surfaces (metal garage and tabletop) varied, whereas non-animal food-contact concrete floor and rubber boot bottoms remained the same (67%, 67%, 83%, 67%, 83%; after positive, after sequence 1, after sequence 2, after sequence 3 and after sequence 4, respectively vs. 100% after positive and sequence 1 to

4, respectively). Dust suspensions from animal food-contact surfaces were challenged in pigs and failed to produce infectivity.

Discussion

The recent enacting of the Food Safety Modernization Act (FSMA) requires animal food manufacturers to identify and control animal food safety hazards because feed is considered animal food and a part of the human food safety system [1]. Hazard characterization includes biological hazards, such as *Salmonella* spp. and *Listeria monocytogenes* [13]; however viral pathogens were not traditionally considered common biological hazards in animal food until after the introduction of PEDV to North America. Recent research identified swine food as one of many potential vectors for virus transmission, and confirmed PEDV contaminated foodstuffs may cause disease [14, 15]. While animal food is not likely the predominant vector, it was one of the remaining potential vectors for PEDV transmission that was not previously controlled by on-farm biosecurity measures. This is concerning because little is known about virus contamination during the manufacturing of animal food. Likewise, viral transmission in animal food manufacturing facilities is not well characterized, nor are tested control methods available to reduce contamination on animal food-contact surfaces. While there are no currently identified similar cases of viral transmission through the human food chain, its potential exists and information gleaned from studying PEDV transmission may be applicable if a virus impacting human health were to enter the human food manufacturing system.

For these reasons, an established protocol for monitoring viral transmission is needed to model animal and human food hazards if additional pathogenic viruses are discovered in our food supply. This is the first study of its kind to fully observe environmental contamination of an animal food-manufacturing facility during a proposed control method after manufacturing viral-

inoculated swine food. Objectives were met by monitoring the extent of virus contamination in an animal food manufacturing facility and investigating a control method to decrease virus contamination on animal food-contact surfaces.

In general, environmental contamination of a virus in any food manufacturing facility has not been well-documented. In human food, norovirus is a known cause of foodborne illness with contamination presumed at point-of-service [16, 17]. However, there is little information regarding norovirus-contaminated food at the manufacturing level due to inadequate surveillance or facility control measures [18]. Even less is known about viral contamination in animal food manufacturing facilities.

The results from this study clearly demonstrate the extent of the widespread viral contamination that occurs in an animal food manufacturing facility following production of virus-inoculated animal food. All of the animal food-contact surfaces and most of the non-animal food-contact surfaces were qPCR positive when swabbed after the contaminated animal food was manufactured and remained qPCR positive after multiple batches of animal food were mixed and conveyed. Therefore it seems that the proposed mitigation technique (feed batch sequencing) did not mitigate environmental PEDV contamination. Additionally, detectible PEDV seemed to persist on some animal food-contact surfaces, such as plastic and rubber conveyors, more than others such as metal. Previous studies have investigated the survivability of virus on inanimate surfaces and determined viral persistence in the environment can be affected by several factors including surface type [19-21]. Additionally, different surface types can have different characteristics such electrostatic, hydrophobic or ionic strength which may impact virus detectability on these surfaces [22, 23]. For example, it has been reported that electrostatic forces impact virus attachment to lettuce [24]. Therefore, it is possible that physical

properties contributed to the persistence of PEDV on animal food-contact surfaces sampled in the current study. This is interesting because most animal food manufacturing equipment have been designed for electrical efficiency and physical cleanout, but not sanitization. For example, plastic conveyer buckets are preferred not only because they are light and more energy efficient, but they are also safer for workers due to elimination of sparking that is a concern with sheeted metal buckets [25].

In pet food manufacturing, equipment surfaces are easy-to-clean with non-porous equipment surfaces selected in order to prevent biofilms or the prevalence of *Salmonella* spp. or *Listeria monocytogenes*. They are also routinely sanitized with steam or chemical sanitizers. Other animal food manufacturing facilities have not selected equipment for these purposes due to previously limited risk for biological hazards. Thus, other strategies, such as use of chemical additives in animal food, may need to be employed to reduce cross-contamination of PEDV in animal food or ingredients [26].

Alternatively, the difference in rate of contamination between the metal mixer or plastic and rubber in the conveyor may be due to equipment design. For example, mixers are typically designed to self-clean with little residual material from one batch to the next compared to conveyors. This is particularly true of bucket elevators, which is the conveyor type used in this experiment. The large rubber belt of a bucket elevator is suspended vertically, and plastic buckets convey feed upward until the feed is flipped from the buckets into a discharge chute. The boot pit, which is the area at the bottom of the bucket elevator, must be large enough for buckets to clear the bottom without coming into contact with the guard or cover. This area typically fills with residual feed and may lead to batch-to-batch cross contamination, which has been demonstrated by carryover of animal drugs [27]. Therefore, it is reasonable to extrapolate that

batch-to-batch carryover of feed residue may also exist when the hazard is an undesirable microorganism.

This research concludes that differences exist in viral contamination rates on different equipment surfaces, which may be due to differences in surface type, equipment design, or other phenomena. Regardless of the source of these differences, animal food manufacturing facilities at risk for PEDV contamination should consider these findings when choosing manufacturing equipment. The results of the current experiment are applicable to other species of animal food and to human food manufacturing facilities because entry of a viral pathogen may cause widespread contamination that is difficult to eliminate. Even with wet chemical cleaning and facility heating, PEDV proved difficult to decontaminate from our facility [8]. This is concerning because extreme methods were used, which are impractical in commercial animal food manufacturing settings.

In the current study, environmental surfaces were swabbed for dust following production of PEDV inoculated animal food and animal-food contact surfaces were evaluated for infectivity. A previous proof-of-concept-study demonstrated that animal food dust can be infectious [6]. Although the exact cause for lack of infectivity in this study is unknown, storage time may have impacted virulence in these samples since long-term low temperature storage has been reported to affect virus fitness and recoverability [28-30]. Additionally, although the minimum infectious dose is low in animal food [10], perhaps not enough viral particles were collected by or eluded from swabs to cause an infection in the present study. Although we were unsuccessful at finding evidence of infectivity in this study, the hypothesis that environment dust is infectious after animal food batch sequencing is still conceivable and remains to be proven.

Another result from this study is that some non-food contact swabs from a repetition were qPCR positive after the negative animal food was manufactured, although importantly, animal food tested was qPCR negative. We hypothesize this genetic material remained on the boot due to inadequate cleaning after a previous replicate and was tracked then detected on the concrete floor. Due to the chemical cleaning between repetitions, the viral material should not have been infective [31]. However, we believe contaminated rubber boot bottoms worn during the experiment helped track and spread the virus as genetic material was consistently detected on concrete floor surfaces. This underscores the importance of foot traffic biosecurity in any facility, including animal food manufacturing facilities [7]. This is especially true as demonstrated in one study, PEDV and porcine deltacorona virus was detected from multiple locations within and around animal food manufacturing facilities [32] which again illustrates foot traffic can be a biosecurity problem. Therefore, key implications from these findings is that foot traffic should be limited across receiving pits or in hand-add areas that have direct access to animal food contact equipment and boots should be cleaned regularly to minimize risk of inadvertent contamination.

As the current study demonstrates, widespread contamination of PEDV occurred and was detected on most surfaces. Material collected from dust collection systems and sweepings should be collected and disposed instead of added to the product flow as per traditional measures [7, 33]. Therefore, animal food manufacturing facilities should re-consider before using dust collected from dust disposal systems and instead consider including procedures to minimize and control dust since it could be a vector of possibly infectious PEDV. Again, once an animal food manufacturing facility is contaminated with an undesired microorganism, it is difficult to eliminate and thus prevention protocols should be implemented [34, 35].

In conclusion, this study clearly demonstrates widespread contamination occurred in an animal food manufacturing facility following PEDV swine food production. Furthermore, the proposed mitigation method of feed batch sequencing was not effective to reduce environmental contamination, although the potential impact of PEDV contamination and importance to prevent virus entry in such facilities was better understood. It is concerning once an animal food manufacturing facility is contaminated with PEDV, it appears to harbor PEDV until chemically cleaned. This research indicates animal food manufacturing facilities potentially contaminated with PEDV can be a central point for virus transmission and the quantification for this risk should be assessed. As a result, the practicality of decontamination is a new challenge facing our animal food manufacturing facilities.

References

1. FDA. Background on the FDA Food Safety Modernization Act (FSMA) 2011. Available from: http://www.fda.gov/Food/GuidanceRegulation/FSMA/ucm247546.htm. Accessed 23 May 2016.
2. Davies PR. The dilemma of rare events: porcine epidemic diarrhea virus in North America. Prev Vet Med. 2015;122(1-2):235-41. doi: 10.1016/j.prevetmed.2015.08.006
3. Huang Y-W, Dickerman AW, Piñeyro P, Li L, Fang L, Kiehne R, et al. Origin, evolution, and genotyping of emergent porcine epidemic diarrhea virus strains in the United States. MBio. 2013;4(5):e00737-13. doi: 10.1128/mBio.00737-13
4. Alvarez J, Sarradell J, Morrison R, Perez A. Impact of porcine epidemic diarrhea on performance of growing pigs. PLoS One. 2015;10(3):e0120532. doi: 10.1371/journal.pone.0120532
5. Jung K, Saif LJ. Porcine epidemic diarrhea virus infection: Etiology, epidemiology, pathogenesis and immunoprophylaxis. Vet J. 2015;204(2):134-43. doi: 10.1016/j.tvjl.2015.02.017
6. Dee S, Clement T, Schelkopf A, Nerem J, Knudsen D, Christopher-Hennings J, et al. 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 Vet Res. 2014;10(1):176. doi: 10.1186/s12917-014-0176-9
7. Cochrane RA, Dritz SS, Woodworth JC, Stark CR, Huss AR, Cano JP, et al. Feed mill biosecurity plans: A systematic approach to prevent biological pathogens in swine feed. J Swine Health Prod. 2016;24(3):154-64

8. Huss AR, Schumacher LL, Cochrane RA, Poulsen EG, Bai J, Woodworth JC, et al. Proof-of-concept method to sanitize a feed mill contaminated with porcine epidemic diarrhea virus. Proc Mid Sec Amer Soc Anim Sci; 2016.

9. Chen Q, Li G, Stasko J, Thomas JT, Stensland WR, Pillatzki AE, et al. Isolation and characterization of porcine epidemic diarrhea viruses associated with the 2013 disease outbreak among swine in the United States. J Clin Microbiol. 2014;52(1):234-43. doi: 10.1128/JCM.02820-13

10. Schumacher LL, Woodworth JC, Jones CK, Chen Q, Zhang J, Gauger PC, et al. Evaluation of the minimum infectious dose of porcine epidemic diarrhea virus in a virus-inoculated feed. Am J of Vet Res (In press). 2016

11. McCoy RA. Mixer Testing. In: Schofield EK, editor. Feed manufacturing technology V. Arlington, VA: American Feed Industry Association; 2005. pp. 620-2.

12. Thomas JT, Chen Q, Gauger PC, Gimenez-Lirola LG, Sinha A, Harmon KM, et al. Effect of porcine epidemic diarrhea virus infectious doses on infection outcomes in naive conventional neonatal and weaned pigs. PLoS ONE. 2015;10(10):e0139266. doi: 10.1371/journal.pone.0139266

13. Current good manufacturing practice, hazard analysis, and risk-based preventive controls for food for animals, 21 C.F.R. Part 11, 116, 117, 500, 507, and 579 (2015).

14. Dee S, Clement T, Schelkopf A, Nerem J, Knudsen D, Christopher-Hennings J. An evaluation of contaminated complete feed as a vehicle for porcine epidemic diarrhea virus infection of naïve pigs following consumption via natural feeding behavior: Proof of concept. BMC Vet Res. 2014;10. doi: 10.1186/s12917-014-0176-9

15. Pasick J, Berhane Y, Ojkic D, Maxie G, Embury-Hyatt C, Swekla K, et al. Investigation into the role of potentially contaminated feed as a source of the first-detected outbreaks of porcine epidemic diarrhea in Canada. Transbound Emerg Dis. 2014;61(5):397-410. doi: 10.1111/tbed.12269

16. Widdowson MA, Sulka A, Bulens SN, Beard RS, Chaves SS, Hammond R, et al. Norovirus and foodborne disease, United States, 1991-2000. Emerging Infectious Diseases. 2005;11(1):95-102

17. de Graaf M, van Beek J, Koopmans MP. Human norovirus transmission and evolution in a changing world. Nat Rev Microbiol. 2016;14(7):421-33. doi: 10.1038/nrmicro.2016.48

18. Malek M, Barzilay E, Kramer A, Camp B, Jaykus L-A, Escudero-Abarca B, et al. Outbreak of norovirus infection among river rafters associated with packaged Delicatessen meat, Grand Canyon, 2005. Clin Infect Dis. 2009;48(1):31-7. doi: 10.1086/594118

19. Vasickova P, Pavlik I, Verani M, Carducci A. Issues concerning survival of viruses on surfaces. Food Environ Virol. 2010;2(1):24-34. doi: 10.1007/s12560-010-9025-6

20. Bean B, Moore BM, Sterner B, Peterson LR, Gerding DN, Balfour HH. Survival of influenza viruses an environmental surfaces. J of Infect Dis. 1982;146(1):47-51

21. Kramer A, Schwebke I, Kampf G. How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. BMC Infect Dis. 2006;6(1):1-8. doi: 10.1186/1471-2334-6-130

22. Hurst CJ, Gerba CP, Cech I. Effects of environmental variables and soil characteristics on virus survival in soil. Appl Environ Microbiol. 1980;40(6):1067-79

23. Lacroix-Gueu P, Briandet R, Lévêque-Fort S, Bellon-Fontaine M-N, Fontaine-Aupart M-P. In situ measurements of viral particles diffusion inside mucoid biofilms. Comptes Rendus Biologies. 2005;328(12):1065-72

24. Vega E, Garland J, Pillai SD. Electrostatic forces control nonspecific virus attachment to lettuce. J Food Prot. 2008;71(3):522-9

25. Fairchild F. Bucket Elevators. In: Schofield EK, editor. Feed manufacturing technology V. Arlington, VA: American Feed Industry Association; 2005. pp. 203-7.

26. Cochrane RA. Chemical mitigation of microbial pathogens in animal feed and ingredients. MS Thesis, Kansas State Univ. 2015. Available: http://krex.k-state.edu/dspace/handle/2097/20105.

27. Herrman T, Behnke K, Loughin T. Mixing and clean-out properties of sulfamethazine and carbadox in swine feed. J Swine Health and Prod. 1995;3(5):127-31

28. Nelson J, Okda F, Parmar R, Singrey A, Lawson S, Liu X, et al. Environmental stability of cell culture adapted U.S. isolate of PEDV. 23rd Intern Pig Vet Soc Congress; 2014 June 8-11; Cancun, Quintana Roo, Mexico.

29. Gerba CP. Applied and theoretical aspects of virus adsorption to surfaces. Adv Appl Microbiol. 1984;30:133

30. Hurst C, Gerba CP, Lance J, Rice R. Survival of enteroviruses in rapid-infiltration basins during the land application of wastewater. Appl Environ Microbiol. 1980;40(2):192-200

31. Bowman AS, Nolting JM, Nelson SW, Bliss N, Stull JW, Wang Q, et al. Effects of disinfection on the molecular detection of porcine epidemic diarrhea virus. Vet Microbiol. 2015;179(3-4):213-8. doi: 10.1016/j.vetmic.2015.05.027

32. Greiner LL. Evaluation of the likelihood of detection of porcine depidemic diarrhea viurs or porcine delta coronavirus ribonucleic acid in areas within feed mills. J Swine Health and Prod. 2016;24(4):198-204

33. McDaniel GL. Dust collection systems. In: Schofield EK, editor. Feed manufacturing technology V. Arlington, VA: American Feed Industry Association; 2005. pp. 238.

34. Huss AR, Cochrane RA, Deliephan A, Stark CR, Jones CK. Evaluation of a biological pathogen decontamination protocol for animal feed mills. J Food Prot. 2015;78(9):1682-8. doi: 10.4315/0362-028X.JFP-15-052

35. Huss AR, Schumacher LL, Cochrane RA, Poulsen E, Bai JF, Woodworth JC, et al. Proof-of-concept method to sanitize a feed mill contaminated with porcine epidemic diarrhea virus [abstract]. J. Anim. Sci. 2016;94 2. doi: 10.2527/msasas2016-217

Tables

Table 3.1 Diet composition of porcine epidemic diarrhea virus (PEDV) inoculated animal food, as fed basis

Ingredient, %	Composition
Corn	79.30
Soybean meal, 46.5% CP	15.70
Choice white grease	1.00
Calcium phosphate (monocalcium)	1.40
Limestone	1.15
Salt	0.50
L-Threonine	0.03
Trace mineral premix[a]	0.15
Sow add pack[b]	0.50
Vitamin premix[c]	0.25
Phytase[d]	0.02
Total	100.00
Formulated analysis[e], %	
DM	91.4
CP	17.1
Crude fiber	3.7
Ca	0.78
P	0.52
Fat	3.5

[a]Each kilogram of premix contains 73 g Fe, 73 g Zn, 22 g Mn, 11g Cu, 0.198 mg I, and 0.198 mg Se.

[b]Each kilogram of premix contains 4,409 IU vitamin E, 44 mg biotin, 992 mg pyridoxine, 331 mg folic acid, 110,229 mg choline, 40 mg chromium, 9,920 mg L-carnitine.

[c]Each kilogram of premix contains 4,409,171 IU vitamin A, 551,146 IU vitamin D_3, 17,637 IU vitamin E, 1,764 mg menadione, 3,300 mg riboflavin, 11,023 mg d-pantothenic acid, 19,841 mg niacin, 15 mg vitamin B_{12}.

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

[e]One sample was analyzed by Ward Laboratories Inc., Kearney, NE.

Table 3.2 Effect of contamination on animal food-contact zone and their types after porcine epidemic diarrhea virus (PEDV) inoculated animal food manufacturing†

	Treatment					
Item	Negative	Positive	After sequence 1	After sequence 2	After sequence 3	After sequence 4
Contact Zone, Detectable RNA/Total‡						
Animal food-contact						
Metal mixer¶	0/9	9/9	9/9	6/9	4/9	4/9
Plastic conveyor bucket#	0/12	12/12	12/12	12/12	12/12	12/12
Rubber conveyor belt††	0/12	12/12	12/12	12/12	12/12	12/12
Swab, Ct*						
Metal mixer	45.0^{a}	29.2^{h}	33.9^{de}	38.2^{c}	40.7^{b}	40.5^{b}
Plastic conveyor buckets	45.0^{a}	30.8^{h}	32.1^{efg}	34.2^{d}	32.8^{def}	32.1^{efg}
Rubber conveyor belt	45.0^{a}	30.8^{gh}	31.5^{fg}	31.5^{fg}	32.2^{efg}	32.1^{efg}

^{a,b,c,d,e,f,g,h}Means with different superscripts differ (P <0.05).

†Tissue culture fluid containing 4.5×10^{6} $TCID_{50}$/ ml of PEDV was inoculated into 45 kg of PEDV negative food to form the positive treatment. For each negative, positive and sequenced batch, food was mixed for 5 min, discharged for 10 min into a conveyer and collected upon exit. Dust was then collected from surfaces using swabs pre-wetted with 2 ml of PBS. Equipment was not cleaned between treatments. Sequences were formed by sequentially adding 50 kg of PEDV negative food to the mixer after the prior batch was processed. This experiment was replicated 3 times. For swab Ct analysis, surface × treatment P <0.0001 and pooled SEM = 0.67.

‡Count of swabs with detectible PEDV RNA/number of swabs analyzed.

¶Metal includes one sample each from the mixer paddle, mixer interior lid, and mixer interior bottom.

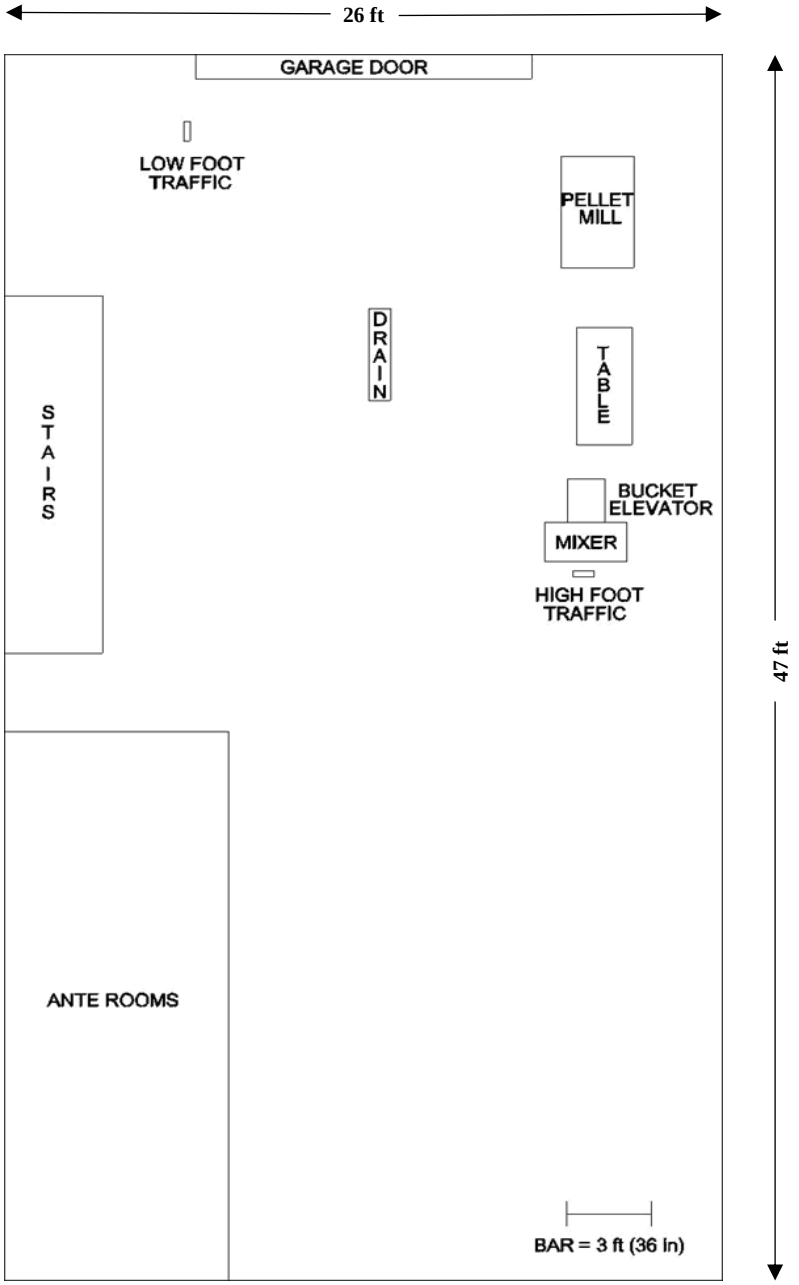
#Plastic includes one swab each from 4 randomly chosen interior conveyor buckets.

††Rubber includes one sample each from 4 belt areas adjacent to chosen conveyor buckets.

*Mean cycle threshold (Ct) value of samples. A value of 45.0 was used for samples with no detectible PEDV RNA.

Figure

Figure 3.1 Arrangement of the first floor of the Kansas State University Cargill Food Safety Research Center. Designated areas swabbed for PEDV qPCR analysis include high and low foot traffic areas (concrete), drain (concrete) , garage door (metal), pellet mill (equipment), table ledge (metal), conveyer (equipment), and food mixer (equipment). Not shown are rubber boot bottoms (rubber).



Chapter 4 - Assessment of RNA Detection in Porcine Epidemic Diarrhea Virus (PEDV)-Inoculated Feed and Spray Dried Porcine Plasma Across 5 Diagnostic Laboratories

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Abstract

Porcine epidemic diarrhea virus (PEDV) is a highly transmissible enteric swine pathogen that has devastated swine herds in North America since its recognized emergence in 2013. Real time quantitative PCR (qPCR) is a widely used detection method for PEDV and is used for analyzing non-clinical samples such as feed, ingredients, and environmental surfaces since animal feed and spray-dried porcine plasma (SDPP) has been discovered as a vector for viral transmission. However, qPCR performance on these matrixes is not well characterized. Therefore, five diagnostic laboratories were selected to analyzed feed and SDPP samples inoculated with a high virus load and low virus load of PEDV prototype strain cell culture isolate USA/IN19338/2013 containing an initial titer of 4.5×10^6 $TCID_{50}$/ml. Results indicated laboratory, matrix, and virus load and their interactions were found to impact the detection of PEDV RNA ($P < 0.05$). One laboratory generated lower ($P < 0.05$) Ct values as compared to the others. Ct values differed ($P < 0.05$) across laboratories, however with the exception of one

laboratory, the magnitude of the difference was small and may be not biologically significant. When matrixes were inoculated with the low virus load (100-fold dilution of stock virus), the intra-assay variation increased as compared to the intra-assay variation from the matrixes inoculated with the high virus load (10-fold dilution of stock virus). Overall, it appears qPCR PEDV RNA detection in feed and SDPP was precise as quantified by low coefficient of variation across laboratories, with the exception of one %CV from SDPP inoculated with low virus load from one laboratory. Although the magnitude of the Ct value difference was large in only 1 of 5 laboratories, comparisons of Ct values across laboratories should be interpreted cautiously. Finally, qPCR can be a useful surveillance tool for detection of PEDV RNA in non-clinical samples such as feed and SDPP.

Introduction

Infectious porcine epidemic diarrhea virus (PEDV) has been confirmed in feed and spray dried porcine plasma (SDPP), a common feed ingredient in swine diets [1, 2]. Animal feed and feed ingredients were unexpected routes of PEDV transmission because in the past, these matrixes were not considered a major risk factor for viral disease transmission and seldom evaluated as biologic hazards. Quantitative real-time reverse transcription PCR (qPCR) assays were rapidly developed for clinical samples in the US for PEDV RNA detection [3] and then adapted for evaluation of feed and environmental samples. Since the US PEDV outbreak, veterinary diagnostic laboratories serving swine clientele have seen an increase in feed, feed ingredients and environmental sample submission for PEDV qPCR analysis. For example, the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL) analyzed a total of 36,983 of feed, feed ingredient and environmental samples for PEDV qPCR from May 2013 through May 2016 (ISU VDL, unpublished data). From May 2013 to April 2014, there were 9,980 of

these sample types analyzed by PEDV qPCR. From May 2014 to April 2015, submission of these sample types to ISU VDL increased 51% (9,980 to 15,059) (ISU VDL; unpublished data). However, the performance of molecular diagnostics on feed and feed ingredients is not well characterized especially between diagnostic laboratories. A challenge for qPCR PEDV diagnostics is different protocols and primers are used across veterinary diagnostic laboratories. Inter-laboratory assay comparisons for different pathogens have been conducted to proficiency test clinical samples such as tissues and feces, however we are unaware of any formal surveys that use feed samples. Therefore, the objective of this study was to assess reproducibility of PEDV RNA detection in feed and SDPP inoculated at 2 virus load levels by qPCR analysis across five diagnostic laboratories.

Materials and Methods

Stock virus

Virus isolation, propagation, and titration were performed in Vero cells (ATCC CCL-81) as previously described [4]. The United States (US) PEDV prototype strain cell culture isolate USA/IN19338/2013 previously demonstrated as pathogenic [5] was used to inoculate a corn soybean meal-based swine feed with identical formation as previously described [5] and spray dried porcine plasma (SDPP) (American Proteins, Cumming, GA). The stock PEDV was cell passage 8 with a titer of 4.5×10^6 50% tissue culture infectious dose/ml ($TCID_{50}$/ml) and had a corresponding PCR cycle threshold (Ct) value of 11. A subsample of the feed and SDPP was obtained prior to inoculation and confirmed negative for the presence of PEDV by qPCR in Laboratory A.

Sample Inoculation and Laboratory Submission

Stock virus was first diluted to either 1:10 or 1:100 with tissue culture media (Gibco cell culture media, Life Technologies, Grand Island, NY). Then 111 ml of the diluted inoculum was added to 100 g of swine diet (feed) or SDPP in 1 L glass jars. Therefore, 2 jars contained swine diet or SDPP that were inoculated with 1:10 diluted inoculum and 2 jars contained swine diet or SDPP inoculated with 1:100 diluted inoculum for a total of 4 treatment jars. The 1:10 inoculated matrixes (high virus load) had an estimated 2.36×10^5 $TCID_{50}$/g and the 1:100 inoculated matrixes (low virus load) had an estimated 2.36×10^4 $TCID_{50}$/g. Next, 400 ml of 1 × PBS (Life Technologies, Grand Island, NY) was added to each jar and then jars were sealed, shaken until homogenous (1-2 minutes), and rested overnight at 4°C. The supernatant eluded from the high virus load inoculated matrixes was estimated at 8.15×10^4 $TCID_{50}$/ml and the supernatant from the low virus load was estimated at 8.15×10^3 $TCID_{50}$/ml. Without remixing, supernatant from each jar was then divided into three, 1 ml aliquot samples placed in 2 ml microfuge tubes. Each laboratory submission contained four treatments with three replicates from each treatment for a total of 12 aliquots submitted per laboratory. Samples were sent to 5 laboratories including the following: Kansas State University Veterinary Diagnostic Laboratory A and B in Manhattan, KS; Iowa State University Veterinary Diagnostic Laboratory in Ames, IA; South Dakota State University Veterinary Diagnostic Laboratory in Brookings, SD; and University of Minnesota Veterinary Diagnostic Laboratory in St. Paul, MN. The selected laboratories analyzed the samples by qPCR using protocols routinely used in their laboratory. Results from laboratories were blinded upon receipt and for this publication, and are reported as laboratory A, B, C, D and E. Samples for laboratory A and B were immediately submitted to the laboratory. Samples for

laboratory C, D, and E were shipped overnight on dry ice. None of the samples were frozen prior to submission.

Diagnostic Laboratory Procedures

Laboratory A

Nucleic acids were extracted from a 50 µL sample of supernatant. Automated extraction was carried out on a KingFisher magnetic particle processor (Thermo Scientific, Waltham, MA) using a MagMAX-96 Viral RNA Isolation Kit (Life Technologies, Grand Island, NY). All manufacturer's instructions were followed, with the exception of a final elution volume of 60 µl. Each 96-well extraction run included an extraction positive control (PEDV stock virus) and an extraction negative control (1 × PBS). An in-house-developed duplex qPCR assay targeting the spike gene (S) of PEDV and host 18S rRNA (internal control) was used for the detection and quantification of PEDV. The 20 µl reaction mixture comprised 1 × Path-ID Multiplex One-Step RT-PCR buffer, 2 µl Path-ID Multiplex One-Step Enzyme Mix (Thermo Scientific, Waltham, MA), 500 nM each of three PEDV primers, 500 nM each of two 18S primers, 62.5 nM of each probe (PEDV and 18S), and 4 µl of nucleic acid extract template. Amplification was performed on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The thermal cycling parameters were: 10 min reverse transcription at 48°C, 10 min of reverse transcriptase inactivation/initial denaturation at 95°C followed by 45 cycles of 10 sec at 95°C and 40 sec at 60°C.

Laboratory B

Nucleic acid extraction was carried out as described for laboratory A. Again, the same qPCR assay procedures were used except the assay targeted primers in the nucleoprotein (N)

gene of PEDV. Amplification was performed on ABI 7500 (Thermo Scientific, Waltham, MA) using parameters as described for laboratory A.

Laboratory C

Extraction procedures and qPCR assay targeting the N gene of PEDV were previously described [6]. Briefly, the extraction of nucleic acids was performed using MagMAX Pathogen RNA/DNA kit (Thermo Scientific, Waltham, MA) following manufacturer's instructions. Five µl of extracted RNA was used in the PCR setup in the 25 µl total reaction using TaqMan Fast 1-Step Master Mix (Thermo Scientific, Waltham, MA). Amplification reactions were performed on an ABI 7500 Fast thermal cycler (Thermo Scientific, Waltham, MA) with the following parameters: 1 cycle of 50°C for 5 min, 1 cycle of 95°C for 20 sec, and 40 cycles of 95°C for 3 sec and 60°C for 30 sec.

Laboratory D

Nucleic acids were extracted from a 175 µl sample using MagMAX-96 viral isolation kit (Life Technologies, Grand Island, NY). Automated extraction was carried out on a KingFisher96 magnetic particle processor (Thermo Scientific, Waltham, MA) as previously described [2]. The commercial multiplex qPCR assay targeting the N gene of PEDV was performed according to manufacturer's instructions (Tetracore, Rockville, MD) as previously described [2]. Briefly, 7 µl of extracted RNA was added to 18 µl of the master mix. Amplification reactions were performed on ABI 7500 instrumentation (Thermo Scientific, Waltham, MA) with the following perimeters: 15 min at 48°C, 2 min at 95°C, 38 cycles of 95°C at 5 sec and then 40 sec at 60°C. Positive and negative controls were included in each run.

Laboratory E

Nucleic acid extraction was performed using the MagMAX-96 viral RNA isolation kit (Life Technologies, Grand Island, NY) according to manufacturer's instructions. The in-house multiplex qPCR assay targeting the S gene of PEDV was based on an assay described elsewhere [7] . Five µl of extracted RNA was used in the PCR setup using AgPath-ID One-Step RT-PCR kit (Life Technologies, Grand Island, NY). Amplification reactions were performed on an ABI 7500 thermal cycler (Thermo Scientific, Waltham, MA) on Fast Mode setting with the following parameters: reverse transcription for 10 min at 48°C, Taq activation for 10 min at 95°C for 10 min and 40 cycles of 15 sec at 95°C and 60°C for 45 sec.

Statistical Analysis

Results were analyzed using the PROC GLIMMIX procedure of statistical software (SAS Institute, Inc., Cary, NC). The statistical model evaluated the main effects of laboratory, matrix, and virus load and their interaction with aliquot as the experimental unit. The LSMEANS procedure was used to compare Ct value differences by pairwise comparison. The intra-assay coefficient of variation (CV) was calculated as standard deviation divided by the mean and reported as a percentage. Results for the response criteria were considered significant at $P \leq 0.05$.

Results

The intra-assay variability (calculated as %CV) across laboratories was greater for low virus load samples as compared to high virus load samples, except for laboratory B SDPP (Table 4.1). For Ct values, there was a matrix × laboratory × virus load interaction ($P = 0.023$). Within all cases of laboratory and matrix, the low virus load had a higher Ct compared to the high virus load ($P < 0.05$; Table 4.1). However, the Ct difference between virus loads for the SDPP analyzed in Laboratory A was much larger than the remaining matrix × laboratory × virus load combinations. Within feed or SDPP inoculated at low or high virus load, laboratory C had

lower ($P < 0.05$) Ct values compared to the other laboratories. Within feed inoculated at the low or high virus load, laboratory D had a higher ($P < 0.05$) Ct value compared to the other laboratories. For SDPP inoculated at high virus load, laboratory A, B, and D had the highest ($P < 0.05$) Ct value compared to laboratory C and E, whereas laboratory A had the highest Ct compared to all others for the low virus load SDPP samples. In feed, the virus load Ct difference ranged from 2.6 to 3.4 and in SDPP this difference was higher ranging from 3.4 to 5.6.

A matrix × laboratory interaction ($P < 0.0001$) occurred, where SDPP had a lower mean Ct than feed, except for laboratory A (Table 4.2). The main effect of virus load ($P < 0.0001$) had a higher Ct in the low virus load as compared to the high virus load which resulted in a 3.4 Ct difference (Table 4.2) which was expected based on the 10 fold dilution between the high and low virus load samples. Within mean Ct across laboratories, Ct values differed however laboratory C had a lower ($P < 0.05$) Ct value compared to all other laboratories. There was matrix × virus load interaction ($P = 0.0029$; Table 4.3) where feed with high virus load had a mean Ct that was higher compared to the SDPP samples. However, for the low virus load, the mean Ct values were similar.

Discussion

Molecular testing of clinical samples has been critical for diagnosing and monitoring PEDV [8]. Since confirmation of additional routes for PEDV transmission, molecular testing on feed, feed ingredients and environmental surfaces are playing an increasingly important role. For example, qPCR has been used to investigate the presence of PEDV RNA in and around feed manufacturing facilities [9]. Generally, qPCR is preferred for direct virus detection due to its quick turnaround time, high sensitivity, specific target quantification, high throughput capability, and allows for additional virus characterization by use of sequence analysis [3, 10]. Although

many improvements have been applied to the qPCR procedure itself, pre-PCR steps such as sampling, extraction, and reverse transcription along with other factors like sample type, instrumentation and laboratory personnel vary between laboratories and can introduce variation in the quantification method [11-13]. Additionally, commercial premixes (PCR master mixes) and nucleic acid extraction/isolation kits afford higher-throughput for routine analysis yet differences in Ct values between kits is possible (J. Zhang, personal communication. 2016). Since feed and feed ingredients have not been analyzed routinely in the past, little is known how this matrix impacts the outcome of PEDV qPCR results. Therefore, we assessed for the first time reproducibility conditions (i.e. results from using the same method on aliquot replicates in different laboratories that have different operators and equipment) of qPCR assays from different diagnostic laboratories using PEDV inoculated feed matrixes.

Challenges analyzing feed and feed ingredients

Feed and feed ingredients can present special challenges for molecular analysis similar to challenges seen with human food diagnostic samples [14]. First, little is known about the performance of qPCR assays analyzed on samples of different matrixes beyond the sample types validated for quality control purposes. Samples for molecular testing are usually validated on clinical samples most commonly derived from infected animal tissues, serum, oral fluids and feces. According to the American Association of Veterinary Laboratory Diagnosticians (AAVLD), "validated tests must have ongoing documentation of laboratory performance using known reference standard(s) for the species or diagnostic specimen of interest and at least either be endorsed or published by a reputable technical organization, be published in a peer-reviewed journal, or document intra- or inter-laboratory comparison to an accepted method/protocol" [15]. Thus, feed and their derivatives would not be considered validated sample types for PEDV

qPCR. Laboratories can analyze other sample types like feed, feed ingredients and environmental samples by qPCR assay not originally included in the validation protocol, but consider them not fully validated and typically urge caution when interpreting results.

While qPCR has the potential to be a good surveillance tool because of its high sensitivity, other tests are not as sensitive and detection of PEDV by other means like cell culture infectivity (virus isolation) has proven difficult since the virus does not seem to readily replicate in host cell systems. In one study to optimize PEDV isolation, only two PEDV isolates from 17 tissue homogenates samples were successfully obtained [4] and in another optimization study, 11 PEDV isolates from 63 intestinal contents were recovered [16]. Thus, it seems virus isolation has low sensitivity for PEDV clinical isolates which contain much higher virus loads than in feed samples. This challenged is not limited to PEDV and is observed with human foodborne viral pathogens as well [17]. The low sensitivity underscores the need for continued improvement of diagnostic *in vitro* isolation methods.

The current study shows Ct values differed lab-to-lab but the magnitude of the difference was small with the exception of laboratory C. However, future research on assay performance should be done using these matrixes types containing low amount of virus near the limit of detection to further assess PEDV qPCR assays. This may help to identify if further standardization of protocols is needed. For example, in our experience feed and feed ingredient samples from the field often have high Ct values which is concerning since the infectious dose to transmit PEDV in feed is low. In fact, the detectible minimum infectious dose in feed challenged in pigs was determined to be as low as 5.6×10^1 $TCID_{50}$/g with a feed Ct = 37 which corresponded to a dose of 112 $TCID_{50}$ when eluded and orally gavaged in pigs [5]. This Ct value in feed at the minimum infectious dose is considered high and depending on the diagnostic

laboratory's threshold of Ct values, may impact interpretation of results (Table 4.4). Furthermore, unless protocols are standardized across laboratories, comparisons of values from different laboratories should be interpreted with caution.

Another challenge when testing feed matrixes is that most will have a non-homogenous distribution and small quantity of detectible virus in the contaminated material. This results in higher Ct values and detectible virus from these samples which can be near the limit of qPCR detection. Therefore, more variation occurs between samples analyzed by qPCR as explained by Poisson statistics. According to Poisson's law of small numbers, if there is a random distribution of quantifiable independent events (i.e. detectible RNA) then predictions can be made when these events occur [18]. Therefore, Poisson distribution is expected in samples containing very low detectible PEDV copies and predicts in a large number of replicates containing an average of one copy of starting template, approximately 37% should have no copies, approximately 37% should have one copy, and approximately 18% should have two copies (Life Technologies, 2011, Real-time PCR: understanding Ct. Available at: https://www.thermofisher.com/content/dam/LifeTech/migration/en/filelibrary/nucleic-acid-amplification-expression-profiling/pdfs.par.70657.file.dat/understanding%20ct%20application%20note.pdf). As a result, repeatability is not consistent with samples at or near the level of detection. Ultimately, sensitivity for qPCR diagnostics would require the test's ability to effectively amplify and detect one starting template copy; however in reality with rigorous quality assurance, most diagnostic qPCR assays are sensitive but at least 5 to 10 copies are needed in a sample to detect the presence of RNA (J. Bai, personal communications. 2014).

The sudden onset of the PEDV epidemic gave rise to several different in-house developed qPCR assays among veterinary diagnostic laboratories. Although these assays meet

accredited standards or are based on a protocol initially provided to the National Animal Health Laboratory Network, [8] variation in protocols is present as illustrated in the materials and methods section of this study. For example, in the current study, 4 of the 5 laboratories used the same extraction kit, 3 of the 5 used different PCR amplification kits and all but one laboratory had the same thermocycler. Again, significant differences in Ct values were observed across laboratories, however with the exception of laboratory C, the magnitude of the difference detected was small and importantly, may be not be biologically significant. Therefore, it appears that although different qPCR assay protocols were used across laboratories, qPCR is precise for PEDV RNA detection in feed and SDPP.

Variation can occur in pre-PCR quality control steps which can also be a challenge associated with qPCR assays. In our case, sampling variability was minimized across laboratories by eluding samples with PBS and then submitting aliquots of supernatant. Elution for feed and feed ingredients is necessary because they are in solid and/or dry form and must be suspended in liquid to encourage viral disassociation; a process also commonly done with human food diagnostic samples [14]. Differences in elution protocols occur between laboratories which may alter the amount of recoverable virus from as sample. For example, some may allow the suspension to rest overnight while others may agitate the suspension over a period of time. Therefore, by standardizing the elution process in this study, better comparison of reproducibility conditions could be done which includes the RNA extraction step. Another problem associated with comparing qPCR assays is maintaining RNA stability in shipped samples. In our case, supernatant samples were placed on dry ice and shipped the same day when harvested (estimated at least 24 hr difference from shipment to analysis) to maintain RNA stability. Although RNA stability was not assessed in the current study, in the future, samples could also be inoculated

with an internal standard and frozen prior shipping to further assess and minimize of RNA degradation as previously done by others [19].

In the current study, there was low intra-assay variation with the exception of SDPP inoculated at low virus load from laboratory A. There is limited information in the diagnostic literature comparing assay variation for US PEDV qPCR. One report indicated intra-assay CV of 2.76% for one method and 2.73 for another PEDV assay [20]. In comparison, the %CV from the current study was at 3.3% or lower with the exception of SDPP low virus load from laboratory A. The high intra-assay CV for laboratory A was the result of a high Ct value from 1 of 3 SDPP low virus load replicates. Therefore, this single sample is responsible for the high CV and is the driver of the 3-way interaction of matrix, laboratory and virus load. Also, it is important to note samples inoculated with the high virus load had lower intra-assay variation compared to those with inoculated with low virus load. This is similar to results reported by others when comparing qPCR assays [20].

Diagnostic implications

Two strategies for testing feed samples can best be applied when 1) sampling suspect feed or feed ingredients in support of clinical cases and 2) continuing surveillance of feed or ingredients as part of a quality assurance plan. It is best to use qPCR results from suspect feed matrixes in context of the entire clinical case since qPCR cannot differentiate infectious from noninfectious RNA. However, discrepancy exists between qPCR and bioassay results. For example, we and others have had infectious samples that tested beyond assay limits of qPCR detection [21, 22]. Regardless, qPCR is a sensitive and specific diagnostic tool which may be best applied to epidemiologic investigations and for biosecurity measures [17]. When surveilling feed or ingredients by qPCR, one may consider adopting a risk-based sampling strategy and

analyze matrixes containing porcine derived proteins which potentially have a higher risk of PEDV contamination rather than regular testing of all feed and feed ingredients. This approach therefore may be a strategic method to mitigate the chance of contamination or entry of virus into a facility.

References

1. Pasick J, Berhane Y, Ojkic D, Maxie G, Embury-Hyatt C, Swekla K, et al. Investigation into the role of potentially contaminated feed as a source of the first-detected outbreaks of porcine epidemic diarrhea in Canada. Transbound Emerg Dis. 2014;61(5):397-410. doi: 10.1111/tbed.12269
2. Dee S, Clement T, Schelkopf A, Nerem J, Knudsen D, Christopher-Hennings J, et al. 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 Vet Res. 2014;10(1):176. doi: 10.1186/s12917-014-0176-9
3. Diel DG, Lawson S, Okda F, Singrey A, Clement T, Fernandes MH, et al. Porcine epidemic diarrhea virus: an overview of current virological and serological diagnostic methods. Virus Res. 2016. doi: 10.1016/j.virusres.2016.05.013
4. Chen Q, Li G, Stasko J, Thomas JT, Stensland WR, Pillatzki AE, et al. Isolation and characterization of porcine epidemic diarrhea viruses associated with the 2013 disease outbreak among swine in the United States. J Clin Microbiol. 2014;52(1):234-43. doi: 10.1128/JCM.02820-13
5. Schumacher LL, Woodworth JC, Jones CK, Chen Q, Zhang J, Gauger PC, et al. Evaluation of the minimum infectious dose of porcine epidemic diarrhea virus in a virus-inoculated feed. Am J of Vet Res (In press). 2016
6. Zhang J, Tsai YL, Lee PY, Chen Q, Zhang Y, Chiang CJ, et al. Evaluation of two singleplex reverse transcription-Insulated isothermal PCR tests and a duplex real-time RT-PCR test for the detection of porcine epidemic diarrhea virus and porcine deltacoronavirus. J Virol Methods. 2016;234:34-42. doi: 10.1016/j.jviromet.2016.03.016

7. Alonso C, Goede DP, Morrison RB, Davies PR, Rovira A, Marthaler DG, et al. Evidence of infectivity of airborne porcine epidemic diarrhea virus and detection of airborne viral RNA at long distances from infected herds. Vet Res. 2014;45(1):73. doi: 10.1186/s13567-014-0073-z

8. GAO. Emerging animal diseases: actions needed to better position USDA to address future risks. Washington, DC: U.S. Government Accountability Office (GAO), 2015 GAO-16-132.

9. Greiner LL. Evaluation of the likelihood of detection of porcine depidemic diarrhea viurs or porcine delta coronavirus ribonucleic acid in areas within feed mills. J Swine Health and Prod. 2016;24(4):198-204

10. Black EM, Lowings JP, Smith J, Heaton PR, McElhinney LM. A rapid RT-PCR method to differentiate six established genotypes of rabies and rabies-related viruses using TaqMan technology. J Virol Methods. 2002;105(1):25-35

11. Svec D, Tichopad A, Novosadova V, Pfaffl MW, Kubista M. How good is a PCR efficiency estimate: Recommendations for precise and robust qPCR efficiency assessments. Biomol Det and Quant. 2015;3(1):9-16. doi: 10.1016/j.bdq.2015.01.005

12. Tichopad A, Bar T, Pecen L, Kitchen RR, Kubista M, Pfaffl MW. Quality control for quantitative PCR based on amplification compatibility test. Methods. 2010;50(4):308-12. doi: 10.1016/j.ymeth.2010.01.028

13. Pfaffl MW. The ongoing evolution of qPCR. Methods. 2010;50(4):215-6. doi: 10.1016/j.ymeth.2010.02.005

14. Cliver D, Ellender R, Fout G, Shields P, Sobsey M. Compendium of methods for the microbiological examination of foods. 3rd ed. Washington, DC: American Public Health Association; 1992.

15. AAVLD. Requirements for an accredited veterinary medical diagnostic laboratory; approved guideline. American Association of Veterinary Laboratory Diagnosticians (AAVLD), 2014 AAVLD Document No.:AC-201.

16. Oka T, Saif LJ, Marthaler D, Esseili MA, Meulia T, Lin CM. Cell culture isolation and sequence analysis of genetically diverse US porcine epidemic diarrhea virus strains including a novel strain with a large deletion in the spike gene. Vet Microbiol. 2014;173. doi: 10.1016/j.vetmic.2014.08.012

17. Cliver DO. Control of Viral Contamination of Food and Environment. Food Environ Virol. 2009;1(1):3-9. doi: 10.1007/s12560-008-9005-2

18. Barbour AD, Holst L, Janson S. Poisson approximation. 1st ed. Oxford, UK: Oxford University Press; 1992.

19. Hoffmann B, Depner K, Schirrmeier H, Beer M. A universal heterologous internal control system for duplex real-time RT-PCR assays used in a detection system for pestiviruses. J Virol Methods. 2006;136(1-2):200-9. doi: 10.1016/j.jviromet.2006.05.020

20. Miller LC, Crawford KK, Lager KM, Kellner SG, Brockmeier SL. Evaluation of two real-time polymerase chain reaction assays for porcine epidemic diarrhea virus (PEDV) to assess PEDV transmission in growing pigs. J Vet Diagn Invest. 2016;28(1):20-9. doi: 10.1177/1040638715621949

21. Thomas JT, Chen Q, Gauger PC, Gimenez-Lirola LG, Sinha A, Harmon KM, et al. Effect of porcine epidemic diarrhea virus infectious doses on infection outcomes in naive

conventional neonatal and weaned pigs. PLoS ONE. 2015;10(10):e0139266. doi: 10.1371/journal.pone.0139266

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

Tables

Table 4.1 Interactive means of porcine epidemic diarrhea virus (PEDV) detection across five veterinary diagnostic laboratories utilizing quantitative real-time reverse transcription PCR (qPCR)[*]

	Laboratory				
Item	A	B	C	D	E
Intra-assay variation[‡]					
Feed[†]					
High virus load	0.4%	0.1%	0.3%	0.5%	0.3%
Low virus load	0.5%	1.0%	0.3%	1.5%	0.8%
SDPP§					
High virus load	2.0%	3.3%	0.0%	0.3%	0.6%
Low virus load	7.1%	0.2%	0.7%	0.8%	1.3%
Ct value					
Feed					
High virus load	24.0^{f}	24.3^{f}	19.4^{j}	26.0^{de}	22.9^{gh}
Low virus load	26.7^{cd}	26.9^{bc}	22.6^{h}	29.2^{a}	26.3^{cde}
SDPP					
High virus load	24.3^{f}	23.6^{fg}	18.0^{k}	24.2^{f}	22.1^{ih}
Low virus load	29.9^{a}	27.0^{cb}	21.5^{i}	27.6^{b}	25.6^{e}

*An initial tissue culture containing 4.5 x 10^6 $TCID_{50}$/ml of PEDV with Ct of 11 was diluted 1:10 (high virus load) and 1:100 (low virus load) using tissue culture media. The 2 inoculum levels were used to inoculate jars containing 100 g of feed or SDPP. PEDV was then eluted with 400 ml of PBS to form supernatant estimated at 8.15×10^4 $TCID_{50}$/ml for the high virus load and 8.15×10^3 $TCID_{50}$/ml for the low virus load. Then 3 aliquots of each matrix × virus load combination supernatant were submitted for PEDV qPCR analysis to 5 different diagnostic laboratories, resulting in 12 samples submitted per laboratory. For Ct values: Matrix × laboratory × virus load $P = 0.023$ and SEM = 0.22.

[‡]Coefficient of variation (CV) was calculated as standard deviation divided by the mean of the triplicate assays.

[†]Corn-soybean meal swine diet.

[§]Spray dried porcine plasma.

[a,b,c,d,e,f,g,h,i,j,k] Within Ct, means lacking common superscript differ $P < 0.05$.

Table 4.2 Effect of porcine epidemic diarrhea virus (PEDV)-inoculated matrixes on virus detection across veterinary diagnostic laboratories[*]

	Laboratory, mean Ct (cycle threshold)				
Item	A	B	C	D	E
Matrix					
Feed[†]	25.3[b]	25.6[b]	21.0[e]	27.6[a]	24.6[c]
SDPP[§]	27.1[a]	25.3[b]	19.8[f]	25.9[b]	23.9[d]
Virus load					
High	24.1	23.9	18.7	25.1	22.5
Low	28.3	27.0	22.1	28.4	25.9
Laboratory main effect	26.2[b]	25.4[c]	20.4[e]	26.7[a]	24.2[d]

*An initial tissue culture containing 4.5 x 10^6 $TCID_{50}$/ml of PEDV with Ct of 11 was diluted 1:10 (high virus load) and 1:100 (low virus load) using tissue culture media. The 2 inoculum levels were used to inoculate jars containing 100 g of feed or SDPP. PEDV was then eluted with PBS to form supernatant estimated at 8.15×10^4 $TCID_{50}$/ml for the high virus load and 8.15×10^3 $TCID_{50}$/ml for the low virus load. Then 3 aliquots of each matrix × virus load combination supernatant were submitted for PEDV qPCR analysis to 5 different diagnostic laboratories, resulting in 12 samples submitted per laboratory. Matrix × laboratory $P < 0.0001$ and SEM = 0.22. Virus load × laboratory $P = 0.20$ and SEM = 0.22. Main effect of laboratory $P < 0.0001$ and SEM = 0.16

[†]Corn-soybean meal swine diet.

[§]Spray dried porcine plasma.

[a,b,c,d,e,f] For matrix, means lacking common superscript differ $P < 0.05$.

[a,b,c,d] For laboratory main effect, means lacking common superscript differ $P < 0.05$.

Table 4.3 Effect of (PEDV)-inoculated matrixes and virus load from veterinary diagnostic laboratories utilizing quantitative real-time reverse transcription PCR (qPCR)[*]

	Virus load	
Item	High	Low
Matrix		
Feed[†]	23.3[b]	26.3[a]
SDPP[§]	22.4[c]	26.3[a]

*An initial tissue culture containing 4.5 x 10^6 $TCID_{50}$/ml of PEDV with Ct of 11 was diluted 1:10 (high virus load) and 1:100 (low virus load) using tissue culture media. The 2 inoculum levels were used to inoculate jars containing 100 g of feed or SDPP. PEDV was then eluted with PBS to form supernatant estimated at 8.15×10^4 $TCID_{50}$/ml for the high virus load and 8.15×10^3 $TCID_{50}$/ml for the low virus load. Then 3 aliquots of each matrix × virus load combination supernatant were submitted for PEDV qPCR analysis to 5 different diagnostic laboratories, resulting in 12 samples submitted per laboratory. Matrix × virus load $P = 0.0029$ and SEM = 0.14.

[†]Corn-soybean meal swine diet.

[§]Spray dried porcine plasma.

[a,b,c] Means within row lacking common superscript differ $P < 0.05$.

Table 4.4 Comparison of reported cycle threshold (Ct) values for porcine epidemic diarrhea virus (PEDV) quantitative real-time PCR (qPCR) across five diagnostic laboratories.

	Veterinary Diagnostic Laboratory			
	A & B	C	D	E
Cycle threshold, Ct	< 37 = positive > 39 = negative 37-39 = suspect	< 35 = positive ≥ 35 = negative	≤ 35 = positive ≥ 40 = negative 35.01-39.99 = suspect	< 38 = positive ≥ 38 = negative

Appendix A

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