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Determining the Minimum Infectious Dose of Porcine Epidemic Diarrhea Virus (PEDV) in a Feed Matrix[1]

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Summary

Understanding the magnitude of transmissible risk Porcine Epidemic Diarrhea Virus (PEDV)-infected feed imposes and establishing the minimum infectious dose of PEDV in a feed matrix are important components in strengthening virus prevention and control methods. In this study, an experiment was performed involving 30 crossbred, 10-d-old pigs that were used as a bioassay model for the minimum infectious dose of PEDV in feed. The PEDV was first diluted using tissue culture media to form 8 serial 10-fold dilutions. An aliquot of the original stock virus at 5.6×10^5 tissue culture infectious dose/ml ($TCID_{50}$/ml), each serial PEDV dilution, and one virus-negative culture medium were mixed into separate 4.5 kg batches of swine diet to form 10 experimental treatments. The feed was then subsequently evaluated for infectivity using bioassay. Fecal swabs were collected at 0, 2, 4, 6, and 7 d after challenge for PCR testing. At 7 d after challenge, all pigs were necropsied. Cecum contents, ileum and jejunum were collected for PCR, histologic and immunohistochemistry (IHC) evaluation. Overall, the results indicate 5.6×10^1 $TCID_{50}$/g was the minimum PEDV dose in which infection was detected. This feed had a corresponding PCR cycle threshold (Ct) of 37. This is a relatively low dose. To illustrate, using this dose, approximately 1 g of PEDV-infected baby piglet feces could contaminate up to 500 tons of feed. The data confirm that detectable Ct values in feed can result in pig infection. Our results also illustrate that the Ct in feed that was detected as infectious can be above the detection threshold used by some diagnostic laboratories.

Key words: PEDV, minimum infectious dose, feed

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Introduction

Porcine Epidemic Diarrhea Virus (PEDV) suddenly and profoundly affected the United States swine industry since its recognized emergence in May 2013. Although the direct route of transmission is fecal-oral, several PEDV outbreaks were suspected to be associated with the consumption of PEDV positive feed or feed ingredients (Pasick et al., 2014)[5]. Subsequently, feed has been confirmed as a potential vehicle for PEDV transmission and has prompted investigations into reducing infectivity risk in contaminated diets or feed ingredients (Dee et al., 2014)[6].

PEDV proved to be highly transmissible in the United States, however little is known about the overall magnitude of transmissible risk PEDV-infected feed imposes. Furthermore, no data are available to define the minimum infectious dose of PEDV in the feed matrix. The objective of this experiment was to determine the minimum infectious dose of PEDV in a feed matrix by use of the 10-d-old pig bioassay.

Procedures

Virus isolation, propagation, and titration were performed in Vero cells (ATCC CCL-81) as previously described (Chen et al., 2014)[7]. The United States (US) PEDV prototype strain cell culture isolate USA/IN/2013/19338, passage 8 (PEDV19338) was used to inoculate feed in this study. The stock PEDV used in the experiment contained 5.6×10^5 tissue culture infectious dose/ml ($TCID_{50}$/ml).

The feed used was a corn soybean meal-based swine diet manufactured at Kansas State University's O. H. Kruse Feed Mill (Table 1). A subsample of this feed was obtained before inoculation and confirmed negative by real-time quantitative polymerase chain reaction (PCR) for the presence of PEDV RNA at the Kansas State University Veterinary Diagnostic Laboratory (Manhattan, KS).

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

Feed and virus were mixed using a manual, bench-top stainless steel paddle mixer (Stainless Steel Meat Mixer, Cabela's Inc., Sidney, NB), which had been validated for mixing efficiency testing using a standard protocol (McCoy, 2005)[8]. The optimal mixing time was determined as 2 minutes or greater to achieve uniform mixing. First

[5] Pasick, J., Y. Berhane, D. Ojkic, G. Maxie, C. Embury-Hyatt, K. Swekla, K. Handel, J. Fairles, and S. Alexandersen. 2014. 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. 61: 397-410.
[6] Dee, S., C. Neill, T. Clement, J. Christopher-Hennings, and E. Nelson. 2014. 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. 10: 220.
[7] Chen, Q., G. Li, J. Stasko, J. T. Thomas, W. R. Stensland, A. E. Pillatzki, P. C. Gauger, K. J. Schwartz, D. Madson, K. J. Yoon, G. W. Stevenson, E. R. Burrough, K. M. Harmon, R. G. Main, and J. Zhang. 2014. Isolation and characterization of porcine epidemic diarrhea viruses associated with the 2013 disease outbreak among swine in the United States. J. Clin. Microbiol. 52: 234-243.
[8] McCoy, R. A. 2005. Mixer testing. In: E. Schofield, editor, Feed manufacturing technology V. American Feed Industry Association, Arlington, VA. p. 620-622.

the 4.5 kg of feed was added to the mixer, and then the 500 ml of media was added slowly while operating the mixer. After the addition, the feed was mixed for 2.5 minutes. Batches of feed were mixed in order of lowest to highest virus concentration with a batch of non-inoculated feed (flush/sequence) mixed between each batch. Subsamples of each batch of feed and each of the flush batches were analyzed for presence of PEDV RNA by PCR. After each inoculated batch and subsequent flush, the mixer was cleaned of any residual feed particulate matter before beginning the mixing process for the next batch.

Three subsamples (100 g/sample) of PEDV-inoculated feed were obtained after mixing each batch and were used to make a 20% suspension. Briefly, the 100-g sample of feed was added to 400 ml of cold phosphate buffered saline (PBS, pH 7.4) in 500-ml bottles, thoroughly mixed and stored at 40°F (4°C) for approximately 12 h. The feed suspension was evaluated using a PEDV N-gene based PCR assay (Chen et al., 2014). Aliquots were then harvested and frozen at -112°F (-80°C) until use in the pig bioassay.

All procedures involving pigs were approved by the Iowa State University Institutional Animal Care and Use Committee. Thirty crossbred, 10-d-old pigs of mixed sex were sourced from a single commercial, crossbred farrow-to-wean herd with no prior exposure to PEDV. Upon arrival, piglets were ear tagged, weighed and administered a dose of cefitiofur. Also, upon arrival fecal swabs were obtained and confirmed negative for PEDV, porcine delta coronavirus (PDCoV) and transmissible gastroenteritis virus (TGEV) using PCR assay conducted at the Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL). To further confirm PEDV-negative status, serum was collected and confirmed negative for PEDV antibody by an indirect fluorescent antibody (IFA) assay and TGEV antibody by enzyme-linked immunosorbent assay (ELISA) conducted at ISU-VDL. Pigs were allowed 2 days of adjustment to the new pens before the bioassay began.

Pigs were randomly allocated into 1 control and 9 challenge groups (3 pigs per treatment for a total of 30 pigs). The bioassay procedures were similar to and conducted in the same facilities as those previously described (Madson et al., 2014)[9]. Briefly, pigs from each experimental treatment were housed in separate rooms with independent ventilation systems. Rooms had solid flooring that was minimally rinsed to reduce PEDV aerosols. Pig were fed liquid milk replacer twice daily and offered a commercial pelleted swine diet ad libitum with free access to water. Each pig was administered 10 ml of the PBS feed suspension supernatants by orogastric gavage using an 8-gauge French catheter 0 days post inoculation (dpi).

Rectal swabs were collected on d 0, 2, 4, 6, and 7 dpi from all piglets and tested for PEDV RNA by PCR. Fresh small intestine, cecum, and colon were collected at necropsy at 7 dpi, along with an aliquot of cecal content. One section of formalin-fixed proximal, middle, distal jejunum and ileum were collected for histopathology. Cecal content was evaluated for PEDV by PCR.

[9] Madson, D. M., D. R. Magstadt, P. H. Arruda, H. Hoang, D. Sun, L. P. Bower, M. Bhandari, E. R. Burrough, P. C. Gauger, A. E. Pillatzki, G. W. Stevenson, B. L. Wilberts, J. Brodie, K. M. Harmon, C. Wang, R. G. Main, J. Zhang, and K. J. Yoon. 2014. Pathogenesis of porcine epidemic diarrhea virus isolate (US/Iowa/18984/2013) in 3-week-old weaned pigs. Vet. Microbiol. 174: 60-68.

Tissue was routinely processed and fixed in neutral buffered formalin, embedded, sectioned, and stained with hematoxylin and eosin stain. One section of proximal, middle, and distal jejunum; and three serial sections from the piece of ileum (for a total of six sections of intestine) were evaluated by a veterinary pathologist blind to the treatments. In each of the sections, one full-length villus and crypt were measured, based on tissue orientation, using a computerized image system (Nikon Eclipse TI-U Microscope, Nikon Instruments Inc., Melville, NY). The mean villi length and crypt depth from each intestinal segment were used to determine statistical differences. Thus, one crypt and villus was measured per section of jejunum and ileum for a total of six values per pig. The values were averaged for calculating the villi height to crypt-depth ratio. Porcine Epidemic Diarrhea Virus immunohistochemistry slides were prepared on the sections of ileum as previously described (Madson et al., 2014). Antigen detection was scored based on the following criteria: no signal (0), mild (1 to 10% signals), moderate (11 to 25% signals), abundant (26 to 50% signals), and diffuse (>50 to 100%).

The effect of PEDV dose on PEDV RNA in feed, fecal shedding, and fecal content for those doses in which PEDV RNA was detected was determined using SAS, version 9.3 (SAS Institute Inc., Cary, NC). The association between the PEDV innocula Ct and feed after inoculation was evaluated using linear regression for those doses in which PEDV RNA was detected in feed. An analysis of variance (ANOVA) was also performed for villi height, crypt depth, villi height to crypt depth ratio, and immunohistochemistry. For these response criteria a single degree of freedom polynomial contrast was used to compare PEDV doses in which PEDV shedding was evident to those where it was not detectable.

Results and Discussion

Proximate analysis of the corn soybean meal-based gestation diet was similar to formulated values (Table 1).

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

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

Fecal shedding of PEDV was not detected in rectal swabs from negative control pigs for the duration of the study (Table 2). Porcine epidemic diarrhea virus PCR analysis of

fecal swabs from pigs challenged with PEDV-inoculated feed resulted in fecal shedding and clinical disease detected in all pigs challenged with 5.6×10^2 $TCID_{50}$/g to 5.6×10^4 $TCID_{50}$/g by 2 dpi and continued through 7 dpi. For the pigs challenged with 5.6×10^1 $TCID_{50}$/g, two of the three pigs had PEDV-positive fecal swabs at 2 dpi, but all three pigs had PEDV-positive fecal swabs at 4 dpi and continued to shed through the termination of the study. Pigs challenged with the PEDV treatments ranging from 5.6×10^0 $TCID_{50}$/g to 5.6×10^{-4} $TCID_{50}$/g had no PEDV-positive fecal swabs collected for the 7 dpi, nor was any of the cecum content at 7 dpi PEDV-positive. The minimum dose of PEDV where infectivity was detected was 5.6×10^1 $TCID_{50}$/g in a feed matrix. This corresponded to a Ct of 37 when the feed was analyzed by PCR.

When comparing pigs fed doses that had fecal RNA shedding compared to those from which RNA was not detected, the villus height was shorter (371.8 ± 25.4 vs 470.8 ± 22.7 mm, $P < 0.01$), crypt depth tended to be greater (152.8 ± 9.3 vs 131.5 ± 8.3 mm, $P = 0.10$), and villus height to crypt depth ratio was lower (2.6 ± 0.3 vs 3.7 ± 0.2 mm, $P < 0.01$; Table 3). Positive immunoreactivity was seen in enterocytes of pigs challenged with any of the four highest doses of PEDV, which confirmed that infection was established.

In summary, our study confirmed that PEDV can be transmitted via feed and that 5.6×10^1 $TCID_{50}$/g was the minimum PEDV dose from which infection was detected using a feed matrix associated with a Ct of 37. Additionally, 5.6×10^0 $TCID_{50}$/g was the highest noninfectious dose, yet when increased 10-fold, infection was demonstrated by positive PCR and IHC results at a dose of 5.6×10^1 $TCID_{50}$/g. Thus, the actual minimum infectious dose is likely between these two doses. Even though 5.6×10^1 $TCID_{50}$/g is a relatively low dose, it is significant because it is the equivalent of approximately 1 g (0.002 lb) of PEDV-infected piglet feces being diluted in 500 tons of feed with all of the resulting feed potentially capable of causing infection. It is important to note that a Ct of 37 may be higher than the threshold used in some diagnostic laboratories to determine PEDV RNA presence. Interestingly, there was a consistent Ct difference of approximately 10 (3 logs) when PEDV was blended into feed, which equates to a 1,000-fold difference in the amount of PEDV RNA that was detected. More research should be conducted to determine if this loss of detectability when PEDV is placed in a feed matrix is related to a similar loss in infectivity or if there is some aspect of the feed matrix that prevents detection but still supports infectivity. In conclusion, for the first time we have demonstrated that batch-to-batch PEDV contamination can occur. Additional research should be conducted to determine the risk of PEDV cross-contamination in feed mills and to determine appropriate biosecurity procedures to prevent such risks from occurring.

Table 1. Diet composition of feed inoculated with Porcine Epidemic Diarrhea Virus (PEDV)

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

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

Table 2. Influence of Porcine Epidemic Diarrhea Virus (PEDV)-inoculated feed on N-gene PCR cycle threshold (Ct) of feed, fecal swabs, and fecal contents of pigs[1]

			Fecal swabs, Ct					
PEDV Concentration in the feed, $TCID_{50}/g$[2]	Tissue Culture Media, Ct	Feed, Ct	0 dpi[3]	2 dpi	4 dpi	6 dpi	7 dpi	7 dpi Cecum content, Ct
Virus-free feed	–[4]	–	–	–	–	–	–	–
5.6×10^{-4}	–	–	–	–	–	–	–	–
5.6×10^{-3}	38.0	–	–	–	–	–	–	–
5.6×10^{-2}	34.3	–	–	–	–	–	–	–
5.6×10^{-1}	30.6	–	–	–	–	–	–	–
5.6×10^{0}	27.4	–	–	–	–	–	–	–
5.6×10^{1}	24.3	37.1	–	33.2[5]	20.7	19.8	25.3	23.1
5.6×10^{2}	20.7	33.6	–	27.3	22.2	21.3	24.2	26.5
5.6×10^{3}	16.6	29.5	–	30.7	22.4	21.2	25.2	24.0
5.6×10^{4}	14.0	27.0	–	27.4	21.0	21.9	25.2	25.4
SEM		0.3	NA	1.9	1.9	2.1	2.8	2.4

[1]An initial tissue culture containing 5.6×10^5 $TCID_{50}$/ml of PEDV was serially diluted using tissue culture media. These dilutions were then used to inoculate batches of feed. Three feed samples per batch were collected and diluted in PBS. The supernatant from each sample was then collected for pig bioassay. The supernatant was administered one time via oral gavage on d 0 to each of three pigs per feed dilution (10 ml per pig). Thus each value represents the mean of three replicates. Pigs were initially 10 d old and 7.9 lb.

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

[3]Day post inoculation.

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

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

Table 3. Morphologic and immunohistochemistry evaluation of small intestine from pigs that were challenged with Porcine Epidemic Diarrhea Virus (PEDV)-inoculated feed[1]

	Morphology[2]			
PEDV Concentration in the feed, $TCID_{50}/g$[3]	Villus height, mm	Crypt depth, mm	Villus height to crypt depth ratio	Immunohistochemistry (IHC)[4]
Virus-free feed	485.8	132.8	3.7	0
5.6×10^{-4}	527.7	136.3	4.3	0
5.6×10^{-3}	464.3	120.7	3.9	0
5.6×10^{-2}	491.3	116.3	4.3	0
5.6×10^{-1}	436.0[5]	136.3	3.2	0
5.6×10^{0}	434.7	147.7	3.0	0
5.6×10^{1}	390.0	191.0	2.3	0.7
5.6×10^{2}	302.0	151.7	2.1	0.3
5.6×10^{3}	365.3	141.3	2.6	0.7
5.6×10^{4}	333.6	183.5	1.8	1
SEM	51.5	17.4	0.5	0.3

[1]An initial tissue culture containing 5.6×10^5 $TCID_{50}$/ml of PEDV was serially diluted using tissue culture media. These dilutions were then used to inoculate batches of feed. Three feed samples per batch were collected and diluted in PBS. The supernatant from each sample was then collected for pig bioassay. The supernatant was administered one time via oral gavage on d 0 to each of three pigs per feed dilution (10 ml per pig). Thus, each value represents the mean of three pigs per dilution and 3 villi measured per pig. Pigs were initially 10 d old and 7.9 lb.

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

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

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