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Prevalence and Distribution of African Swine Fever Virus in Swine Feed After Mixing and Feed Batch Sequencing¹

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Summary

As the United States maintains trade with countries where African swine fever virus (ASFV) is endemic, it is critical to have methods that can detect and mitigate the risk of ASFV in potentially contaminated feed or ingredients. Therefore, the objectives of this study were to 1) evaluate feed batch sequencing as a mitigation technique for ASFV contamination in a feed mill, and 2) determine if a feed sampling method could identify ASFV following experimental inoculation. Batches of feed were manufactured in a BSL-3Ag room at Kansas State University's Biosafety Research Institute in Manhattan, KS. First, the pilot feed manufacturing system mixed, conveyed, and discharged 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 TCID₅₀/g. Then, four subsequent ASFV-free batches of feed were manufactured. After discharging each batch into a biohazard tote, 10 samples were collected in a double 'X' pattern. Samples were analyzed using a qPCR assay specific for the ASFV p72 gene to determine the cycle threshold (Ct) and log₁₀ genomic copy number (CN)/g of feed. Batch of feed affected the qPCR Ct values ($P < 0.0001$) and the log₁₀ genomic CN/g ($P < 0.0001$) content of feed. Feed samples obtained after manufacturing the ASFV-contaminated diet contained the greatest ($P < 0.05$) amounts of ASFV p72 DNA across all criteria. Quantity of ASFV p72 DNA decreased sequentially as additional batches of initially ASFV-free feed were manufactured, but it was still detectable after batch sequence 4, suggesting cross contamination between batches. This subsampling method was able

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to identify ASFV genetic material in feed samples using the PCR assay specific for the ASFV p72 gene. In summary, sequencing batches of feed decreases concentration of ASFV contamination in feed, but does not eliminate it. Bulk ingredients or feed can be accurately evaluated for ASFV contamination by collecting 10 evenly distributed subsamples, representing 0.05% of the volume of the container, using the sampling method described herein.

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 pathogen transmission in pigs.⁶ This hypothesis was never unequivocally proven. Recently, biosecurity practices have been heavily emphasized to protect the supply chain for swine feed in the United States (US). Research has continued to demonstrate that the risk for feed-based virus transmission extends beyond PEDV and could include viruses such as African swine fever virus (ASFV), foot and mouth disease virus (FMDV), or classical swine fever virus (CSFV).^{7,8} The US maintains trade relationships with a number of countries that are now in ASFV-endemic regions, leading to concerns that ASFV may enter the US through the feed supply chain or other avenues. It has been hypothesized that the same methods which demonstrated appropriate sensitivity and specificity for PEDV detection in feed may be applicable to ASFV. 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, both of these strategies have never been evaluated. Therefore, the objectives of this study were to 1) determine if a common sampling strategy could consistently detect ASFV in feed, and 2) evaluate if feed batch sequencing could serve as a potential mitigation technique for ASFV contamination during feed manufacturing.

Materials and Methods

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 biosafety level (BSL)-3Ag animal room; the 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 ethylenediaminetetraacetic acid (EDTA) from ASFV-infected pigs was mixed in RPMI media to

⁶ USDA-APHIS. United States Department of Agriculture Animal and Plant Health Inspection Service Veterinary Services (2015). Swine enteric coronavirus introduction to the United States: Root cause investigation report. Accessed February 24, 2021.

⁷ Dee, S. A., Bauermann, F. V., Niederwerder, M. C., Singrey, A., Clement, T., de Lima, M., ... & Petrovan, V. (2018). Survival of viral pathogens in animal feed ingredients under transboundary shipping models. *PloS ONE*, 13(3). doi:10.1371/journal.pone.0194509.

⁸ Stoian, A. M. M., Petrovan V., Constance, L. A., Olcha, M., Dee, S., Diel, D. G., Sheahan, M. A., Rowland, R. R. R., Patterson, G., & Niederwerder, M. C. (2020). Stability of classical swine fever virus and pseudorabies virus in animal feed ingredients exposed to transpacific shipping conditions. *Transboundary and Emerging Diseases*, 00, 1-10. doi:10.1111/tbed.13498.

prepare 530 mL of virus inoculum at the final concentration of 2.7×10^6 TCID₅₀/mL of ASFV genotype II virus (strain Armenia 2007).

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 yard³) steel electric paddle mixer (H.C Davis Sons Manufacturing, model # SS-L1; Bonner Springs, KS). The feed was mixed for 5 minutes 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 TCID₅₀/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 TCID₅₀/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 collected as previously described by Jones et al.⁹ Briefly, the 10 samples, up to 100 g in weight, were taken from the feed that had been discharged in a biohazard tote through two 'X' patterns. To achieve this pattern, the biohazard tote was divided into two halves and in each half, two diagonal lines were imagined in the researcher's mind from corner to corner to make an 'X'. Samples were taken from the corners of each half along with a sample from the middle where the two imaginary diagonal lines crossed. The 10 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.

Laboratory analysis

Feed samples were tested at a BSL-3+ laboratory in the BRI. Ten g of each feed sample was put in a tube, suspended with 35 mL of phosphate buffered saline (PBS), and the tube was capped, inverted, and incubated overnight at 40°F. Approximately 10 mL of supernatant was recovered, aliquoted into 5 mL cryovials, and stored at -112°F until qPCR analysis. 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. 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

⁹ 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.

extraction was performed according to the manufacturer's instructions with modifications. Briefly, 200 μ L of AL/sample lysate was transferred to column A of the Taco deep-well extraction plate which contained 500 μ L of the GeneReach lysis buffer and 50 μ L of magnetic beads, followed by addition of 200 μ L of molecular grade isopropanol (ThermoFisher Scientific, Waltham, MA). The extraction consisted of two washes with 750 μ L of wash buffer A, one wash with 750 μ L wash buffer B, and a final wash with 750 μ L of 200 proof molecular grade ethanol (ThermoFisher Scientific). After a 5-min drying time, DNA was eluted with 100 μ L 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 was 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 μ L of template DNA, 0.2 μ L (200nM) of each primer (Integrated DNA Technology, Coralville, IA), and 0.4 μ L (200nM) of FAM probe (Thermo Fisher Scientific) in a total reaction volume of 20 μ L. 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 was mathematically determined using the PCR-determined cycle threshold (C_t) 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 were calculated from CN/mL.

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]. The experimental unit for this study was the feed sample. Each feed sample had one extraction for the qPCR assay and each extraction was run in duplicate for qPCR analysis with the exception of samples from batch 2 in which each feed sample had two extractions for the qPCR assay. Both extractions were run in duplicate for qPCR analysis as an initial assessment to evaluate the variability present within the extraction and amplification procedures. Samples were considered qPCR-positive if 2 of 2 qPCR reactions had detectable ASFV DNA, suspect if 1 of 2 qPCR reactions had detectable ASFV DNA, and non-detected if 0 of 2 qPCR reactions had detectable ASFV DNA.

Response values for the ASFV p72 gene were analyzed using a linear mixed model fit using the lme function in the nlme package using a normal distribution with a fixed effect of batch and a random effect of sample to indicate the appropriate level of experimental replication given the duplicate qPCR analysis of feed samples. Results of C_t and genomic CN/g are reported as least squares means $\pm$ 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 normalized prior to analysis using a $\log_{10}$ transformation. 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 $P \leq 0.05$.

Results and Discussion

After the ASFV-positive batch of feed was manufactured, all feed samples had detectable ASFV p72 genetic material (Table 1). 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. In terms of the presence of ASFV DNA, the batch of feed affected the Ct value ($P < 0.0001$) and the $\log_{10}$ genomic CN/g ($P < 0.0001$) of samples, with samples taken from the feed manufactured with direct contamination with ASFV containing the greatest amount of ASFV p72 genetic material ($P < 0.05$). Sequence 1 had lower levels of ASFV DNA detected compared to the positive control batch ($P < 0.05$), and sequence 4 had a lower ASFV DNA quantity than both the positive control and sequence 1 ($P < 0.05$). The levels of detectable ASFV DNA in sequence 2 and 3 were intermediate between sequence 1 and 4.

In general, the quantity of detected ASFV p72 DNA decreased sequentially as additional batches of feed were manufactured. However, detection of ASFV p72 DNA was still possible after 4 sequences of ASFV-free feed showing that cross contamination between batches occurred. This suggests that flushing a feed mill with ASFV-free feed after an ASFV-contaminated feed batch will reduce the amount of ASFV in the feed but won't eliminate the virus entirely. The current study's findings also suggest that the X pattern sampling technique used was able to identify ASFV contamination within batches of feed.

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Table 1. Detection and quantification of African swine fever virus (ASFV) p72 DNA in feed samples¹

Item	Batch of feed					
	Negative	Positive	Sequence 1	Sequence 2	Sequence 3	Sequence 4
Count of PCR result						
Positive	0/10	10/10	10/10	9/10	9/10	7/10
Suspect	0/10	0/10	0/10	1/10	1/10	3/10
Non-detected	10/10	0/10	0/10	0/10	0/10	0/10
Cycle threshold ²	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 ³	0.0	4.7 ± 0.08 ^a	3.6 ± 0.09 ^b	3.1 ± 0.23 ^{b,c}	3.1 ± 0.23 ^{b,c}	2.8 ± 0.23 ^c

¹Swine gestation feed was inoculated with ASFV to achieve 5.6×10^4 TCID₅₀/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) that were initially free of ASFV. Ten feed samples were collected from each batch of feed and analyzed using an ASFV p72-specific qPCR assay with each sample analyzed in duplicate. Samples were considered qPCR-positive if 2 of 2 qPCR reactions had detectable ASFV DNA, suspect if 1 of 2 qPCR reactions had detectable ASFV DNA, and non-detected if 0 of 2 qPCR reactions had detectable ASFV DNA. Statistical analysis for cycle threshold and genomic copy number includes all treatment groups except for negative control where samples were collected prior to ASFV inoculation.

²Cycle threshold values for qPCR reactions with no detectable ASFV p72 gene expression were assigned a value of 45 within the statistical analysis. Batch, $P < 0.0001$.

³Log₁₀ transformed genomic copies for the ASFV p72 gene per g of feed. Batch, $P < 0.0001$.

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