RESEARCH REPORT
SWINE HEALTH

Title: Evaluating the prevalence and distribution of African swine fever virus during feed manufacture, as well as feed mill decontamination measures -_**NPB #20-018**

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Industry Summary: The feed supply chain can be a significant risk of pathogen transfer. While historically, farms have evolved to have biosecurity limiting farm-to-farm interaction, there continues to be gaps in swine biosecurity because the feed mill serves as a central point of cross-contact. These biosecurity gaps can lead to pathogens entering the feed supply chain. If a pathogen enters the feed manufacturing environment, it has the potential to be distributed. The objective of this experiment was to evaluate the prevalence and distribution of African swine fever virus (ASFV) if it enters a feed mill as a contaminated ingredient. To meet this objective, a pilot-scale feed mill was built inside a biosafety level-3Ag room in the Kansas State University Biosecurity Research Institute in Manhattan, KS. Equipment included a pilot-scale 50-kg mixer and a bucket elevator with downspout with 74 buckets and discharge rate of 4.5 kg/min. Initially, ASFV-negative ingredients were batched into the mixer, mixed for 5 minutes, and discharged through the bucket elevator and downspout into biohazard totes. Next, a batch of feed was manufactured that contained an ingredient spiked with ASFV. Finally, four subsequent batches of feed were manufactured using ingredients that did not contain ASFV. Between batches, ten samples of feed were collected after discharging into the biohazard tote in a double X pattern. Additionally, environmental samples were collected from feed contact surfaces, non-feed contact surfaces nearby the manufacturing location, non-feed contact surfaces further away from the manufacturing location (> 1 m), and transient surfaces that moved between zones, such as the shoes and clothing of personnel. Results indicated that ASFV was detected in all batches of feed after it was initially introduced into the manufacturing equipment. For example, there was no ASFV detected in feed samples in batch 1, but all ten samples collected in batches 2, 3, 4, 5, and 6 contained detectable levels of ASFV, even though batches 3, 4, 5, and 6 were manufactured with ASFV-free ingredients. This is supported by environmental samples, which demonstrated the interior of manufacturing equipment remained contaminated, even after manufacturing batches of feed with ASFV-free ingredients in an attempt to flush out the contamination. Contamination was distributed throughout the room, with 100% of transient surfaces containing ASFV after batch 2 was manufactured and staying contaminated through batch 6. In summary, this research demonstrates that once introduced to a feed mill, African swine fever virus is widely and relatively uniformly distributed. Manufacturing subsequent batches of feed helped reduce the quantity of ASFV, but some level of contamination remained, even after 4 batches of ASFV-free ingredients. This research indicates that feed mills should prioritize prevention of ASFV entry into the facility because once it enters, the contaminant can impact multiple batches and surfaces. Questions regarding this research or its implications can be directed to the Principal Investigator, Dr. Cassie Jones, at jonesc@ksu.edu.

Key Findings:

- Contamination with ASFV was rapid and widespread after introduction through inoculated feed and presence of ASFV-specific DNA minimally changed with each subsequent batch.

- Sequencing with four batches of feed can decrease overall ASFV contamination within feed samples but not eliminate it entirely
- Collecting 10 evenly distributed samples allows for detection of ASFV under the conditions of the current investigation
- If there is viral contamination within the feed mill environment, it can be found with environmental swabs
- Transient surfaces play an important role in the spread of virus through the feed mill. Moving objects like people, PPE, and trucks should be taken in account when designing feed biosecurity protocols and feed/feed mill surveillance could be pivotal in maintaining appropriate feed biosecurity.
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Keywords: African swine fever virus, distribution, feed, feed mill, prevalence

Scientific Abstract: It is critical to have methods that can detect and mitigate the risk of African Swine Fever Virus (ASFV) in potentially contaminated feed or ingredients bound for the United States. The purpose of this experiment was to evaluate feed batch sequencing as a mitigation technique for ASFV and if sampling methods could identify ASFV in feed samples following experimental inoculation. Batches of feed were manufactured in BSL-3Ag room at Biosafety Research Institute in Manhattan, Kansas. First, the pilot feed manufacturing system was used to mix, convey, and discharge an ASFV-free diet. Next, a diet was manufactured using the same equipment, but contained feed inoculated with ASFV for a final concentration of 5.6×10^4 $TCID_{50}$/g. Finally, four subsequent ASFV-free batches of feed were manufactured. After discharging each batch into a collection container, 10 samples were collected in a double 'X' pattern. Environmental swabs from 18 locations were collected after each batch of feed was discharged. The locations of the swabs were categorized into four zones: 1) feed contact surface, 2) non-feed contact surface < 1 meter away from feed, 3) non-feed contact surface > 1 meter from feed, and 4) transient surfaces. Samples were analyzed for ASFV p72 encoding gene PCR assay with response criteria of cycle threshold (Ct) and Log_{10} genomic copy number (CN)/g of feed. Batch of feed impacted Ct value (P<0.0001) and Log_{10} genomic CN/g (P<0.0001) of feed samples. Samples after manufacturing the positive control diet contained greatest amounts of detected p72 genetic material across all response criteria (P<0.05). Quantity of detected p72 genetic material decreased sequentially as additional batches of feed were manufactured but still detectable after sequence 4. The sampling method utilized was able to identify ASFV p72 genetic material in samples of feed. For environmental samples, there was no evidence of a zone × batch interaction for log_{10} genomic CN/mL (P = 0.625) or cycle threshold (Ct) value (P = 0.608). Sampling zone impacted the log_{10} p72 genomic CN/mL (P < 0.0001) and Ct values (P < 0.0001), with a greater amount of viral genome detected on transient surfaces compared to other surfaces (P < 0.05). In summary, sequencing batches of feed helps to decrease the concentration of ASFV contamination in feed, but does eliminate it. Bulk ingredients can be accurately evaluated for ASFV by collecting 10 subsamples using the methods described herein to create a common composite sample for analysis. Future research is needed to evaluate using two mitigation techniques in combination to reduce ASFV contamination in feed. Once ASFV enters the feed mill environment it becomes widespread and movement of people can significantly contribute to the spread of ASFV in a feed mill environment.

Introduction: The porcine epidemic diarrhea virus (PEDV) outbreak of 2013-2014 was the first major disease outbreak to suggest a potential link between contaminated feed and disease in pigs (USDA- APHIS, 2015). As a result, the concept of applying biosecurity practices to the United States (US) swine industry feed manufacturing and delivery systems became heavily emphasized. Due to the US naïve status to PEDV at the time along with the movement of contaminated vehicles associated with feed and animal delivery, the virus became endemic in the US. Another contributing factor to the quick spread of PEDV in the US was the feed mill. Once introduced into the feed mill, PEDV became widely distributed, serving as a continuous source of disease to the workers and feed delivery vehicles. Decontamination methods were often unsuccessful to rid the environment of PEDV but were also expensive and time consuming while sequencing of diets within the feed mill to

dilute the virus within the feed were also unsuccessful at eliminating PEDV. Research has continued to demonstrate that the risk for feed-based viral transmission extends beyond PEDV and includes diseases such as African swine fever virus (ASFV), foot and mouth disease virus (FMDV), or classical swine fever virus (CSFV; Dee at al., 2018, Stoian et al., 2020). This became particularly important in 2018 when a number of historically ASFV-free countries first began to report the disease (Gaudreault et al., 2020). The US maintains trade relationships with a number of countries that are now in ASFV-endemic regions, leading to concern that ASFV may enter the United States through the feed supply chain. There is no active surveillance for ASFV in feed or ingredients imported from endemic regions, nor is there a validated protocol to sample or analyze for the virus in a feed or ingredient matrix (USDA-APHIS-VS, 2019). It has been hypothesized that methods demonstrated appropriate for PEDV detection in feed may be applicable to ASFV, but this has not yet been tested. Furthermore, it has been suggested that mitigation measures common in PEDV, such as feed batch sequencing to reduce viral concentration, may be equally effective with ASFV. However, this has also never been evaluated.

Objectives: The objective of this experiment is to evaluate the prevalence and distribution, as well as the methods to decontaminate the facility.

Materials & Methods: *General*
The study was conducted at the Biosecurity Research Institute (BRI) in Manhattan, KS, with approval by the Kansas State University Institutional Biosafety Committee (project approval #1427.1). The feed manufacturing process was done within a biosafety level (BSL)-3Ag large animal room while laboratory work was done within a BSL-3+ laboratory space. Neither humans nor animals were used as research subjects in this experiment, so relevant approvals were not applicable.

Inoculation
To prepare the inoculum, 8.5 mL of pooled blood treated with ethylendiaminetetraacetic acid (EDTA) from ASFV infected pigs was mixed in RPMI media to prepare 530 mL of the virus inoculum at the final concentration of 2.7×10^6 $TCID_{50}$/mL of ASFV genotype II virus (Armenia 2007).

Manufacture and Sampling
Feed was manufactured as described by Schumacher et al. (2017). The feed manufacturing system was first primed with an ASFV-free batch of feed which was subsequently followed by a second batch of feed that was contaminated with ASFV. Four additional batches of ASFV-free feed were then mixed and discharged through the same equipment without any cleaning or disinfection occurring between batches. For this study, a corn and soybean-meal based diet with a composition normally fed to gestating sows was manufactured at the Kansas State University O.H. Kruse Food Technology Innovation Center (Manhattan, KS) and transported to the BSL-3Ag facility.

Treatments consisted of the following:

- Negative Control (Batch 1) – Priming the feed mill: To initiate the trial, a 25 kg batch of ASFV-free feed was mixed in a 50 kg capacity steel mixer with a 0.113 m^3 electric paddle mixer (H.C Davis Sons Manufacturing, model # SS-L1; Bonner Springs, KS). The feed was mixed for five minutes 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 feed. The feed was conveyed and discharged through a downspout into double-lined bags.

- Positive Control (Batch 2) - ASFV-contaminated feed: Upon completion of priming the system with the initial batch of ASFV-free feed, 530 mL of a genotype II (Armenia 2007) African swine fever virus (2.7×10^6 $TCID_{50}$/mL) was then mixed with 4.7 kg of diet in a 5 kg stainless steel mixer (Cabela's Inc., Sidney, NE) to make 5.23 kg of ASFV-contaminated feed which was subsequently added to 20 kg of feed with a final ASFV concentration of 5.6×10^4 $TCID_{50}$/g, and then mixed, conveyed, and discharged using the same equipment and procedures as previously described for the negative control.
- Sequences 1-4 (Batch 3, 4, 5, and 6) – Manufacture of subsequent batches of feed: Following discharge of the positive control batch of feed, the same process of mixing, conveying, and discharging 25 kg batches of feed was repeated four additional times using ASFV-free diet.

After a batch of feed was discharged, 10 feed samples were taken similar to Jones et al. (2020). Briefly, ten samples were taken from the feed that had been discharged in a biohazard tote through two 'X' patterns. The ten samples were not mixed together but analyzed in separate PCR reactions. This sampling technique resulted in a grand total of 60 feed samples for the entirety of the experiment.

Environmental Sampling

Environmental swabs were taken for the negative control, positive control, and batch sequences 1-4. Negative control samples were taken after priming the feed mill, positive control samples were taken after the usage of ASFV- contaminated feed, and batch sequences 1-4 samples were taken after each subsequent batch. All environmental swabs collected on previously marked environmental surfaces prior to inoculation with ASFV had no detectable ASFV DNA.

After each batch of feed was manufactured, environmental surfaces were swabbed using 10 cm × 10 cm cotton surgical gauze squares pre-moistened with 5 mL of phosphate-buffered solution (PBS) and individually stored in a 50 mL conical tube prior to usage. Prior to sample collection, a clean pair of outside gloves were donned and tubes aseptically opened by a sampling assistant. The previously chosen and marked location was swabbed, the environmental swab placed back in the conical tube, and outside gloves were changed. Once the experiment was concluded, samples were transferred to the BSL-3+ laboratory following appropriate procedures.

Locations for environmental sampling were chosen based off proximity to feed. Feed contact surface locations were the mixer ribbon, mixer barrel, mixer discharge, bucket elevator bucket, bucket elevator belt, and bucket elevator discharge. Non-feed contact surfaces < 1 m from feed locations were wall less than 1 m to mixer, wall less than 1 m to bucket elevator, floor less than 1 m to mixer, floor less than 1 m to bucket elevator, and ceiling less than 1 m to mixer. Non-feed locations > 1 m from feed locations were wall greater than 1 m from mixer, floor greater than 1 m from mixer, floor greater than 1 m from bucket elevator, and ceiling greater than 1 m from mixer. Transient surface locations were the boot soles of researchers walking through all other zones.

Laboratory Analysis

Feed samples and environmental swabs were tested at a BSL-3+ laboratory in the BRI. Briefly, 10 g of each feed sample was put in a tube, suspended with 35 mL of PBS, and the tube was capped and inverted, and incubated overnight at 4°C. Approximately 10 mL of

supernatant was recovered, aliquot into 5 ml cryovials, and stored at -80°C until processed for qPCR. For environmental samples, each swab was placed in a 50 ml conical tube and 20 mL of PBS was added, the tube was capped and inverted, and incubated overnight in 4°C. Tubes were vortexed for about 30 seconds and held upright for 5 minutes. Approximately 10 mL of supernatant was recovered, aliquoted into 5 ml cryovials, and stored at -80°C until processed for qPCR. In preparation for magnetic bead-based DNA extraction, 500µL of PBS eluent was combined with 500µL of Buffer AL (Qiagen, Germantown, MD, USA), briefly vortexed, and incubated at 70°C for 10 minutes in an oscillating heat block. DNA extraction was carried out using the GeneReach DNA/RNA extraction kit on a Taco mini automatic nucleic acid extraction system (GeneReach, Boston, MA, USA). The extraction was performed according to the manufacturer's instructions with modifications. Briefly, 200µL of AL sample lysate was transferred to column A of the taco deep-well extraction plate which contained 500µL of the GeneReach lysis buffer and 50µL of magnetic beads, followed by addition of 200µL of molecular grade isopropanol (ThermoFisher Scientific, Waltham, MA, USA). The extraction consisted of two washes with 750µL of wash buffer A, one wash with 750µL wash buffer B, and a final wash with 750µL of 200 proof molecular grade ethanol (ThermoFisher Scientific). After a five-minute drying time, DNA was eluted with 100 µL elution buffer and subsequently transferred into 1.5mL DNA/RNA- free centrifuge tubes (VWR) for storage. Positive and negative extraction controls were included in sample processing and consisted of the positive extraction control which was a partial sequence of the ASFV p72 gene cloned into plasmid Bluescript II and PCR-grade water, respectively. Real-time quantitative PCR (qPCR) was carried out using primers and probes designed to detect the gene encoding for ASFV p72 and PerfeCTa FastMix II (Quanta Biosciences, Gaithersburg, MD, USA) on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The qPCR reactions were performed in duplicate with each well containing 5µL of template DNA, 0.2µL (200nM) of each primer (Integrated DNA Technology, Coralville, IA, USA), and 0.4µL (200nM) of FAM probe (Thermo Fisher Scientific) in a total reaction volume of 20 µL. Thermocycling conditions were 95°C for 5 minutes, followed by 45 cycles of 95°C for 10 seconds and 60°C for 1 minute.

ASFV p72 genomic copy number (CN) was calculated using reference standard curve methodology using a reference standard curve composed from ten-fold serial dilutions performed in triplicate of the quantitated ASFV p72 plasmid DNA control. The CN for samples were mathematically determined using the PCR-determined cycle threshold (Ct) for ASFV p72 (two PCR well replicates) and the slope and intercept of the ASFV p72 DNA standard curve. Genomic CN/g for each sample was based upon the genomic CN/mL - the response value of genomic CN/mL was multiplied by the value 35 due to feed samples being suspended in 35 mL of PBS then divided by 10 because there was 10 g of feed per suspension to get the respective genomic CN/g.

Statistical Analysis

Statistical analysis for this study was performed using R programming language [Version 3.6.1 (2019-07-05), R Core Team, R Foundation for Statistical Computing, Vienna, Austria]. Experimental unit for this study was the feed sample. Each feed sample had one extraction for PCR assay and each extraction was run in duplicate for PCR analysis with the exception of samples from batch 2 in which each feed sample had two extractions for PCR assay and both extractions were run in duplicate for PCR analysis as an initial assessment to evaluate the variability present within the extraction and amplification procedures. For environmental samples, data were analyzed as 4 × 5 factorial arrangement with 4 sampling surfaces, and 5 batches of feed not including the initial negative control samples. Individual

sample collected from a surface for a specific batch was considered the experimental unit. Response values for the ASFV p72 gene were analyzed using a linear mixed model fit using the lme function in the nlme packing using a normal distribution with the fixed effect as batch with a random effect of sample to indicate the appropriate level of experimental replication given the duplicate qPCR analysis of feed samples. Results of Ct and genomic CN/g are reported as least squares means ± standard error of the mean. Samples not containing detectable ASFV DNA were assigned a value of 45 because that was the greatest number of cycles the qPCR assay performed before concluding a sample did not have detectable ASFV DNA. Genomic CN/g data were Log_{10} transformed and analysis included PCR negative reactions using a value of 0 for the quantified genomic CN/g. All statistical models were evaluated using visual assessment of studentized residuals and models accounting for heterogeneous residual variance were used when appropriate. A Tukey multiple comparison adjustment was incorporated when appropriate. Results were considered significant at $P \leq 0.05$ and marginally significant between $P > 0.05$ and $P \leq 0.10$.

Results: After the ASFV positive batch of feed was manufactured, all samples had detectable ASFV p72 genetic material. The number of samples with detectable ASFV p72 genetic material decreased with each subsequent batch. However, by sequence 4, feed samples still contained detectable ASFV p72 genetic material. For ASFV p72 genetic material, batch of feed impacted the Ct value ($P < 0.0001$) and Log_{10} genomic CN/g ($P < 0.0001$) of samples. Samples after manufacturing the positive control diet contained the greatest amount of detected p72 genetic material across all response criteria ($P < 0.05$). Sequence 1 had slightly lower levels detected compared to the positive control diet ($P < 0.05$), and sequence 4 had a lower detected quantity than both the positive control and sequence 1 diets ($P < 0.05$). The level of detectable ASFV genetic material in sequence 2 and 3 were intermediate between sequence 1 and 4. In general, the quantity of detected p72 genetic material decreased sequentially as additional batches of feed were manufactured. However, detection of p72 genetic material was still possible after 4 sequences of ASFV free feed.

As expected, environmental swabs collected prior to inoculation had no detectable ASFV DNA. Environmental swabs collected after the manufacture of ASFV-contaminated feed showed presence of ASFV-specific DNA in all zones, with 38% to 100% of qPCR reactions resulting in detectable ASFV DNA depending on the contact surface. There was no evidence of a sampling zone × batch of feed interaction for prevalence of qPCR reactions detecting ASFV DNA ($P = 0.912$), log_{10} genomic copies/mL ($P = 0.625$), or Ct value ($P = 0.608$). Additionally, there was insufficient evidence to conclude that the proportion of qPCR positive reactions was affected by sampling zone ($P = 0.701$) or batch of feed ($P = 1.000$).

Batch of feed influenced the Ct value for environmental samples ($P = 0.037$), with samples collected after manufacture of the ASFV- contaminated batch of feed having a lower Ct value compared to the environmental swabs collected after sequence 3 ($P < 0.05$). Environmental swabs collected after other sequences (1, 2, 4) were intermediate in terms of Ct value. There was marginally significant evidence that batch of feed influenced log_{10} p72 genomic copy/mL ($P = 0.059$), however no significant pairwise differences were detected when using a Tukey multiple comparison adjustment.

There was a significant difference in both the Ct value and log_{10} genomic copy/mL values between sampling zones ($P < 0.0001$), with the transient surfaces having lower Ct values ($P < 0.05$) and greater log_{10} p72 genomic copies/mL ($P < 0.05$) compared to all other sampling

zones. This indicates that the soles of worker boots contained a greater quantity of detectable ASFV DNA compared to all other sampling zones, including feed contact and non-feed contact surfaces.

Discussion: These results suggest that flushing a feed mill with ASFV free feed after an ASFV contaminated feed will reduce the amount of ASFV in the feed but won't eliminate the virus entirely. Schumacher et al. (2019) found similar results in their study evaluating sequencing to reduce PEDV contamination. The current study's findings also suggest that this sampling technique was able to identify ASFV contamination within feed samples and supports Jones et al. (2020) who had similar success with this sampling method for various levels of PEDV contamination within feed containers. Probability of infection for this data could be estimated from Neiderwerder et al. (2019) data on ASFV infectious dose and probability of infection. Based off their exposure model, the amount of genomic CN/g found in this study's feed samples from sequence 1-4 has infection probability ranging from 0.25 – 1.00. However, Neiderwerder et al. (2019) used a different ASFV genotype than this study so infectivity based off this model is purely extrapolation.
It is evident that distribution of ASFV into the feed manufacturing environment is widespread and persists even after manufacturing additional feed batches initially free of ASFV. This indicates that it is extremely important for the US to prevent the entry of ASFV into US feed mills since once ASFV is in a feed mill, it will remain in its environment for an extended period of time. This knowledge is important to consider when designing and implementing surveillance and monitoring programs for ASFV as currently being investigated in ASFV endemic regions.

The present study demonstrates that transient surfaces had the highest amount of detectable ASFV DNA across all zones. This indicates that people and personal protective equipment (PPE) have a high potential to spread viruses within the feed mill. This is a consistent finding because it was previously reported that moving objects of a farm, like trucks and feed, contributed to the spread of PEDV, and that PPE and people transmitted PEDV to naïve herds. An understanding of the contamination within the feed mill environment is vital due to how the US manufactures and distributes feed within the swine industry. If a feed truck is contaminated, there is a risk that it could contaminate the production site it is delivering to, but it also could potentially contaminate the feed mill when returning from a production site currently experiencing a disease outbreak. Additionally, recent information from Vietnam has indicated that feed trucks are an area where contamination with ASFV can be found. This current study along with previous studies with PEDV and ASFV highlight the importance of understanding the epidemiological interaction of the US feed delivery system regardless of the virus of concern.

A significant limitation of this study is the lack of infectivity data associated with the feed containing qPCR detectable ASFV-specific DNA. This research utilizes ASFV, a BSL-3 pathogen and select agent in the US, for which there are no validated virus isolation or pig bioassay methods. Validating these infection assays for feed are critically important, but out of the scope of this research. Our primary goal was to evaluate how the manufacture of feed with an ASFV-contaminated ingredient impacts the spread of that contamination throughout subsequent feed batches and the feed mill environment, which we have demonstrated with the response criteria selected in this study. We believe that the data herein provide significant value to the literature through establishing distribution

characteristics of ASFV within a feed manufacturing facility which can provide critical background knowledge to assist with epidemiological investigations.
In conclusion, sequencing with four batches of feed can decrease overall ASFV contamination within feed samples but not eliminate it entirely. In addition, collecting 10 evenly distributed samples allows for detection of ASFV under the conditions of the current investigation. These findings highlight the importance of excluding ingredients from ASFV endemic countries but also highlights that proper sampling can be an effective tool to detect contamination. Additional research is necessary to evaluate the combination of mitigation techniques like chemically treating flush diets (similar to what is done with PEDV) on ASFV contaminated ingredients. Furthermore, this study reveals that ASFV has similar distribution characteristics as PEDV in a feed mill environment. Contamination with ASFV was rapid and widespread after introduction through inoculated feed and presence of ASFV-specific DNA minimally changed with each subsequent batch. This study also proved that if there is viral contamination within the feed mill environment, it can be found with environmental swabs. In areas where ASFV or PEDV is considered endemic, environmental swabs can be incorporated into surveillance programs or feed mill audits to understand the potential contamination within the feed mill and respective delivery system. In the present study it was also demonstrated that transient surfaces play an important role in the spread of virus through the feed mill. Moving objects like people, PPE, and trucks should be taken in account when designing feed biosecurity protocols and feed/feed mill surveillance could be pivotal in maintaining appropriate feed biosecurity.

Table 1. Diet composition (as-fed basis)

Item	Swine gestation diet
Ingredient, %	
Corn	78.41
Soybean meal[1]	17.27
Soybean oil	0.50
Calcium carbonate	1.30
Monocalcium phosphate	1.30
Sodium chloride	0.50
Trace mineral[2]	0.15
Sow add pack[3]	0.25
Vitamin premix[4]	0.25
Phytase[5]	0.08
Total	100
Calculated analysis, %[6]	
Crude protein	14.7
Crude fiber	3.5
Crude fat	2.2
Total calcium	0.91
Total phosphorous	0.61

[1] Conventional dehulled, solvent extracted soybean meal.
[2] Each kg of premix contains 73 g Fe, 73 g Zn, 22 g Mn, 11 g Cu, 198 mg I, and 198 mg Se.
[3] Each kg of premix contains 1,650,000 IU vitamin A, 8,800 IU vitamin E, 88 mg biotin, 396 mg pyridoxine, 880 mg folic acid, 220,000 mg choline, 79 mg chromium, 19,800 mg L-carnitine.
[4] Each kg of premix contains 1,650,000 IU vitamin A, 660,000 IU vitamin D3, 17,600 IU vitamin E, 1,320 mg menadione, 3,300 mg riboflavin, 11,000 mg d-pantothenic acid, 19,800 mg niacin, 13 mg vitamin B12.
[5] HiPhos 2700 (DSM Nutritional Products, Parsippany, NJ).
[6] NRC. 2012. Nutrient Requirements of Swine, 11th ed. Natl. Acad. Press, Washington D.C.

Table 2. Location of environmental swabs and grouping by zone.

Zone type	Location
Feed contact surface	Mixer ribbon
	Mixer barrel
	Mixer discharge
	Bucket elevator bucket
	Bucket elevator belt
	Bucket elevator discharge
Non-feed contact surface < 1 meter away from feed contact surface	Wall close to mixer
	Wall close to bucket elevator
	Floor close to mixer
	Floor close to bucket elevator
	Ceiling close to mixer
Non-feed contact surface > 1 meter away from feed contact surface	Wall far from mixer
	Floor far from mixer
	Floor far from bucket elevator
	Ceiling far from mixer
Transient surface	Boot sole of researcher A
	Boot sole of researcher B
	Boot sole of researcher C

Table 3. Detection of African swine fever virus (ASFV) p72 DNA in feed samples

	Batch of feed[†]

	Negative	Positive	Sequence 1	Sequence 2	Sequence 3	Sequence 4
Positive	0	10	10	9	9	7
Suspect	0	0	0	1	1	3
Non-detected	10	0	0	0	0	0

[†]Swine gestation feed was inoculated with African swine fever virus (ASFV) at 5.6×10^4 $TCID_{50}$/gram inoculated feed (positive) following an initial priming of the feed manufacturing equipment with ASFV free feed (negative). Four subsequent batches of feed were manufactured (sequence 1 to 4) and were initially free of ASFV. Ten feed samples were collected after each batch of feed and were analyzed using an ASFV p72 qPCR assay with each sample analyzed in duplicate for each assay. Samples were considered PCR positive if 2 of 2 PCR reactions had detectable ASFV DNA, suspect if 1 of 2 PCR reactions had detectable ASFV DNA, and non-detected if 0 of 2 PCR reactions had detectable ASFV DNA.

Table 4. Concentration of detectable African swine fever virus (ASFV) p72 DNA in feed samples.

	Batch of feed[†,‡]					
Assay:	Negative	Positive	Sequence 1	Sequence 2	Sequence 3	Sequence 4
Cycle threshold[§]	45.0	33.0 ± 0.37[a]	37.5 ± 0.42[b]	39.5 ± 0.61[b,c]	39.3 ± 0.61[b,c]	40.1 ± 0.61[c]
Log_{10} genomic copies/g[¶]	0.0	4.74 ± 0.08[a]	3.62 ± 0.09[b]	3.11 ± 0.23[b,c]	3.07 ± 0.23[b,c]	2.77 ± 0.23[c]

[†]Swine gestation feed was inoculated with African swine fever virus (ASFV) at 5.6×10^4 $TCID_{50}$/gram inoculated feed (positive) following an initial priming of the feed manufacturing equipment with ASFV free feed (negative). Four subsequent batches of feed were manufactured (sequence 1 to 4) and were initially free of ASFV. Ten feed samples were collected after each batch of feed and were analyzed using an ASFV p72 encoding gene qPCR assay with each sample analyzed in duplicate for each assay.

[‡]Statistical analysis includes all treatment groups except for negative control where samples were collected prior to ASFV inoculation. Values for main effect of batch do not include negative batch of feed.

[§]Cycle threshold values for PCR reactions with no detectable ASFV p72 gene were assigned a value of 45 within the statistical analysis. Batch: $P < 0.0001$

[¶]Log_{10} transformed genomic copies for ASFV p72 encoding gene per g of feed from feed samples. Batch: $P < 0.0001$.

[abc] Means within row lacking common superscript differ ($P < 0.05$) using Tukey multiple comparison adjustment.

Table 5. Interactive effect of feed batch and zone on detection of African swine fever virus (ASFV) during manufacture of virus inoculated

feed[1,2]

Item	Batch of feed					
	Negative	Positive	After sequence 1	After sequence 2	After sequence 3	After sequence 4
Detectable DNA/Total[3]						
Feed contact	0/12	9/12	6/12	5/12	6/12	5/12
Non-feed contact, < 1 m	0/10	8/10	5/10	4/10	1/10	3/10
Non-feed contact, > 1 m	0/8	3/8	4/8	4/8	3/8	3/8
Transient surface	0/6	6/6	6/6	6/6	6/6	6/6
Log_{10} genomic copy number/mL[4]						
Feed contact	0	2.74 ± 0.481	1.51 ± 0.481	1.16 ± 0.481	1.75 ± 0.481	1.32 ± 0.481
Non-feed contact, < 1 m	0	2.70 ± 0.526	1.55 ± 0.526	1.04 ± 0.526	0.28 ± 0.526	0.86 ± 0.526
Non-feed contact, > 1 m	0	0.96 ± 0.589	1.27 ± 0.589	1.45 ± 0.589	0.91 ± 0.589	1.06 ± 0.589
Transient surface	0	4.44 ± 0.455	4.07 ± 0.455	3.92 ± 0.455	3.83 ± 0.455	4.14 ± 0.455
Cycle threshold[5]						
Feed contact	45.0	37.3 ± 1.33	41.1 ± 1.33	42.2 ± 1.33	40.2 ± 1.33	41.5 ± 1.33
Non-feed contact, < 1 m	45.0	37.7 ± 1.46	41.0 ± 1.46	42.8 ± 1.46	44.3 ± 1.46	42.9 ± 1.46
Non-feed contact, > 1 m	45.0	42.8 ± 1.63	42.3 ± 1.63	41.4 ± 1.63	43.0 ± 1.63	42.4 ± 1.63
Transient surface	45.0	31.6 ± 1.40	33.1 ± 1.40	33.7 ± 1.40	34.1 ± 1.40	32.8 ± 1.40

[1] Swine gestation feed was inoculated with African swine fever virus (ASFV) at 5.6×10^4 $TCID_{50}$/gram inoculated feed (positive) following an initial priming of the feed manufacturing equipment with ASFV free feed (negative). Four subsequent batches of feed were manufactured (sequence 1 to 4) and were initially free of ASFV. Environmental samples were collected at multiple locations within the facility following each batch of feed and were analyzed using an ASFV p72 encoding gene qPCR assay.

[2] Statistical analysis includes all treatment groups except for negative control.

[3] Count of PCR reactions with detectible ASFV DNA/number of qPCR reactions for each combination of sampling location and batch with each sampling swab was analyzed by duplicate reactions; Zone × Batch, $P = 0.912$; Zone, $P = 0.701$; Batch, $P = 1.000$.

[4] Log_{10} transformed genomic copies for ASFV p72 encoding gene per mL of solution recovered from environmental swab sample ± standard error of mean. Zone × Batch, $P = 0.625$; Zone, $P < 0.0001$; Batch, $P = 0.059$.

[5] Cycle threshold values with samples having no detectable ASFV DNA (ND) being assigned a value of 45 within the statistical analysis ± standard error of mean. Zone × Batch, $P = 0.608$; Zone, $P < 0.0001$; Batch, $P = 0.037$.

Table 6. Main effect of feed batch and zone on detection of African swine fever virus (ASFV) during manufacture of virus inoculated feed[1,2]

Main effect	Detectable DNA/Total[3]	Log_{10} genomic copy number/mL[4]	Cycle threshold[5]
Batch			
Negative	0/36	0	45.0
Positive	26/36	2.71 ± 0.258	37.4 ± 0.73[a]
After sequence 1	21/36	2.10 ± 0.258	39.4 ± 0.73[a,b]
After sequence 2	19/36	1.89 ± 0.258	40.0 ± 0.73[a,b]
After sequence 3	16/36	1.69 ± 0.258	40.4 ± 0.73[b]
After sequence 4	17/36	1.85 ± 0.258	39.9 ± 0.73[a,b]
Zone			
Feed contact	31/60	1.70 ± 0.215[a]	40.5 ± 0.60[a]
Non-feed contact, < 1 m	21/50	1.29 ± 0.235[a]	41.7 ± 0.65[a]
Non-feed contact, > 1 m	17/40	1.13 ± 0.263[a]	42.4 ± 0.73[a]
Transient surface	30/30	4.08 ± 0.203[b]	33.1 ± 0.63[b]

[1] Swine gestation feed was inoculated with African swine fever virus (ASFV) at 5.6×10^4 $TCID_{50}$/gram inoculated feed (positive) following an initial priming of the feed manufacturing equipment with ASFV-free feed (negative). Four subsequent batches of feed were manufactured (sequence 1 to 4) and were initially free of ASFV. Environmental samples were collected at multiple locations within the facility following each batch of feed and were analyzed using an ASFV p72 encoding gene qPCR assay.

[2] Statistical analysis includes all treatment groups except for negative control where samples were collected prior to ASFV inoculation. Values for main effect of contact surface do not include negative batch of feed.

[3] Count of PCR reactions with detectible ASFV DNA/number of qPCR reactions for each combination of sampling location and batch with each sampling swab was analyzed by duplicate reactions; Batch, $P = 1.000$; Zone, $P = 0.701$.

[4] Log_{10} transformed genomic copies for ASFV p72 encoding gene per mL of solution recovered from environmental swab sample; Batch, $P = 0.059$; Zone, $P < 0.0001$.

[5] Cycle threshold values with samples having no detectable ASFV DNA being assigned a value of 45 within the statistical analysis; Batch, $P = 0.037$; Zone, $P < 0.0001$.

[abc] Means within main effect lacking common superscript differ ($P < 0.05$) using Tukey multiple comparison adjustment.