Evaluation of the minimum infectious dose of porcine epidemic diarrhea virus in virus-inoculated feed

Loni L. Schumacher DVM
Jason C. Woodworth PhD
Cassandra K. Jones PhD
Qi Chen MS, DVM
Jianqiang Zhang PhD
Phillip C. Gauger DVM, PhD
Charles R. Stark PhD
Rodger G. Main DVM, PhD
Richard A. Hesse PhD
Mike D. Tokach PhD
Steve S. Dritz DVM, PhD

Received November 18, 2015.
Accepted December 21, 2015.

From the Department of Diagnostic Medicine/Pathobiology, College of Veterinary Medicine (Schumacher, Hesse, Dritz), and the Departments of Animal Sciences and Industry (Woodworth, Tokach) and Grain Science and Industry (Jones, Stark), College of Agriculture, Kansas State University, Manhattan, KS 66506; and the Department of Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA 50011 (Chen, Zhang, Gauger, Main).

Address correspondence to Dr. Schumacher (loni1@vet.k-state.edu).

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 X 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, histologic examination, and immunohistochemical analysis.

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 the 4 highest concentrations (lowest concentration to cause infection, 5.6 X 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 the limit of detection established for PEDV PCR assays used by some diagnostic laboratories. Overall, results indicated 5.6 X 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;77:1108–1113)

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.

ABBREVIATIONS

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

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, they received identification ear tags; pigs were then weighed and administered a dose of ceftiofur.[a] Fecal

swab specimens were obtained and confirmed negative for PEDV, porcine delta coronavirus, and transmissible gastroenteritis virus by use of virus-specific RT-qPCR assays conducted at the Iowa State University Veterinary Diagnostic Laboratory. To further verify 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 ELISAs, 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. The stock solution of PEDV contained 5.6×10^5 $TCID_{50}$/mL.

Feed

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).** 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 PEDV inoculation and confirmed to have negative results for PEDV RNA by use of a RT-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, 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 RT-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 RT-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 by use of a spreadsheet-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 room 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 received 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 an RT-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, as previously described[14]; only the ileum samples were evaluated. Cecal content was evaluated for PEDV by use of an RT-qPCR assay.

RNA extraction and RT-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] 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 RT-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 RT-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

RT-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 RT-qPCR assay Ct values of 16.6 to > 45 **(Table 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 (log_{10}) 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 RT-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

Table 1—The Ct values for an RT-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					Cecal contents†
			Day 0	Day 2	Day 4	Day 6	Day 7	
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. ND = Not determined. Neg = Negative result because a Ct value > 45 was established as the cutoff for a negative result.

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.

RT-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). The RT-qPCR assay 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 on day 2, but all 3 of these pigs had PEDV-positive fecal swab specimens on 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 PEDV was transmitted 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 RT-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 2**). 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 higher doses was confirmed by use of various assays. Results for RT-qPCR assay of fecal samples of pigs challenge exposed with PEDV-inoculated feed indicated the presence of detectable RNA. Shortened villi in infected pigs were 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 RT-qPCR assay Ct value of 37, which may be considered higher than the cutoff Ct used for a negative result at some veterinary diagnostic laboratories.[14] Infectivity above the Ct detection limit of the RT-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, that 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 clari-

Table 2—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).

PEDV in feed ($TCID_{50}$/g)*	Histologic findings			
	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 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). The mean was calculated (3 samples/pig × 3 pigs/treatment) for each treatment.

See Table 1 for remainder of key.

fied 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 resulted 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 the RT-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 one 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.[17,1] 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.[18]

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 [log_{10}]) difference in the amount of PEDV RNA, assuming that a reduction of 1 (log_{10}) 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 differed. 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 an RT-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 the RT-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 evaluated 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 those differences. We hypothesized 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 adheres to plant cell wall material via carbohydrate moieties, which is a method that may enhance viral persistence and thwart decontamination efforts.[19] 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 the highest 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.

Acknowledgments

Supported in part by the National Pork Board (Grant No. 14-159).
Presented in abstract form at the 2015 American Society of

Animal Science Midwest Section Annual Meeting, Des Moines, Iowa, March 2015.

Footnotes

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, Iowa.
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.
l. Cochrane RA, Woodworth JW, Dritz SS, et al. Evaluating chemical mitigation of porcine epidemic diarrhea virus in swine feed and ingredients (abstr), in *Proceedings*. Am Soc Anim Sci Midwest Sect Am Dairy Sci Assoc Midwest Branch Joint Meet 2015;41–42.

References

1. Huang Y-W, Dickerman AW, Piñeyro P, et al. Origin, evolution, and genotyping of emergent porcine epidemic diarrhea virus strains in the United States. *MBio* 2013;4:e00737–e00713.
2. Saif L, Pensaert MB, Sestak K, et al. Coronaviruses. In: Straw B, ed. *Diseases of swine.* 10th ed. Chichester, England: Wiley-Blackwell, 2012;501–524.
3. Alonso C, Goede DP, Morrison RB, 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;45:73.
4. Lowe J, Gauger P, Harmon K, et al. Role of transportation in spread of porcine epidemic diarrhea virus infection, United States. *Emerg Infect Dis* 2014;20:872–874.
5. Pasick J, Berhane Y, Ojkic D, 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;61:397–410.
6. Jones CK, Bai J, Dritz SS, et al. Post-processing contamination chemical mitigation strategies to control PEDV in feed and feed ingredients. Available at: http://research.pork.org/FileLibrary/ResearchDocuments/14-158-JONES-KSt.pdf. Accessed Mar 15, 2015.
7. Dee S, Clement T, Schelkopf A, 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;10:176.
8. Lee JH, Park JS, Lee SW, et al. Porcine epidemic diarrhea virus infection: inhibition by polysaccharide from *Ginkgo biloba* exocarp and mode of its action. *Virus Res* 2014;195:148–152.
9. Dee S, Neill C, Clement T, et al. 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;10:220.
10. Chen Q, Li G, Stasko J, 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;52:234–243.
11. McCoy RA. Mixer testing. In: Schofield E, ed. *Feed manufacturing technology V.* Arlington, Va: American Feed Industry Association, 2005;620–622.
12. Madson DM, Magstadt DR, Arruda PH, et al. Pathogenesis of porcine epidemic diarrhea virus isolate (US/Iowa/18984/2013) in 3-week-old weaned pigs. *Vet Microbiol* 2014;174:60–68.
13. Thomas JT, Chen Q, Gauger PC, et al. Effect of porcine epidemic diarrhea virus infectious doses on infection outcomes in naive conventional neonatal and weaned pigs. *PLoS ONE.* 2015;10:e0139266.
14. Schumacher LL, Dritz SS. Feed diagnostics: what does it really mean?, in *Proceedings.* 46th Annu Meet Am Assoc Swine Vet 2015;21–24.
15. Goyal SM. Environmental stability of PED (porcine epidemic diarrhea virus). Available at: www.pork.org/wp-content/uploads/2014/05/goyal-13-215-main.pdf. Accessed Dec 15, 2014.
16. Jung K, Annamalai T, Lu Z, et al. 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;178:31–40.
17. Dee S, Neill C, Clement T, et al. An evaluation of porcine epidemic diarrhea virus survival in individual feed ingredients in the presence or absence of a liquid antimicrobial. *Porcine Health Manage* 2015;1:1–10.
18. Greiner, L. Evaluation of the risk of a feed mill being contaminated with PEDV or SdCV. Available at: www.pork.org/wp-content/uploads/2014/05/greiner-14-165-main-020615.pdf. Accessed Jul 28, 2015.
19. 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;78:786–794.

Appendix

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 4,400,000 U 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}.