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Understanding the Reduction of Porcine Epidemic Diarrhea Virus, Porcine Reproductive and Respiratory Syndrome Virus, and Seneca Valley Virus 1 RNA in Inoculated Feed and the Environment Following Thermal Processing

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Summary

Pelleting of feed has been demonstrated to be an effective mitigation strategy for porcine epidemic diarrhea virus (PEDV) contaminated feed but has not been evaluated for other endemic swine viruses like porcine reproductive and respiratory syndrome virus (PRRSV) or Seneca Valley virus 1 (SVV1). Therefore, the objective of this experiment was to evaluate the efficacy of pelleting to inactivate PEDV, PRRSV, and SVV1 inoculated feed. Ten replicates were conducted in the Cargill Feed Safety Research Center at Kansas State University (K-State) using a pilot scale mixer, bucket elevator, pellet mill (including conditioner and die), and cooler. First, a virus negative batch of gestation feed was run through all equipment to simulate a commercial feed mill, then a positive batch of feed inoculated with all three viruses was run through all feed manufacturing equipment. Feed was conditioned to a minimum of 180°F with a 30 sec retention time; all feed was cooled for 10 min. Feed and environmental samples were taken from each piece of equipment following both the negative and positive batch. Samples were analyzed via PCR at the K-State Veterinary Diagnostic Laboratory. A four-room bioassay was conducted to evaluate the infectivity of the feed samples. Feed from the mixer and bucket elevator had greater quantities of SVV1, PEDV, and PRRSV RNA ($P < 0.05$) than the other sampling locations. Similarly, environmental samples from the mixer and bucket elevator had greater SVV1 detection ($P < 0.05$) than those collected from the conditioner, pellet die, and cooler. Pelleting reduced viral RNA

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($P < 0.05$) for all viruses in both feed and environmental samples. Although SVV1 and PEDV RNA were still detectable following pelleting, no pigs inoculated with the pelleted feed showed signs of SVV1 or PEDV clinical infection. Interestingly, PRRSV RNA was not detectable in pelleted feed samples. However, one pig showed signs of replicating PRRSV virus on d 7 of the bioassay which suggests a greater sensitivity when utilizing a bioassay compared to PCR alone. Overall, pelleting reduced the quantity of detectable viral RNA and reduced the risk of infectivity; yet small quantities of viral RNA remaining in the feed and environment following pelleting may increase the risk of re-contamination.

Introduction

Following the porcine epidemic diarrhea virus (PEDV) outbreak in 2013, feed was identified as a possible vector for swine viruses and research focused on mitigation of this risk in feed and feed ingredients. Pelleting feed is a form of thermal processing and is considered a point-in-time mitigation as it is capable of inactivating viruses during processing but does not protect the feed from cross-contamination. The effect of pelleting on PEDV infectivity has been variable. At low conditioning temperatures (< 130°F), pelleted feed was still able to cause infection in a swine bioassay while feed conditioned at greater than 130°F failed to cause clinical signs of infection even though viral RNA was detected in the initial inoculum.[5] However, dry heating the complete feed at temperatures between 248–293°F took up to 25 min for viral inactivation[6] compared to the relatively short conditioning time of 30 sec used when pelleting.

Experiments conducted that utilized pelleted and inoculated swine feed have rarely been conducted with other swine viruses due to the specialized equipment and space needed for these experiments. The viruses common in commercial swine production such as porcine reproductive and respiratory virus (PRRSV) and Seneca Valley virus 1 (SVV1), have been evaluated for viral mitigation at room temperatures,[7] but feed contaminated with these viruses have not undergone pelleting parameters. Therefore, the objective of this experiment was to evaluate the efficacy of pelleting as a thermal mitigant for PEDV, PRRSV, and SVV1 in contaminated feed.

Procedures

Feed inoculation and sample collection were conducted at the Kansas State University Cargill Feed Safety Research Center (FSRC). Biocontainment was entered 10 separate times, representing 10 inoculation cycles. All protocols were approved by the Kansas State University Institutional Biosafety Committee (IBC-1636).

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Inoculum information

Equal volumes of SVV1 (GenBank: KX7780101.1), PEDV CO-isolate (GenBank KF272920), and PRRSV 1-7-4 were used for feed inoculation. The original stock contained 1×10^8 50% tissue culture infectious dose/mL ($TCID_{50}$/mL) SVV1, 1×10^7 $TCID_{50}$/mL PEDV, and 1×10^8 $TCID_{50}$/mL PRRSV. Viruses were individually packaged into 25 mL aliquots, shipped from South Dakota State University to K-State on dry ice, and stored at -112°F until used in the FSRC. One aliquot of each virus was removed from storage on the day of inoculation, transported the FSRC, and allowed to thaw at room temperature.

Swine diet

A corn-soybean meal mash gestation diet was manufactured at Hubbard Feeds (Beloit, KS). Feed samples were collected from multiple bags after feed delivery and submitted for PCR analysis to confirm SVV1, PEDV, and PRRSV negative status prior to entering the FSRC.

Feed inoculation

All viruses underwent a one log reduction in phosphate buffer solution (PBS) before being added to the feed. To accomplish this, 23 mL of each virus was added to 613 mL of PBS and gently stirred. The 1.5 mL aliquots of each virus prior to dilution and the combined inoculum were retained for analysis. The 682 mL inoculum, with an approximate concentration of 1×10^7 $TCID_{50}$/mL SVV1, 1×10^6 $TCID_{50}$/mL PEDV, and 1×10^7 $TCID_{50}$/mL PRRSV, was added to 4.8 lb of feed in an 11-lb benchtop stainless steel paddle mixer (Cabela's, Inc., Sidney, NE) and mixed for 5 min creating 6 lb of feed with an approximate concentration of 1×10^6 $TCID_{50}$/g SVV1, 1×10^5 $TCID_{50}$/g PEDV, and 1×10^6 $TCID_{50}$/g PRRSV. The contents of the benchtop mixer were added to 44 lb of feed and mixed for 5 min for a final approximate concentration of 1×10^5 $TCID_{50}$/g SVV1, 1×10^4 $TCID_{50}$/g PEDV, and 1×10^5 $TCID_{50}$/g PRRSV. The inoculated feed was discharged and underwent manufacturing as previously described.

Feed manufacturing

Feed manufacturing equipment was primed with a virus negative batch of feed (primer batch), followed by feed inoculated with equal quantities of SVV1, PEDV, and PRRSV (positive batch). During each batch, feed was mixed for 5 min in a 50-lb mixer (Hayes & Stolz Industrial Manufacturing Co. LLC, Burleson, TX) and discharged at a rate of 10 lb/min into a feed bin. Feed was then poured into the hopper of a bucket elevator (Universal Industries, Cedar Falls, IA) with 74 buckets (each 7 $in.^3$) at an equal discharge rate (10 lb/min) and conveyed through a downspout into a fresh feed bin. All feed was then pelleted in a CL-5 Laboratory Pellet Mill (CPM, Waterloo, IA) reaching a conditioning temperature of 180°F with a 30 sec retention time. Feed was dispensed into 6 trays each holding approximately 8.8 lb of pelleted feed per tray. As trays were filled, they were then placed in a double door experimental pellet cooler for 10 min before being sampled and discarded into double-lined autoclave totes. Trays 1–3 contained pellets which had not been conditioned to a minimum of 180°F, while trays 4–6 contained pellets which had reached the conditioning temperature of 180°F. Tray 4 was considered the low-temperature (LT) tray, as it shared circulating air in the pellet cooler with trays that had not reached the goal conditioning temperature. Tray 6 was considered the high-temperature (HT) tray as it was placed in the pellet cooler only with trays that had met or exceeded the minimum conditioning temperature of 180°F.

Feed sample collection

Feed samples were collected from the mixer, the bucket elevator, the pellet mill conditioner, the pellet die, and two from the pellet cooler (LT tray and HT tray) during the positive batch of each replication for a total of 60 feed samples. Samples from the mixer, bucket elevator, conditioner, and die were collected using the cut stream method. During discharge a 2 oz plastic cup (Great Value Cups, 2 oz, Walmart, Bentonville, AR) was filled; this process was repeated twice more every 90 seconds. Three individual feed samples were taken from the LT tray in a "V" pattern, two 2 oz cups were filled from the top of the tray and one from the bottom of the tray. This was repeated for the HT tray. The three individual samples from each piece of equipment were combined into one composite sample weighing 50 g in a Whirl-pak bag (Nasco, Fort Atkinson, WI).

Environmental sample collection

Environmental samples were collected from the ribbon of the mixer, the boot of the bucket elevator, a bucket of the bucket elevator, the conditioner of the pellet mill, the pellet die, and the interior of the pellet cooler during the positive batch of each replication for a total of 60 environmental samples. Environmental samples were taken using a 4- × 4-in. cotton surgical gauze pre-moistened with 5 mL of PBS that was prepared and stored in a 50 mL conical tube prior to sampling. The designated area was swabbed, and the gauze was returned to the conical tube.

Sample processing

Upon leaving the FSRC, samples underwent disinfection protocols before being placed on ice and transported to a biosafety level-2 laboratory in the K-State Veterinary Diagnostic Laboratory for further processing. Feed samples were transferred to 500 mL high density polyurethane bottles with 200 mL of PBS to create a 20% suspension. Feed samples were shaken for approximately 10 sec and stored at 39°F overnight. The next day, 25 mL of the supernatant from the feed samples were transferred to fresh 50 mL conical tubes. The supernatant was centrifuged at 4,000 × g for 10 minutes at 46.5°F. Two 300 µL aliquots were retained for PCR analysis and 20 mL was transferred to a fresh 50 mL conical tube for a bioassay. For environmental samples, 25 mL of PBS was added and each tube was vortexed for 10 sec. Environmental samples were allowed to incubate at room temperature for one hour. After one hour, a 20 mL aliquot was transferred to a fresh 50 mL conical tube. Centrifugation and sample allocation followed the feed sample procedures.

Quantitative viral analysis

Samples were analyzed for detection of SVV1, PEDV, and PRRSV using quantitative reverse transcription real-time polymerase chain reaction (qRT-PCR) at the Kansas State University Veterinary Diagnostic Laboratory. First, 50 µL of supernatant was placed in a deep well plate and RNA was extracted using a Kingfisher Flex magnetic particle processor (Fisher Scientific, Pittsburgh, PA) and a MagMAX-96 Viral Isolation Kit (Life Technologies, Grand Island, NY). The final elution volume was reduced to 60 µL, and extracted RNA was stored at -112°F until analyzed for SVV1, PEDV, or PRRSV using a qRT-PCR triplex assay with a maximum cycle threshold of 45. Results were reported as the number of samples considered positive and the cycle threshold (Ct) below 45 at which either SVV1, PEDV, or PRRSV RNA was detected.

Bioassay

A bioassay was conducted at the Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL) utilizing 4 treatment rooms with 3 mixed sex 10-day old piglets in each room. The day 0 inoculation treatments included 1) negative control; 2) pure virus positive control with 15 µL each of SVV1, PEDV, and PRRSV diluted in 15 mL of PBS which was approximately the concentration of the positive batch of feed; 3) feed samples from the positive batch pre-thermal processing (mixer or bucket elevator); and 4) feed samples from the positive batch post-thermal processing (conditioner, pellet die, or LT/HT cooler). Each pig was inoculated with 2 mL intramuscularly, 2 mL intranasally (1 mL/nostril), and either 10 mL oral gavage (feed samples) or a 10 mL dust slurry fed to the piglet to allow natural swallowing reflexes. Rectal swabs were collected day 1–7 post-inoculation (dpi), blood samples were collected -1, 4, and 7 dpi. Tonsils, lung tissue, jejunal and cecal tissue and cecal contents were collected at necropsy. Day 0 inoculum, rectal (SVV1 and PEDV), and serum (PRRSV) samples were analyzed via PCR at the ISU-VDL.

Statistical analysis

Main effects of location and thermal processing (pre- or post-) were analyzed using the GLIMMIX procedure of SAS (v. 9.4, SAS Institute Inc., Cary, NC). Samples from the mixer and bucket elevator would be classified as pre-thermal processing while the conditioner, pellet die, and cooler were considered post-thermal processing locations. Fixed effects included location and thermal processing. Inoculation cycle was included in the model as a random effect. Data were separated and analysis performed within sample type (feed or environmental) and virus (SVV1, PEDV, and PRRSV). Two response criteria were considered, the proportion of PCR positive samples and the quantity of detectable viral RNA. The proportions of PCR positive samples were analyzed by fitting to a binary distribution, logit link, Laplace approximation, and ridge-stabilized Newton-Raphson algorithm. As a binary distribution model, the data were fit by each main effect – location or thermal processing. To estimate the quantity of detectable viral RNA, the Ct of each sample was used and analyzed using a linear mixed model assuming a normal distribution. If no viral RNA was detected, samples were assigned a value of 45.0. A Kenward-Roger denominator degree of freedom adjustment was used, as well as a Tukey-Kramer multiple comparison adjustment. Results were considered significant at $P \leq 0.05$.

Results and Discussion

There were significant differences ($P < 0.001$) in the quantity of RNA found between the different feed sampling locations regardless of virus (Table 1). Feed from the mixer and bucket elevator had more SVV1, PEDV, or PRRSV RNA ($P < 0.05$) than the other sampling locations. As these were the feed manufacturing pieces prior to thermal processing, the presence of more viral RNA is not surprising. Interestingly, SVV1 RNA was found in feed following thermal processing from the conditioner, pellet die, and from the cooler while PEDV RNA was also identified in the cooler. Viral RNA was found on all pieces of equipment during environmental sampling; however, there was a noticeable decrease in SVV1 RNA ($P < 0.05$) on equipment used during the pelleting process. Thermal processing (Table 2) was able to decrease detectable RNA ($P < 0.05$) for all viruses regardless of sampling matrix. There was also a greater proportion of pre-thermal processing PEDV positive feed samples ($P < 0.05$) and SVV1 positive envi-

ronmental samples ($P < 0.05$) than their respective post-thermal processing samples (Table 3).

The results of the bioassay are shown in Table 4. As expected, the negative control pigs, who were given feed samples from the primer batch before virus was introduced, did not display clinical signs of SVV1, PEDV, or PRRSV infection during the study. Pigs given the pure virus (diluted to the approximate concentration of the feed) were SVV1 and PRRSV positive by d 4. Interestingly, signs of PEDV infection were not noticed in pigs given the pure virus even though the pure inoculum itself was found to be infective via virus isolation. As the pure PEDV was unable to cause infection, it is not surprising that no pigs showed clinical signs of infection in the pre-thermal feed treatment room. Pigs in the pre-thermal feed treatment room were positive for SVV1 on d 4 and PRRSV on d 7; however, it is unclear why persistent infections of both viruses were not observed for the duration of the bioassay. Although there was detectable SVV1 and PEDV RNA in post-thermal processing feed samples, the swine bioassay showed no signs of clinical infection for those pigs inoculated with these samples. Unlike SVV1 and PEDV, PRRSV RNA was not detectable in the post-thermal feed inoculum, but one pig exhibited clinical signs of PRRSV infection on d 7. This phenomenon was observed in treatment rooms that evaluated other objectives of the project within the FSRC, so it is unlikely this pig became infected from a single point of cross-contamination. The identification of a PRRSV positive pig demonstrates the sensitivity of a bioassay over the more cost-effective methods such as PCR. Furthermore, the temperatures used when pelleting these feed samples may not be sufficient to inactivate PRRSV, as infection did occur during this bioassay.

The use of pelleting reduced the quantity of detectable RNA in the feed and environmental surfaces within the feed mill. The remaining SVV1 and PEDV RNA was considered non-infectious when given to pigs but pelleting at a minimum of 180°F did not sufficiently reduce the risk of PRRSV infection. Furthermore, the accumulation of viral RNA throughout the feed manufacturing facility may further increase the risk of recontamination. Overall, pelleting is a suitable technique for reducing the risk of contamination provided care is taken to avoid contact between the pre-thermal and post-thermally processed feed, and sufficient temperatures are utilized.

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Table 1. Effect of location on the relative quantification of Seneca Valley virus 1 (SVV1), porcine epidemic diarrhea virus (PEDV), and porcine reproductive and respiratory syndrome virus (PRRSV) in feed samples during the pelleting process[1]

	Mixer	Bucket elevator	Conditioner	Pellet die	Cooler – Low temperature	Cooler – High temperature	SEM	*P* =
SVV1	27.7^{c}	27.3^{c}	43.5^{ab}	42.2^{ab}	45.0^{a}	41.4^{b}	1.10	< 0.0001
PEDV	36.8^{b}	36.1^{b}	45.0^{a}	45.0^{a}	45.0^{a}	44.4^{a}	1.05	< 0.0001
PRRSV	41.6^{b}	40.9^{b}	45.0^{a}	45.0^{a}	45.0^{a}	45.0^{a}	0.94	< 0.0001

[1] A batch of feed inoculated with SVV1, PEDV, and PRRSV was run through a feed manufacturing system and pelleted at a conditioning temperature of 180°F with a 30 sec retention time. Feed samples from the Cooler – Low Temperature shared circulating air with feed that had not reached the conditioning temperature of 180°F. Cooler – High Temperature samples only shared circulating air with feed that had met or exceeded a conditioning temperature of 180°F. A cycle threshold (Ct) value of 45.0 is considered negative with no detectable viral RNA.

[abc] means with differing superscripts within row differ significantly, $P < 0.05$.

Table 2. Effect of thermal processing on the relative quantification of Seneca Valley virus 1 (SVV1), porcine epidemic diarrhea virus (PEDV), and porcine reproductive and respiratory syndrome virus (PRRSV) in feed and environmental samples during the pelleting process[1]

	Pre-thermal processing	Post-thermal processing	SEM	*P* =
Feed samples				
SVV1	27.5^{b}	43.0^{a}	0.73	< 0.0001
PEDV	36.5^{b}	44.9^{a}	0.62	< 0.0001
PRRSV	41.3^{b}	45.0^{a}	0.56	< 0.0001
Environmental samples				
SVV1	33.6^{b}	42.9^{a}	0.83	< 0.0001
PEDV	42.8^{b}	44.2^{a}	0.58	0.018
PRRSV[2]	45.0	45.0	—	—

[1] A batch of feed inoculated with SVV1, PEDV, and PRRSV was run through a feed manufacturing system and pelleted at a conditioning temperature of 180°F with a 30 second retention time. Pre-thermal processing samples were taken before heat was introduced into the feed manufacturing system (mixer and bucket elevator) while post-thermal processing samples were taken after the introduction of heat (conditioner, pellet die, and cooler). A cycle threshold (Ct) value of 45.0 is considered negative with no detectable viral RNA.

[2] Statistical analysis was not conducted on PRRSV environmental samples as all were considered PCR negative.

[ab] means with differing superscripts within row differ significantly, $P < 0.05$.

Table 3. Effect of thermal processing on the proportion of PCR positive samples of Seneca Valley virus 1 (SVV1), porcine epidemic diarrhea virus (PEDV), and porcine reproductive and respiratory syndrome virus (PRRSV) in feed and environmental samples during the pelleting process[1]

	Pre-thermal processing	Post-thermal processing	*P* =
Feed samples[2]			
SVV1	20/20	11/40	0.843
PEDV	17/20[a]	1/40[b]	0.013
PRRSV	11/20	0/40	0.980
Environmental samples[3]			
SVV1	28/30[a]	9/30[b]	0.001
PEDV	8/30	4/30	0.075
PRRSV[4]	0/30	0/30	—

[1] A batch of feed inoculated with SVV1, PEDV, and PRRSV was run through a feed manufacturing system and pelleted at a conditioning temperature of 180°F with a 30 second retention time.
[2] Pre-thermal feed samples included the mixer and bucket elevator (n = 20), post-thermal samples included the conditioner, pellet die, and two samples from the cooler (n = 40).
[3] Pre-thermal environmental samples included the mixer and two samples from the bucket elevator (n = 30), post-thermal samples included the conditioner, pellet die, and the cooler (n = 30).
[4] Statistical analysis was not conducted on PRRSV environmental samples as all were considered PCR negative.
[ab] means with differing superscripts within row differ significantly, $P < 0.05$.

Table 4. Effect of thermal processing as Seneca Valley virus 1 (SVV1), porcine epidemic diarrhea virus (PEDV), and porcine reproductive and respiratory syndrome virus (PRRSV) mitigation as evaluated by a swine bioassay[1]

	SVV1		PEDV		PRRSV	
	4 dpi	7 dpi	4 dpi	7 dpi	4 dpi	7 dpi
Negative control	– – –	– – –	– – –	– – –	– – –	– – –
Pure virus positive control	+ + +	+ – –	– – –	– – –	+ + +	+ + +
Pre-thermal feed	– – –	+ + +	– – –	– – –	+ + –	– – –
Post-thermal feed	– – –	– – –	– – –	– – –	– – –	+ – –

[1] Three pigs per each treatment were inoculated on day 0 via intramuscular, intranasal, and either oral gavage (feed samples) or a slurry (dust samples) and evaluated for 7 days. Rectal samples were taken daily to evaluate SVV1 and PEDV presence and blood was collected on 4 and 7 dpi to assess PRRSV presence.
+/– corresponds to the viral status of each pig in the treatment room where + pigs signify viral RNA was present for the respective virus and – pigs had no detectable viral RNA.