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Persistence of African Swine Fever Virus in Feed and Feed Mill Environment over Time after Manufacture of Experimentally Inoculated Feed

Grace E. Houston
Kansas State University, cgelijah@k-state.edu

Jessie D. Trujillo
Kansas State University, jdtrujillo@k-state.edu

Cassandra K. Jones
Kansas State University, jonesc@k-state.edu

See next page for additional authors

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Persistence of African Swine Fever Virus in Feed and Feed Mill Environment over Time after Manufacture of Experimentally Inoculated Feed

Abstract

To reduce the risk of disease from harmful feed-based pathogens, some feed manufacturers quarantine high-risk ingredients prior to their inclusion in feed. Data exist that confirms this practice is effective, but to our knowledge there is no information about porcine pathogen survival in mill environments. The objective of this study was to determine survival of African swine fever virus (ASFV) in swine feed and on mill surfaces after manufacture of experimentally inoculated swine feed. A pilot-scale feed mill was placed within a biosecurity level (BSL) 3 facility to manufacture batches of feed. The priming batch, Batch 1, was ASFV-free feed and was followed with Batch 2 which was experimentally inoculated with ASFV (5.6×10^4 $TCID_{50}$/gram). Four subsequent ASFV-free batches were then manufactured (Batch 3-6). After each batch of feed, 10 feed samples were aseptically collected in a double 'X' pattern. During feed manufacturing, 24 steel coupons were placed on the floor of the manufacturing area and feed dust was allowed to settle onto them overnight. Once feed manufacturing was completed, feed samples and steel coupons were stored at room temperature. On the day of (day 0) and d 3, 7, 14, 28, 60, 90, and 180 after feed manufacturing, feed samples and 3 steel coupons were randomly selected, taken out of storage, and analyzed for ASFV DNA. For feed samples there was a statistically significant ($P = 0.023$) batch × day interaction for $\log_{10}$ genomic copies per gram of feed, and a marginal statistical significance ($P = 0.072$) for batch × day interaction for cycle threshold (Ct) values. This indicates that the batch of feed and days held at room temperature impacted the amount of the detectable ASFV DNA in feed samples. There was no evidence ($P = 0.433$) of ASFV degradation on environmental coupons over the 180-d storage period. This study found that quarantine time can help reduce, but not eliminate ASFV DNA in feed over time. Surprisingly, ASFV DNA is detectable on feed manufacturing surfaces for at least 180 days.

Keywords

African swine fever virus, dust, feed mill, feed samples, persistence

Cover Page Footnote

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Authors

Grace E. Houston, Jessie D. Trujillo, Cassandra K. Jones, Taeyong Kwon, Charles R. Stark, Konnor R. Cool, Chad B. Paulk, Natasha N. Gaudreault, Jason C. Woodworth, Igor Morozov, Carmina Gallardo, Jordan T. Gebhardt, and Juergen A. Richt

K-STATE Research and Extension

SWINE DAY 2022

KANSAS STATE UNIVERSITY SWINE INDUSTRY DAY 2022

Persistence of African Swine Fever Virus in Feed and Feed Mill Environment over Time after Manufacture of Experimentally Inoculated Feed[1]

Grace E. Houston,[2] Jessie D. Trujillo,[2,3] Cassandra K. Jones, Taeyong Kwon,[2,3] Charles R. Stark,[4] Konner Cool,[2,3] Chad B. Paulk,[4] Natasha N. Gaudreault,[2,3] Jason C. Woodworth, Igor Morozov,[2,3] Carmina Gallardo,[5] Jordan T. Gebhardt,[2] and Juergen A. Richt[2,3]

Summary

To reduce the risk of disease from harmful feed-based pathogens, some feed manufacturers quarantine high-risk ingredients prior to their inclusion in feed. Data exist that confirms this practice is effective, but to our knowledge there is no information about porcine pathogen survival in mill environments. The objective of this study was to determine survival of African swine fever virus (ASFV) in swine feed and on mill surfaces after manufacture of experimentally inoculated swine feed. A pilot-scale feed mill was placed within a biosecurity level (BSL) 3 facility to manufacture batches of feed. The priming batch, Batch 1, was ASFV-free feed and was followed with Batch 2 which was experimentally inoculated with ASFV (5.6×10^4 $TCID_{50}$/gram). Four subsequent ASFV-free batches were then manufactured (Batch 3-6). After each batch of feed, 10 feed samples were aseptically collected in a double 'X' pattern. During feed manufacturing, 24 steel coupons were placed on the floor of the manufacturing area and feed dust was allowed to settle onto them overnight. Once feed manufacturing was completed, feed samples and steel coupons were stored at room temperature. On the day of (day 0) and d 3, 7, 14, 28, 60, 90, and 180 after feed manufacturing, feed samples and 3 steel coupons were randomly selected, taken out of storage, and analyzed for ASFV DNA. For feed samples there was a statistically significant ($P = 0.023$) batch × day interaction for $\log_{10}$ genomic copies per gram of feed, and a marginal statistical significance ($P = 0.072$) for batch × day interaction for cycle threshold (Ct) values. This indicates that the batch of feed and days held at room temperature impacted the amount of the detectable ASFV DNA in feed samples. There was no evidence

[1] Funding was provided the National Pork Board and U.S. Department of Agriculture National Bio and Agro-Defense Facility Transition Funds.

[2] Department of Diagnostic Medicine/Pathobiology, College of Veterinary Medicine, Kansas State University.

[3] Center of Excellence for Emerging and Zoonotic Animal Disease, College of Veterinary Medicine, Kansas State University.

[4] Department of Grain Science and Industry, College of Agriculture, Kansas State University.

[5] Instituto Nacional de Investigación y Technología Agraria y Alimentaria, Animal Health Research Centre, Madrid, Spain.

($P = 0.433$) of ASFV degradation on environmental coupons over the 180-d storage period. This study found that quarantine time can help reduce, but not eliminate ASFV DNA in feed over time. Surprisingly, ASFV DNA is detectable on feed manufacturing surfaces for at least 180 days.

Introduction

In 2018, African swine fever virus (ASFV) was confirmed for the first time in Asia and is now considered endemic in several regions globally.[6] As ASFV continues to spread, there are some concerns that ASFV could potentially be introduced into naïve countries via trade of feed ingredients sourced from these areas. Given this potential risk, it is important to understand strategies that could be implemented to mitigate any potential viral contamination introduced through imported feed ingredients. One way to mitigate the risk of potentially contaminated feed ingredients from these areas, is to hold or quarantine the ingredients at arrival so that any potential virus present within these feed ingredients could naturally decay over time. Recent research investigating ASFV characteristics in swine feed and feed mill environments has shown that ASFV can persist within the feed mill environment and swine feed after manufacture of 6 batches of feed and people can play an important part in spreading ASFV within the feed mill.[7,8] However, this research evaluated quantity of ASFV on the day of feed manufacturing and not the impacts of ASFV quantity over time. Given the recent incursion of ASFV within the western hemisphere for the first time in 40 years,[9] it is critical to understand the long-term implications and a potential risk mitigation technique if ASFV were to be introduced within a feed mill. Therefore, this study aimed to evaluate holding times at room temperature for experimentally inoculated complete swine feed and dust generated during feed manufacturing.

Procedures

General

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

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[6] Gaudreault, N.N., Madden, D.W., Wilson, W.C., Trujillo, J.D., and Richt, J.A. (2020). African Swine Fever Virus: An Emerging DNA Arbovirus. *Front. Vet. Sci. 7:215.* doi:10.3389/fvets.2020.00215.

[7] Elijah, C.G., Trujillo, J.D., Jones, C.K., Kwon, T., Stark, C.R., Cool, K., Paulk, C.B., Gaudreault, N.N., Woodworth, J.C., Morozov, I., Gallardo, C., Gebhardt, J.T., and Richt, J.A. (2021). Effect of mixing and feed batch sequencing on the prevalence and distribution of African swine fever virus in swine feed. *Transboundary and Emerging Diseases:* 1-6. doi:10.1111/tbed.14177.

[8] Elijah, C.G., Trujillo, J.D., Jones, C.K., Gaudreault, N.N., Stark, C.R., Cool, K.R., Paulk, C.B., Kwon, T., Woodworth, J.C., Morozov, I., Gallardo, C., Gebhardt, J.T., and Richt, J.A. (2021). Evaluating the distribution of African swine fever virus within a feed mill environment following manufacture of inoculated feed. *PLoS ONE 16(8):*e0256138. doi:10.1371/journal.pone.0256138.

[9] Paulino-Ramirez, R. and Jimenez, J.A. (2021). Food Security and Research Agenda in African Swine Fever Virus: a new Arbovirus Threat in the Dominican Republic. *InterAmerican Journal of Medicine and Health,* 4. doi:10.31005/iajmh.v4i.210.

Inoculation

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

Feed manufacture and sampling

Feed was manufactured in the following order of events:

Negative control (Batch 1): To prime the feed mill, a 55-lb batch of ASFV-free feed was mixed in a 110 lb capacity (0.12 $yard^3$) steel electric paddle mixer (H.C. Davis Sons Manufacturing, model # SS-L1; Bonner Springs, KS). The feed was mixed for 5 min then discharged at a rate of approximately 10 lb/min into the bucket elevator conveyor (Universal Industries, Cedar Falls, IA) that carried 74 buckets (each 44.8 $in.^3$) of feed. The feed was conveyed and discharged through a downspout into a biohazard tote.

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 ASFV (strain Armenia 2007) at a concentration of 2.7×10^6 $TCID_{50}$/mL was then mixed with 10.3 lb of feed in an 11-lb stainless steel mixer (Cabela's Inc., Sidney, NE) to make 11.5 lb of ASFV-contaminated feed. This mixture was subsequently added to 44 lb of feed resulting in 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): Following discharge of the ASFV-contaminated batch of feed, the same process of mixing, conveying, and discharging 55-lb batches of ASFV-free feed was repeated 4 additional times.

After a batch of feed was discharged, 10 feed samples were taken similar to that described by Jones et al.[10] Briefly, ten samples were taken from the feed that had been discharged in a biohazard tote through two 'X' patterns. This sampling pattern was done eight separate times after every batch to account for the day of and 3, 7, 14, 28, 60, 90, and 180 days of holding time in room temperature (RT) storage. Once feed manufacturing was completed, all feed samples were transported to a BSL-3+ laboratory for ASFV DNA analysis. Day of manufacturing feed samples were analyzed as previously described and included from Elijah et al.[6] as part of the data analysis. All other feed samples were stored at RT for the intended storage time. On 3, 7, 14, 28, 60, 90, and 180 days after feed manufacturing, the 10 corresponding feed samples for each batch were removed from RT storage and 3 of the feed samples were randomly selected for ASFV DNA analysis while the remaining 7 feed samples were discarded.

[10] Jones, C., Stewart, S., Woodworth, J., Dritz S., & Paulk, C. (2020). Validation of sampling methods in bulk feed ingredients for detection of swine viruses. *Transboundary and Emerging Diseases,* 67, 1-5. doi:10.1111/tbed.13326.

Dust sampling

Nine stainless steel coupons, referred to as environmental discs, were placed in three different locations during the feed manufacturing process, allowed to collect dust during the milling process, and rested overnight. The next morning, the environmental discs were placed into a storage container and stored at RT in a locked cabinet. On the day of and 3, 7, 14, 28, 60, 90, and 180 days after manufacturing, one environmental disc from each of the three locations was randomly selected and swabbed using a 10 × 10 cm cotton gauze as previously described.[7]

Laboratory analysis

Feed samples and environmental swabs from environmental discs were tested at a BSL-3+ laboratory in the BRI. For the feed samples, 10 g of feed was put in a tube, suspended with 35 mL of PBS, and the tube was capped and inverted, and incubated overnight in 39°F. Approximately 10 mL of supernatant was recovered, aliquoted into 5 mL cryovials, and stored at -112°F until processed for qPCR. For environmental swabs from the environmental discs, each swab was placed in a 50 mL conical tube, 20 mL of PBS was added, the tube was capped and inverted, and incubated overnight in 39°F. 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 -112°F until processed for qPCR. After this step, feed samples and environmental disc samples were processed in a similar manner.

In preparation for magnetic bead-based DNA extraction, 500 µL of PBS eluent was combined with 500 µL of Buffer AL (Qiagen, Germantown, MD), briefly vortexed, and incubated at 158°F for 10 min in an oscillating heat block. The DNA extraction was carried out using the GeneReach DNA/RNA extraction kit on a Taco mini automatic nucleic acid extraction system (GeneReach, Boston, MA). 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 500 µL of the GeneReach lysis buffer and 50 µL of magnetic beads, followed by addition of 200 µL of molecular grade isopropanol (ThermoFisher Scientific, Waltham, MA). The extraction consisted of two washes with 750 µL of wash buffer A, one wash with 750 µL wash buffer B, and a final wash with 750 µL of 200 proof molecular grade ethanol (ThermoFisher Scientific). After a 5 min drying time, DNA was eluted with 100 µL elution buffer and subsequently transferred into 1.5 mL DNA/RNA-free centrifuge tubes for storage. A partial sequence of the ASFV p72 gene cloned into plasmid Bluescript II and PCR-grade water were included in sample processing as a positive and negative control, 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) on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The qPCR reactions were performed in duplicate with each well containing 5 µL of template DNA, 0.2 µL (200nM) of each primer (Integrated DNA Technology, Coralville, IA), and 0.4 µL (200nM) of FAM probe (ThermoFisher Scientific) in a total reaction volume of 20 µL. Thermocycling conditions were 203°F for 5 min, followed by 45 cycles of 203°F for 10 s, and 140°F for 1 min.

The ASFV p72 genomic copy numbers (CN) were calculated using reference standard curve methodology using a reference standard curve composed from 10-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 calculated from CN/mL. Data are reported as PCR determined copy number per mL. Genomic CN/g for each sample was based upon the genomic CN/mL of solution recovered during sample processing, multiplied by the volume of PBS added during sample processing (35 mL), then divided by the amount of feed per suspension (10 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]. Feed sample or environmental sample was considered the experimental unit. Each feed and environmental sample had one extraction for PCR assay and each extraction was run in duplicate for PCR analysis. However, for feed sample results from batch 2 on d 1, each feed sample had two extractions for PCR assay and both extractions were run in duplicate for PCR analysis.

For feed samples, response values for the ASFV p72 gene were analyzed using a linear model fit using the lme function in the nlme packing using a normal distribution with the fixed effect as batch, day, and the associated interaction 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.

For environmental discs, response values for the ASFV p72 gene were analyzed using a linear model fit using the lme function in the nlme packing using a normal distribution with the fixed effect as day with a random effect of sample to indicate the appropriate level of experimental replication given the duplicate qPCR analysis of environmental samples. Results of Ct and genomic CN/mL are reported as least squares means ± standard error of the mean.

Any 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 and CN/mL data were transformed with $\log_{10}$ function and analysis included PCR negative reactions using a value of 0 for the quantified genomic CN/g or CN/mL. 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 $P \leq 0.10$. For genomic CN/g or CN/mL, the higher value indicates there are greater ASFV DNA quantities within the samples. For Ct values, the lower value indicates there are greater ASFV DNA quantities within the sample.

Results and Discussion

The batch of feed and days held at RT storage impacted the amount of detectable ASFV DNA within feed samples (Table 1). The count of feed samples with detectable ASFV DNA generally decreased as holding times increased for each subsequent batch of feed. However, ASFV DNA was still detectable in feed samples from Batch 6 that were stored at RT for 90 days. In terms of quantity of ASFV DNA, the batch × day interaction for Ct values was marginally significant ($P = 0.072$) and the batch × day interaction for $\log_{10}$ genomic copies per g of feed was significant ($P = 0.023$). For $\log_{10}$ genomic copies per g of feed, there were no differences over time in early batches (Batches 1-3; $P > 0.05$), but the quantity of detectable ASFV decreased with increasing storage time after collection for Batches 4-6. For Batch 4, the quantity of ASFV detected was lower ($P < 0.05$) on d 7 compared to d 1 with other days of analysis being intermediate. In Batch 5, the quantity of ASFV detected was lower ($P < 0.05$) on d 60 compared to d 1 with other days of analysis being intermediate. While in Batch 6, the quantity of ASFV detected was lower ($P < 0.05$) on d 28 and 180 compared to d 1 with other days of analysis being intermediate.

For Ct values, the main effect of batch ($P < 0.0001$) and day ($P = 0.0001$) were statistically significant (Table 2). In Batch 2, the quantity of ASFV detected was greater than Batch 4, 5, and 6 ($P < 0.05$) indicating that the Batch that was experimentally inoculated had the greatest detectable amount of ASFV DNA while subsequent produced batches without contamination with ASFV had lower detectable amounts of ASFV DNA. For holding dates, d 1 had the lowest Ct value and d 7, 60, and 180 the greatest Ct values with all other holding dates being intermediate ($P < 0.05$), indicating that feed samples analyzed on the day of feed manufacturing had greater amounts of ASFV DNA and as feed was held for periods of times, quantity of ASFV DNA decreased but was not eliminated.

For $\log_{10}$ genomic copies/g of feed, main effects of batch ($P < 0.0001$) and day ($P < 0.0001$) were statistically significant. In Batch 2, the quantity of ASFV detected was greater than Batches 4, 5, and 6 ($P < 0.05$), indicating that the Batch that was experimentally inoculated with ASFV had the greatest detectable amount of ASFV DNA while subsequent batches had lower detectable amounts of ASFV DNA. For holding dates, d 1 had the greatest amount of genomic copies with all other holding dates having lower amounts of genomic copies except for d 7 which was intermediate ($P < 0.05$), indicating that amount of ASFV DNA was greatest on day of manufacturing ASFV contaminated feed and decreased as feed samples were held due to natural decay.

The impact of storage time on the detectable amount of ASFV DNA in dust samples was not statistically significant ($P = 0.449$; Table 3), indicating that holding dust samples at room temperature for a specified time did not impact the detectable ASFV DNA within the samples.

In this study, holding feed samples at room temperature over time reduced detectable ASFV DNA within feed samples but did not eliminate the presence of ASFV DNA in the feed after 180 days of room temperature storage. This work demonstrated that once ASFV was introduced into a feed mill environment, ASFV DNA is detectable in dust collected on steel coupons for at least 180 days after feed manufacture, which could become a source of pathogen persistence in the environment of sample collection

if concerned about viral presence within feed mills. Additional research is necessary to determine if ASFV DNA present in feed at various time points represents an infectious pathogen. However, this study does document the dissemination and persistence of pathogen nucleic acids in a feed mill environment, which could help biosafety and biosecurity efforts in regard to efforts to prevent pathogen dissemination through animals' feed.

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Table 1. Proportion PCR positive and interactive means of cycle threshold value, and log_{10} genomic copies/g of feed samples for ASFV DNA survival after experimental inoculation of swine feed and subsequent feed batch sequencing[1]

	Batch of feed				
Item	2	3	4	5	6
Proportion PCR Positive					
d 1	40/40	20/20	19/20	19/20	17/20
d 3	6/6	4/6	3/6	3/6	1/6
d 7	6/6	5/6	1/6	2/6	2/6
d 14	6/6	6/6	4/6	5/6	3/6
d 28	6/6	6/6	3/6	3/6	0/6
d 60	6/6	6/6	3/6	1/6	2/6
d 90	6/6	6/6	2/6	2/6	1/6
d 180	6/6	5/6	2/6	2/6	0/6
Cycle threshold[2]					
d 1	33.0^{a}	37.5^{b,c,d,e}	39.5^{e,f,g,h,i}	39.3^{e,f,g,h,i}	40.1^{e,f,g,h,i}
d 3	31.7^{a,b}	39.5^{c,d,e,f,g,h,i}	42.4^{e,f,g,h,i}	41.4^{e,f,g,h,i}	43.8^{g,h,i}
d 7	31.6^{a}	37.8^{a,b,c,d,e,f,g,h}	44.3^{h,i}	42.2^{e,f,g,h,i}	43.5^{f,g,h,i}
d 14	31.8^{a,b}	36.9^{a,b,c,d,e,f,g}	40.6^{e,f,g,h,i}	39.6^{d,e,f,g,h,i}	41.4^{e,f,g,h,i}
d 28	31.3^{a}	36.5^{a,b,c,d,e,f}	42.7^{e,f,g,h,i}	42.5^{e,f,g,h,i}	45.0^{i}
d 60	32.4^{a,b,c}	37.8^{a,b,c,d,e,f,g,h}	42.3^{e,f,g,h,i}	44.3^{h,i}	43.2^{e,f,g,h,i}
d 90	32.6^{a,b,c,d}	36.2^{a,b,c,d,e}	43.0^{e,f,g,h,i}	43.6^{f,g,h,i}	43.6^{f,g,h,i}
d 180	32.0^{a,b}	39.7^{d,e,f,g,h,i}	43.5^{f,g,h,i}	41.5^{e,f,g,h,i}	45.0^{i}
Log_{10} genomic copies/g[3]					
d 1	4.7^{i}	3.6^{f,g,h,i}	3.1^{d,e,f,g,h}	3.1^{c,d,e,f,g,h}	2.8^{b,c,d,e,f,g,h}
d 3	5.0^{h,i}	2.5^{a,b,c,d,e,f,g,h,i}	1.5^{a,b,c,d,e,f}	1.7^{a,b,c,d,e,f,g}	0.6^{a,b,c}
d 7	5.0^{h,i}	3.2^{b,c,d,e,f,g,h,i}	0.5^{a,b}	1.3^{a,b,c,d,e,f}	0.9^{a,b,c,d,e}
d 14	4.9^{h,i}	3.7^{b,c,d,e,f,g,h,i}	2.2^{a,b,c,d,e,f,g,h}	2.7^{a,b,c,d,e,f,g,h,i}	1.7^{a,b,c,d,e,f,g}
d 28	5.1^{h,i}	3.8^{b,c,d,e,f,g,h,i}	1.4^{a,b,c,d,e,f}	1.5^{a,b,c,d,e,f}	0.00^{a}
d 60	4.8^{g,h,i}	3.5^{e,f,g,h,i}	1.5^{a,b,c,d,e,f}	0.5^{a,b}	1.0^{a,b,c,d,e}
d 90	4.7^{g,h,i}	3.8^{b,c,d,e,f,g,h,i}	1.1^{a,b,c,d,e}	0.9^{a,b,c,d,e}	0.6^{a,b,c,d}
d 180	4.9^{h,i}	2.7^{e,f,g,h,i}	0.9^{a,b,c,d,e}	1.4^{a,b,c,d,e,f}	0.0^{a}

[1]Swine gestation feed was inoculated with African swine fever virus (ASFV) at 5.6×10^{4} $TCID_{50}$/gram inoculated feed (batch 2) following an initial priming of the feed manufacturing equipment with ASFV-free feed. Four subsequent batches of feed were manufactured (batch 3-6) and were initially free of ASFV. On the day of and 3, 7, 14, 28, 60, 90, and 180 days after manufacture following room temperature storage, three samples were mixed with approximately 35 mL of phosphate buffered solution, incubated for 2 hr at room temperature then centrifuged at 1000 × g for 3 min. Samples were then analyzed using qRT-PCR for detection of the gene encoding for the p72 protein. Analysis of d 1 feed samples have been reported by Elijah et al. (2021b) and are included in the current analysis of ASFV detection over time.

[2]Samples that had no detectable ASFV DNA were assigned a Ct value of 45.0. Batch × day: $P = 0.072$. SEM for batch 2, d 1 = 0.64; SEM for batch 3-6, d 1 = 0.69; All other SEM = 1.27.

[3]Log_{10} genomic copies/g of feed. Batch × day, $P = 0.023$. SEM for batch 2, d 1 = 0.27; SEM for Batch 3-6, d 1 = 0.30; All other SEM = 0.56.

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

Table 2. Proportion PCR positive and main effects of batch and day on cycle threshold value and log_{10} genomic copies/g for feed held in room temperature storage for ASFV DNA survival after experimental inoculation of swine feed and subsequent feed batch sequencing[1]

Item	Proportion PCR positive	Cycle threshold value[2]	Log_{10} genomic copies/g[3]
Batches of feed			
2	82/82	32.1[a]	4.9[c]
3	58/62	37.7[b]	3.3[b]
4	37/62	41.8[c]	1.5[a]
5	37/62	42.3[c]	1.6[a]
6	26/62	43.2[c]	1.0[a]
Day			
1	115/120	37.9[a]	3.5[b]
3	17/30	39.8[a,b]	2.2[a]
7	16/30	39.9[b]	2.2[a]
14	24/30	38.1[a,b]	3.0[a,b]
28	18/30	39.6[a,b]	2.3[a]
60	18/30	40.0[b]	2.2[a]
90	17/30	39.8[a,b]	2.2[a]
180	15/30	40.3[b]	2.0[a]

[1]Swine gestation feed was inoculated with African swine fever virus (ASFV) at 5.6×10^4 $TCID_{50}$/gram inoculated feed (batch 2) following an initial priming of the feed manufacturing equipment with ASFV-free feed. Four subsequent batches of feed were manufactured (batch 3-6) and were initially free of ASFV. On the day of and 3, 7, 14, 28, 60, 90, and 180 days after manufacture following room temperature storage, three samples were mixed with approximately 35 mL of phosphate buffered solution, incubated for 2 hr at room temperature then centrifuged at 1000 × g for 3 min. Samples were then analyzed using qRT-PCR for detection of the gene encoding for the p72 protein. Analysis of d 1 feed samples have been reported by Elijah et al. (2021) and are included in the current analysis of ASFV detection over time.

[2]Samples that had no detectable ASFV DNA were assigned a Ct value of 45.0. Batch, $P < 0.0001$, SEM = 0.43; Day, $P = 0.0001$, SEM for d 1 = 0.31, otherwise SEM = 0.57.

[3]Log_{10} genomic copies/g feed. Batch, $P < 0.0001$, SEM = 0.19; Day, $P < 0.0001$, SEM for d 1 = 0.13; otherwise SEM = 0.25.

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

Table 3. Proportion PCR positive and main effects of day of storage on cycle threshold (Ct), and log_{10} genomic copies/mL of environmental discs for ASFV DNA survival after experimental inoculation of swine feed and subsequent feed batch sequencing[1]

Day	Proportion PCR positive	Cycle threshold value[2]	Log_{10} genomic copies/g[3]
1	6/6	33.8	3.9
3	6/6	34.0	3.9
7	6/6	35.3	3.6
14	5/6	36.7	3.0
28	6/6	33.9	3.9
60	6/6	37.7	2.9
90	6/6	35.5	3.5
180	4/6	39.3	2.2

[1]Twenty-seven stainless steel coupons were randomly placed in location (9 coupons in each of 3 corners of the room) and allowed to collect feed dust produced during manufacturing. Stainless steel coupons remained sealed in a secondary container and stored at room temperature (RT) in a locked cabinet. On the day of and 3, 7, 14, 28, 60, 90, and 180 days after feed manufacturing, one sample from each of the three location blocks following RT storage were randomly selected, opened within a biosafety cabinet (BSC), swabbed using a 10 × 10 cm cotton gauze, prepared and analyzed as for ASFV DNA via PCR.

[2]Samples that had no detectable ASFV DNA were assigned a Ct value of 45.0. Day: $P = 0.449$, SEM = 1.98.

[3]Genomic copies/mL of sample processing lysate. Day: $P = 0.433$, SEM = 0.60.

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