RESEARCH REPORT
SWINE HEALTH

Title: Understanding the survivability and infectivity of African swine fever virus in various environments, **NPB #19-098**

Investigator: Dr. Megan Niederwerder

Institution: Kansas State University

Date Submitted: May 9, 2022

Industry Summary: African swine fever (ASF) is the most significant foreign animal disease threat to the US pork industry. ASF virus (ASFV) not only causes high case fatality rates of infected pigs but is a trade-limiting disease resulting in significant economic losses to pork exports. Over the last 4 years, ASFV has spread rapidly into new countries and regions of the world, including China in 2018 and the Dominican Republic in 2021. The virus is resistant to environmental degradation and maintains infectivity in swine feed ingredients exposed to transoceanic shipment conditions. As ASFV is transmissible through consumption of contaminated feed, imported feed and ingredients from ASFV-positive countries are a risk for introduction and spread of the virus to US swine. Storage of high-risk feed ingredients to allow for virus degradation over time has been recommended as a mitigation strategy to reduce this risk. However, holding times for feed at risk for ASFV contamination have historically been calculated based on conditions with fluctuating temperature and humidity. The objective of this study was to evaluate the infectivity of ASFV Georgia 2007 over time in feed stored at stable environmental temperatures to develop holding time recommendations. Three feed matrices (complete feed, soybean meal, ground corncobs) along with positive and negative controls were exposed to three environmental storage temperatures (40°F, 68°F, 95°F) for up to 1 year. Additionally, medium chain fatty acid and formaldehyde-based feed additives were evaluated for temperature-dependent efficacy against ASFV in stored feed. ASFV DNA was highly stable across feed matrices and was detectable by qPCR in almost all samples through the conclusion of each study. Infectious ASFV was most stable in soybean meal, maintaining infectivity for as long or longer than ASFV infectivity in laboratory media. All three feed additives tested in this study reduced ASFV infectivity in stored complete feed. This data helps define risk mitigation of ASFV introduction and transmission through feed ingredients. Further, this data is being used to characterize ASFV decay patterns for time by temperature feed storage. This study underscores the longevity of ASFV survival in contaminated soybean meal and supports the concept of feed quarantine for targeted high-risk ingredients as part of swine biosecurity programs.

Key Findings:

- ASFV viability was dependent on feed ingredient, being most stable in soybean meal, followed by complete feed, and was least stable in ground corncob particles
- ASFV DNA was highly stable, being detected in almost all feed samples for up to 1 year when stored at 40°F and 68°F
- ASFV remained infectious in soybean meal for at least 112 days at 40°F, at least 21 days at 68°F, and at least 7 days at 95°F
- Storage time of feed ingredients at risk for ASFV contamination is temperature-dependent with longer times required for feed stored at colder temperatures

Keywords: African swine fever virus, ASFV, feed, soybean meal, pigs, virus stability, environmental storage

Scientific Abstract: African swine fever (ASF) causes high case fatality in pigs and is a trade-limiting disease that causes significant economic losses to pork production. The ASF virus (ASFV) is resistant to environmental degradation and maintains infectivity in swine feed ingredients exposed to transoceanic shipment conditions. As ASFV is transmissible through consumption of contaminated feed, the objective of this study was to evaluate the stability of ASFV Georgia 2007 in three feed matrices (complete feed, soybean meal, ground corncobs) exposed to three environmental storage temperatures (40°F, 68°F, 95°F) for up to 365 days. ASFV DNA was highly stable across feed matrices and was detectable by qPCR in almost all samples through the conclusion of each study. Infectious ASFV was most stable in soybean meal, maintaining infectivity for at least 112 days at 40°F, at least 21 days at 68°F, and at least 7 days at 95°F. This data helps define risk of ASFV introduction and transmission through feed and ingredients.

Introduction: African swine fever (ASF) is the most significant threat to global pork production. The causative agent, ASF virus (ASFV), is an enveloped double-stranded DNA virus and the only member of the family *Asfarviridae* (Alonso et al., 2018). High environmental stability of ASFV compared to other porcine viruses (Niederwerder and Rowland, 2017) contributes to the challenges of transboundary spread and disease control. Since the 2007 introduction of ASFV into the country of Georgia (Rowlands et al., 2008), ASFV has continued to rapidly emerge into new continents and countries, including China in 2018 (Zhou et al., 2018), Germany in 2020 (Sauter-Louis et al., 2020), and the Dominican Republic in 2021 (Gonzales et al., 2021). With no commercially available vaccine and depopulation of infected herds being the primary route for disease control, ASFV is a constant risk to pork producing countries negative for the virus. Should ASFV be introduced into the US, economic losses are estimated to be between $15 – $50 billion (Carriquiry et al., 2020).

Since the introduction of porcine epidemic diarrhea virus (PEDV) into the US in 2013, feed and feed ingredients have been recognized as a potential route of virus introduction and spread onto pig farms (Niederwerder and Hesse, 2018). Contaminated feed and ingredients have also been identified as a risk factor for ASFV introduction and spread into new regions and farms in Europe and Asia (Niederwerder, 2021, Nielsen et al., 2021). ASFV is transmissible through the natural consumption of complete feed (Niederwerder et al., 2019) and the virus is highly stable across commonly imported feed ingredients exposed to transoceanic environmental conditions consistent with global trade (Dee et al., 2018).

Although the half-life of ASFV in feed ingredients has been reported between 9.6 – 14.2 days (Stoian et al., 2019), these estimates were based on shipment conditions with fluctuating temperature and humidity lasting 30 days. Storage or holding times for high-risk feed ingredients after importation is recommended to reduce the risk of virus contamination prior to incorporation into complete swine diets (Patterson et al., 2019, Calvin et al., 2022). Therefore, characterizing the time required for ASFV inactivation in feed under stable environmental storage conditions is needed. The objectives of this study were to define the time course of ASFV infectivity, ASFV DNA detection, and additive efficacy in feed stored at three constant environmental temperatures.

Objectives:
Objective 1. Prepare a time and temperature calculator for the inactivation of ASFV Georgia 2007.
Objective 2: Evaluate antiviral treatments in feed.

Materials & Methods:
Virus

All use of virus and pigs was performed in accordance with the Federation of Animal Science Societies Guide for the Care and Use of Agricultural Animals in Research and Teaching, the USDA Animal Welfare Act and Animal Welfare Regulations, and approved by the Kansas State University Institutional Animal Care and Use and the Institutional Biosafety Committees. All ASFV work was performed at the Biosecurity Research Institute (BRI) under Biosafety Level 3 (BSL-3) containment

conditions. ASFV Georgia 2007 isolate was derived from the spleen of an acutely infected pig as previously described (Niederwerder et al., 2019). Briefly, splenic tissue was minced and passed through a cell strainer using sterile phosphate-buffered saline (PBS) with penicillin/streptomycin and fungizone. Splenic suspensions were centrifuged at 4,000xg for 30 min and supernatant stored at 4°C. Pellets were resuspended in PBS followed by 3 freeze-thaw cycles. Suspensions were centrifuged and supernatant pooled to create inoculum.

Porcine alveolar macrophages (PAMs) collected from 3 or 4-week-old pigs through bronchoalveolar lavage were used for ASFV titration. PAMs were cultured for 24 hours in RPMI media (Roswell Park Memorial Institute; HyClone™ 1640, Fisher Scientific) supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37°C in 5% CO_2 prior to use in titration assays. Ten-fold serial dilutions of the splenic homogenate inoculum were prepared in triplicate and dilutions added to confluent monolayers of PAMs in a 96-well plate. After 3 days of incubation at 37°C, PAMs were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) and permeabilized with 0.1% Triton-X. Monoclonal antibody directed at ASFV p30 was added at 1:6,000 (mouse ascites fluid) or 1:400 (hybridoma supernatant) followed by 30 min incubation at 37°C. After washing the cells with PBS, goat anti-mouse antibody (Alexa Fluor™ 488, Invitrogen, Thermofisher) was added at 1:400 and the plate was incubated for 30 min at 37°C. Infection of cells was viewed with an inverted fluorescence microscope and the 50% tissue culture infectious dose ($TCID_{50}$/ml) was calculated using the method of Spearman and Karber (Finney, 1964).

Feed Inoculation and Environmental Storage

Feed matrices included conventional soybean meal, grow-finish complete feed in meal form, and ground corncob particles. Prior to inoculation, all feed was gamma-irradiated (Neutron Products, Inc.; minimum absorbed dose 25 kilograys). Five grams of each irradiated ingredient were added to 50-ml conical tubes in duplicate. Controls included 5 ml RPMI media (positive control) and 5 grams of complete feed inoculated with 100µL sterile PBS (negative control). Feed ingredients and media were inoculated with 100µL of ASFV Georgia 2007 (10^6 $TCID_{50}$/ml resulting in 10^5 $TCID_{50}$/tube). After inoculation, tubes were capped and vortexed for 10 s prior to incubation in each respective temperature.

Antimicrobial feed additives included 1% inclusion of a medium chain fatty acid blend (MCFA; Purina Animal Nutrition, LLC), 0.33% inclusion of an aqueous formaldehyde and propionic acid product (Sal CURB®, Kemin Industries, Inc.), and 0.9% inclusion of a medium chain fatty acid blend (MCFA, Vigilex; Cargill, Inc.). Feed mitigants were added to complete feed in meal form 3 – 5 days prior to ASFV inoculation. Dry powder or liquid mitigants were thoroughly mixed in ≥100 g volumes of complete feed prior to aliquoting into 5 g samples.

Temperature conditions representing environmental storage of feed included 40°F (cool storage, Fisher Scientific Isotemp Refrigerator), 68°F (ambient storage, laboratory cabinet), and 95°F (hot storage, Environmental Chamber Model 3911, Thermo Scientific Forma). A total of 506 feed samples were included. For 40°F, 130 samples without additives were created for each feed matrix and control representing 1, 3, 5, 7, 14, 21, 30, 60, 90, 112, 210, 269, and 365 days post-inoculation (dpi; 5 matrices x 2 replicates x 13 days). For 68°F, 140 samples without additives were created for each feed matrix and control representing 1, 2, 3, 5, 7, 14, 21, 30, 60, 90, 112, 210, 269, and 365 dpi (5 matrices x 2 replicates x 14 days). For 95°F, 110 samples without additives were created for each feed matrix and control representing 1, 2, 3, 5, 7, 14, 21, 30, 60, 90, and 112 dpi (5 matrices x 2 replicates x 11 days). Per temperature, 42 complete feed samples with additives were tested on 1, 3, 7, 14, 30, 60, and 112 dpi (3 additives x 2 replicates x 7 days x 3 temperatures).

On the designated dpi, duplicate samples of each feed matrix and control were removed from the respective temperature condition for batch processing. Samples were processed by adding 15 mL of sterile PBS with antibiotics/antimycotics and vortexing for 10 s. Following centrifugation at 10,000xg for 10 min at 4°C, supernatant was collected and stored at -80°C for diagnostic testing.

DNA Extraction and qPCR

Nucleic acid was extracted from feed supernatant or porcine biological samples using the MagMAX™ 96 Viral RNA Isolation Kit (ThermoFisher) as previously described (Niederwerder et al., 2021, Stoian et al., 2020, Khanal et al., 2021). Paramagnetic beads were mixed with a bead enhancer solution and 20µL of the bead mix was added to wells on a U-bottom 96-well plate or 1.5mL snap-cap

microcentrifuge tubes (FisherScientific). Samples (50µL) were added and mixed for 1 min prior to adding 130µL lysis/binding solution (containing lysis/binding concentrate, carrier RNA, and 100% isopropanol). Samples were mixed for 5 min before placing on a magnetic stand to capture the beads. Beads were washed twice with 150µL wash solution 1 and 150µL wash solution 2. DNA was eluted from the beads with 50µL elution buffer.

Conserved regions of ASFV p72 were amplified using two PCR protocols. First, PCR primers and probe were designed (King et al., 2003) and utilized as previously described (Khanal et al., 2021). Second, the PCR protocol was designed (Zsak et al., 2005) and utilized according to the manufacturer's instructions (*VetAlert*™ African Swine Fever Virus DNA Test Kit, Tetracore). Briefly, extracted DNA samples (5µL) were added to 20µL of master mix (containing 19.25µL ASF Mastermix and 0.75µL Enzyme Solution) in a Hard-Shell® 96-well PCR plate (Bio-Rad Laboratories). The plate was sealed, centrifuged briefly to remove air bubbles, and placed in a CFX96 Real-Time System (Bio-Rad Laboratories). The PCR conditions consisted of an initial incubation of 48°C for 15 min, then 95°C incubation for 2 min, followed by 45 cycles of 95°C incubation for 10 sec, and 60°C for 40 sec.

PCR data were analyzed using CFX Manager software version 3.1 (Bio-Rad Laboratories) and results are generated as the cycle threshold (Ct). Each PCR plate included a standard curve created with seven 1:10 serial dilutions of ASFV Georgia 2007 splenic homogenate (10^6 $TCID_{50}$/ml). Starting quantity was calculated for each sample based on the standard curve and all results are reported as the $\log_{10}$ $TCID_{50}$ equivalents/PCR reaction.

Virus Isolation from Feed

Virus isolation (VI) of ASFV from feed supernatant samples was performed on confluent monolayers of PAMs. Two-fold or ten-fold serial dilutions of supernatant samples were prepared in RPMI media and added to PAMs in 96-well plates. After incubation for 1 h at 37°C, PAMs were washed and RPMI media replaced prior to a 3-day incubation at 37°C. Following incubation, cells were fixed and immunofluorescence assays (IFA) were performed as described above to classify samples as positive or negative for infectious ASFV.

Swine Bioassays

Swine bioassays were performed to detect infectious ASFV in feed supernatant samples that had detectable ASFV DNA on qPCR but a lack of detectable ASFV using virus isolation on PAMs. Nursery age barrows (n = 32; average 24.8 ± 1.4 days old) were obtained from a single high-health commercial source. Pigs were housed in individual pens (1.9 m^2) in one of two identical rooms (66 m^2) at the BRI and maintained under BSL-3Ag containment conditions. Raised stainless-steel pens were separated by 1.5 m with six pens/room. Each room contained up to six swine bioassays per replicate, with one pig maintained as a negative control to confirm the lack of cross-contamination and aerosol transmission between pens. Inoculum was prepared by centrifuging duplicate feed supernatant samples at 6,000rpm for 5 min. Following centrifugation, supernatant from duplicate samples were mixed and administered as a 1ml intramuscular injection. Each pig received one sample type collected from a single time and temperature condition with negative control pigs receiving a representative negative complete feed sample.

After 3 days of acclimation post-arrival to BRI, pigs were inoculated (n = 27) or mock-inoculated (n = 5; supernatant from negative control complete feed) with 1 ml suspension intramuscularly. Pigs were monitored once daily by a veterinarian for clinical signs associated with ASF. Pigs with significant clinical disease were treated or humanely euthanized. At 7 dpi, all remaining pigs were humanely euthanized and assessed for the presence or absence of ASF. ASFV infection was determined based on the results of 3 diagnostic assays: qPCR of serum, qPCR of splenic homogenate, and VI of splenic homogenate. Splenic homogenates from bioassay pigs were prepared using the Omni Bead Ruptor 4 (Omni International). Briefly, spleen samples were diced into 2 mm pieces, mixed with an equal volume of PBS containing antibiotics/antimycotics, and homogenized over four cycles of 30 sec at speed 4. Splenic homogenates were centrifuged at 6,000rpm for 30 min prior to storing supernatant for diagnostic testing. Feed samples were classified as positive for infectious ASFV if ≥2 diagnostic assay results from porcine biological bioassay samples had detectable ASFV.

Results:

Detection of ASFV DNA

Extraction efficiency of ASFV DNA was ingredient dependent with efficiency typically highest in soybean meal and lowest in ground corncob particles. No ASFV DNA was detected in negative control complete feed samples. At 40°F, all inoculated samples maintained detectable quantities of ASFV DNA through 365 dpi and mitigated samples maintained detectable DNA through the final sampling day of 112 dpi (**Fig 1**). Overall, between 1 – 365 dpi, the quantity of ASFV DNA in soybean meal, RPMI media, and complete feed was consistent with negligible evidence of decay. At 68°F, both replicates of ground corncob particles were negative for ASFV DNA at 210 dpi and remained negative through the conclusion of the study at 365 dpi (**Fig 2**). Additionally, one replicate of complete feed treated with Kemin was negative on PCR at 112 dpi. All remaining samples had detectable ASFV DNA across the 365-day time course. At 95°F, both replicates of ground corncob particles were negative for ASFV DNA at 60 dpi, 90 dpi and 112 dpi (**Fig 3**). Further, both replicates of complete feed treated with Kemin were PCR negative at 112 dpi. All other inoculated feed samples, treated or nontreated, remained positive for ASFV DNA through the conclusion of the hot storage environment study at 112 dpi.

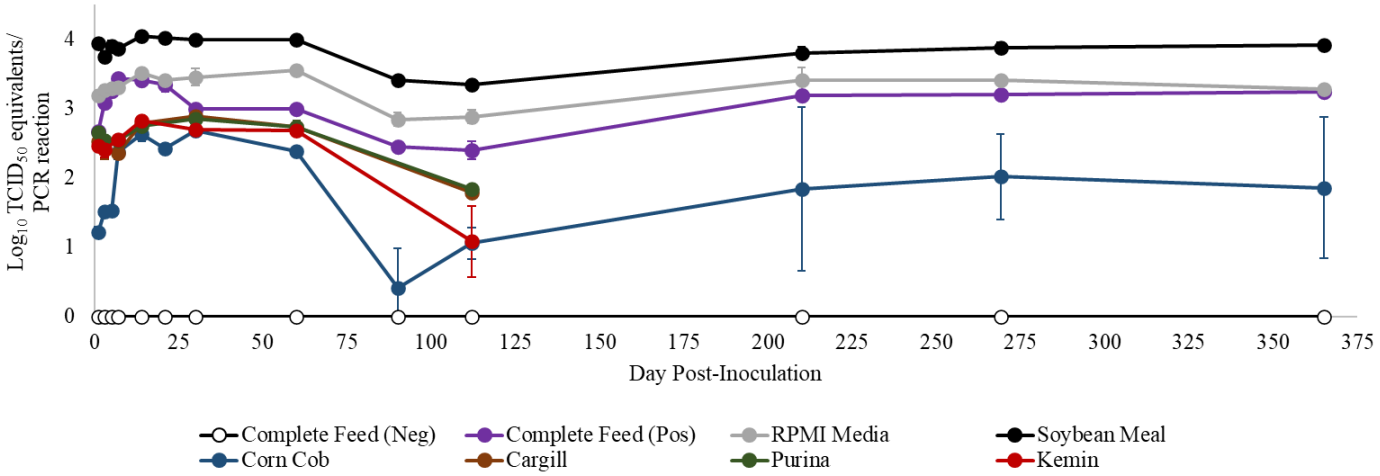

Figure 1. Detection of African swine fever virus (ASFV) DNA by quantitative PCR in feed and ingredients stored at 40°F for up to 365 days. PCR detection based on amplification of ASFV p72. Data calculated as $\log_{10}$ 50% tissue culture infectious dose ($TCID_{50}$) equivalents per PCR reaction based a standard curve of ASFV in splenic homogenate. Data shown as mean $\log_{10}$ $TCID_{50}$ equivalents/PCR reaction for duplicate samples ± standard deviation. Feed additives (Cargill, Purina, Kemin) were added to complete feed prior to inoculation with ASFV. All matrices were inoculated with ASFV Georgia 2007 (10^5 $TCID_{50}$) or PBS (negative control).

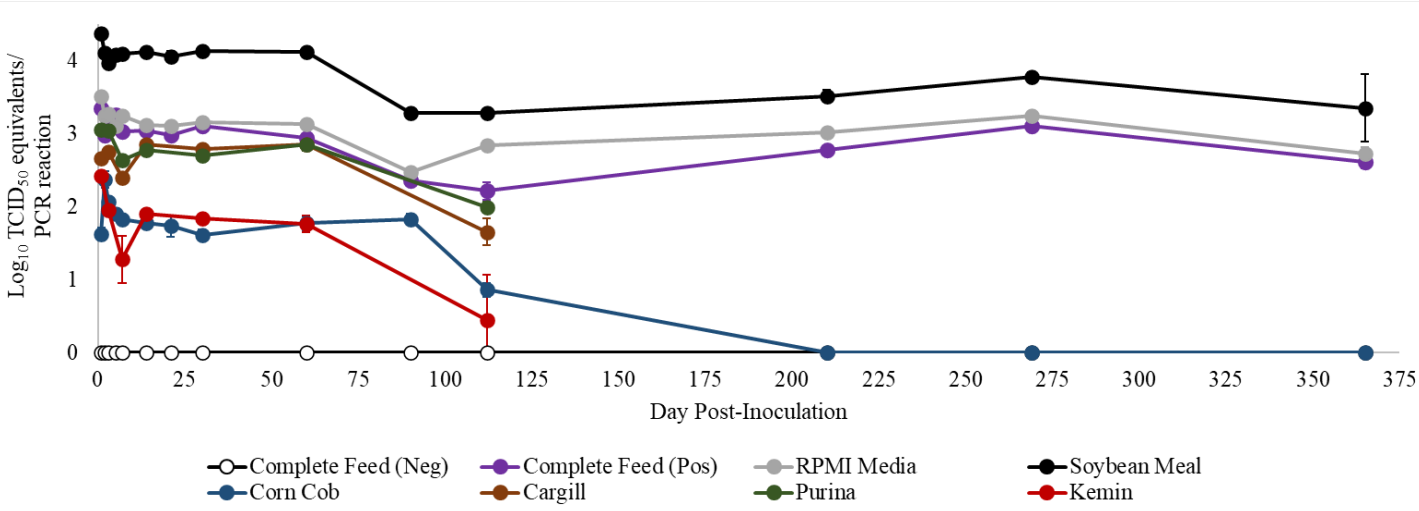

Figure 2. Detection of African swine fever virus (ASFV) DNA by quantitative PCR in feed and ingredients stored at 68°F for up to 365 days. PCR detection based on amplification of ASFV p72. Data calculated as log_{10} 50% tissue culture infectious dose ($TCID_{50}$) equivalents per PCR reaction based a standard curve of ASFV in splenic homogenate. Data shown as mean log_{10} $TCID_{50}$ equivalents/PCR reaction for duplicate samples ± standard deviation. Feed additives (Cargill, Purina, Kemin) were added to complete feed prior to inoculation with ASFV. All matrices were inoculated with ASFV Georgia 2007 (10^5 $TCID_{50}$) or PBS (negative control).

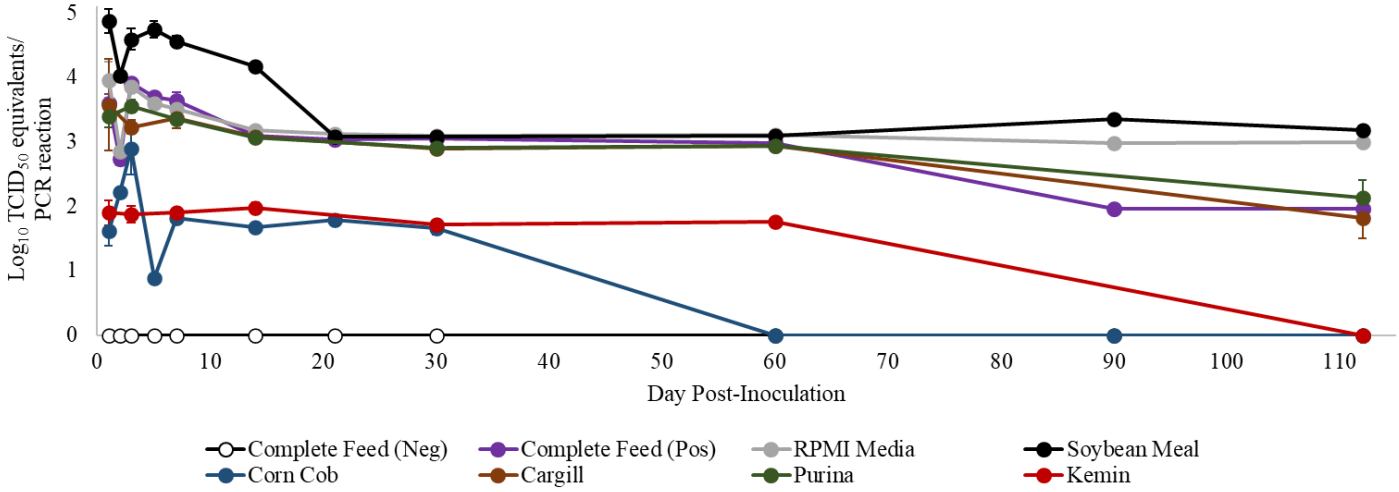

Figure 3. Detection of African swine fever virus (ASFV) DNA by quantitative PCR in feed and ingredients stored at 95°F for up to 112 days. PCR detection based on amplification of ASFV p72. Data calculated as log_{10} 50% tissue culture infectious dose ($TCID_{50}$) equivalents per PCR reaction based a standard curve of ASFV in splenic homogenate. Data shown as mean log_{10} $TCID_{50}$ equivalents/PCR reaction for duplicate samples ± standard deviation. Feed additives (Cargill, Purina, Kemin) were added to complete feed prior to inoculation with ASFV. All matrices were inoculated with ASFV Georgia 2007 (10^5 $TCID_{50}$) or PBS (negative control).

Detection of Infectious ASFV

Feed supernatant samples were classified as positive for infectious ASFV if one or both duplicate samples had immunofluorescence detected on cell culture of PAMs. Most duplicate samples had consistent results across both replicates (**Tables 1 – 3**); however, inconsistency across replicates was found in complete feed treated with Purina and at higher temperatures. No negative control complete feed samples had ASFV detected with IFA on PAMs.

Feed supernatant samples selected for bioassays were from the first or second sequential sample day in which both replicates were negative for ASFV on PAMs. Samples were selected using an adaptive design for each of the 6 bioassay replicates, where results of each replicate were used to select samples to be tested in subsequent replicates. This design allowed testing of sequential samples based on an initial positive bioassay result. A total of 32 feed supernatant samples were tested in pigs, including 11 samples from 40°F, 11 samples from 68°F, and 10 samples from 95°F. Of the 32 pigs utilized for swine bioassays, 2 pigs required euthanasia prior to the conclusion of the study at 6 dpi due to severity of clinical disease. The remaining 30 pigs were either humanely euthanized or died on 7 dpi. Complete necropsies were performed and gross lesions consistent with ASF were documented in positive pigs. Of the 27 ASFV-inoculated samples, 11 were classified as ASFV-positive based on evidence of infection in pigs. All feed supernatant samples classified as positive for infectious ASFV on swine bioassay had ASFV detected across all 3 diagnostic assays (serum qPCR, spleen qPCR, spleen VI). None of the 5 pigs inoculated with negative control complete feed samples were classified as ASFV-positive. These factors provide evidence for accurate classification of samples and adequate biosecurity being maintained between pens.

At 40°F, infectious ASFV was detected in complete feed until 30 dpi (negative at 60 dpi), in RPMI media until at least 112 dpi, in soybean meal until at least 112 dpi, in corncob particles until 5 dpi (negative at 7 dpi), and in complete feed with Purina until 1 dpi (negative at 3 dpi). Complete feed with Cargill or Kemin were negative on the first sample collected at 1 dpi. At 68°F, infectious ASFV was detected in complete feed until 1 dpi (negative at 3 dpi), in RPMI media until at least 21 dpi, in soybean meal until at least 21 dpi, and in corncob particles until 1 dpi (negative at 3 dpi). Complete feed with Cargill, Purina or Kemin were negative on the first sample collected at 1 dpi. At 95°F, infectious ASFV was detected in RPMI media until 2 dpi (negative at 3 dpi) and in soybean meal until at least 7 dpi. Complete feed, corncob particles, and complete feed with additives (Cargill, Purina, or Kemin) were negative on the first sample collected at 1 dpi.

Table 1. Detection of infectious African swine fever virus (ASFV) by cell culture and swine bioassay in feed and ingredients stored at 40°F over time*

		Storage Time of Feed Post-Inoculation (days)												
Matrix	Replicate	1	3	5	7	14	21	30	60	90	112	210	269	365
CF (Negative)	1	-	-	-	-	-	-	-	- Bioassay	-	- Bioassay	NT	NT	NT
	2	-	-	-	-	-	-	-	- Negative	-	- Negative	NT	NT	NT
CF (Positive)	1	+	+	+	+	+	+	+	- Bioassay	-	NT	NT	NT	NT
	2	+	+	+	+	+	+	+	- Negative	-	NT	NT	NT	NT
RPMI Media	1	+	+	+	+	+	+	+	+	- Bioassay	- Bioassay	NT	NT	NT
	2	+	+	+	+	+	+	+	+	- Positive	- Positive	NT	NT	NT
Soybean Meal	1	+	+	+	+	+	+	+	+	- Bioassay	- Bioassay	NT	NT	NT
	2	+	+	+	+	+	+	+	+	- Positive	- Positive	NT	NT	NT
Corncob Particles	1	+	+	+	- Bioassay	-	-	-	-	-	NT	NT	NT	NT
	2	+	+	+	- Negative	-	-	-	-	-	NT	NT	NT	NT
CF + Cargill	1	- Bioassay	-		-	-		-	-		NT			
	2	- Negative	-		-	-		-	-		NT			
CF + Purina	1	+	- Bioassay		-	-		-	-		NT			
	2	-	- Negative		-	-		-	-		NT			
CF + Kemin	1	- Bioassay	-		-	-		-	-		NT			
	2	- Negative	-		-	-		-	-		NT			

*Detection of infectious ASFV was assessed on porcine alveolar macrophages (PAMs) using immunofluorescence assay (IFA) with a p30 ASFV monoclonal antibody. Results of IFA are shown for each of the duplicate samples as positive (+) or negative (-). Samples with detectable ASFV genome on PCR but negative for IFA on PAMs were tested in nursery pig bioassays via intramuscular injection of pooled duplicate samples. Nursery pigs were assessed for the presence of ASFV infection 6 – 7 days post-inoculation of pooled supernatant samples through PCR of serum and splenic homogenate, and IFA of splenic homogenate. Bioassay results are written out as Positive or Negative. Samples are marked red when positive for infectious ASFV and green when negative for infectious ASFV; grey boxes indicate no sample was generated. Key: CF, complete feed; RPMI, Roswell Park Memorial Institute; NT, not tested. Cargill feed additive applied at 0.9% inclusion, Purina feed additive applied at 1.0% inclusion, Kemin feed additive applied at 0.33% inclusion.

Table 2. Detection of infectious African swine fever virus (ASFV) by cell culture and swine bioassay in feed and ingredients stored at 68°F over time*

		Storage Time of Feed Post-Inoculation (days)													
Matrix	Replicate	1	2	3	5	7	14	21	30	60	90	112	210	269	365
CF (Negative)	1	-	-	- Negative	-	-	-	- Negative	NT	NT	NT	NT	NT	NT	NT
	2	-	-	- Bioassay	-	-	-	- Bioassay	NT	NT	NT	NT	NT	NT	NT
CF (Positive)	1	+	-	- Negative	-	-	-	NT	NT	NT	NT	NT	NT	NT	NT
	2	+	-	- Bioassay	-	-	-	NT	NT	NT	NT	NT	NT	NT	NT
RPMI Media	1	+	+	+	+	+	- Positive	- Positive	NT	NT	NT	NT	NT	NT	NT
	2	+	+	+	+	-	- Bioassay	- Bioassay	NT	NT	NT	NT	NT	NT	NT
Soybean Meal	1	+	+	+	+	-	- Positive	- Positive	NT	NT	NT	NT	NT	NT	NT
	2	+	+	+	+	-	- Bioassay	- Bioassay	NT	NT	NT	NT	NT	NT	NT
Corncob Particles	1	+	-	- Negative	-	-	-	NT	NT	NT	NT	NT	NT	NT	NT
	2	+	-	- Bioassay	-	-	-	NT	NT	NT	NT	NT	NT	NT	NT
CF + Cargill	1	- Negative		-		-	-		NT	NT		NT			
	2	- Bioassay		-		-	-		NT	NT		NT			
CF + Purina	1	- Negative		-		-	-		NT	NT		NT			
	2	- Bioassay		-		-	-		NT	NT		NT			
CF + Kemin	1	- Negative		-		-	-		NT	NT		NT			
	2	- Bioassay		-		-	-		NT	NT		NT			

*Detection of infectious ASFV was assessed on porcine alveolar macrophages (PAMs) using immunofluorescence assay (IFA) with a p30 ASFV monoclonal antibody. Results of IFA are shown for each of the duplicate samples as positive (+) or negative (-). Samples with detectable ASFV genome on PCR but negative for IFA on PAMs were tested in nursery pig bioassays via intramuscular injection of pooled duplicate samples. Nursery pigs were assessed for the presence of ASFV infection 6 – 7 days post-inoculation of pooled supernatant samples through PCR of serum and splenic homogenate, and IFA of splenic homogenate. Bioassay results are written out as Positive or Negative. Samples are marked red when positive for infectious ASFV (or sequential samples are positive) and green when negative for infectious ASFV; grey boxes indicate no sample was generated. Key: CF, complete feed; RPMI, Roswell Park Memorial Institute; NT, not tested. Cargill feed additive applied at 0.9% inclusion, Purina feed additive applied at 1.0% inclusion, Kemin feed additive applied at 0.33% inclusion.

Table 3. Detection of infectious African swine fever virus (ASFV) by cell culture and swine bioassay in feed and ingredients stored at 95°F over time*

		Storage Time of Feed Post-Inoculation (days)													
Matrix	Replicate	1	2	3	5	7	14	21	30	60	90	112	210	269	365
CF (Negative)	1	- Negative	-	-	-	-	NT	NT	NT	NT	NT	NT			
	2	- Bioassay	-	-	-	-	NT	NT	NT	NT	NT	NT			
CF (Positive)	1	- Negative	-	-	NT	NT	NT	NT	NT	NT	NT	NT			
	2	- Bioassay	-	-	NT	NT	NT	NT	NT	NT	NT	NT			
RPMI Media	1	-	+	- Negative	-	NT	NT	NT	NT	NT	NT	NT			
	2	-	+	- Bioassay	-	NT	NT	NT	NT	NT	NT	NT			
Soybean Meal	1	+	-	- Positive	- Positive	- Positive	NT	NT	NT	NT	NT	NT			
	2	-	-	- Bioassay	- Bioassay	- Bioassay	NT	NT	NT	NT	NT	NT			
Corncob Particles	1	- Negative	-	-	NT	NT	NT	NT	NT	NT	NT	NT			
	2	- Bioassay	-	-	NT	NT	NT	NT	NT	NT	NT	NT			
CF + Cargill	1	- Negative		-		NT	NT		NT	NT		NT			
	2	- Bioassay		-		NT	NT		NT	NT		NT			
CF + Purina	1	- Negative		-		NT	NT		NT	NT		NT			
	2	- Bioassay		-		NT	NT		NT	NT		NT			
CF + Kemin	1	- Negative		-		NT	NT		NT	NT		NT			
	2	- Bioassay		-		NT	NT		NT	NT		NT			

*Detection of infectious ASFV was assessed on porcine alveolar macrophages (PAMs) using immunofluorescence assay (IFA) with a p30 ASFV monoclonal antibody. Results of IFA are shown for each of the duplicate samples as positive (+) or negative (-). Samples with detectable ASFV genome on PCR but negative for IFA on PAMs were tested in nursery pig bioassays via intramuscular injection of pooled duplicate samples. Nursery pigs were assessed for the presence of ASFV infection 6 – 7 days post-inoculation of pooled supernatant samples through PCR of serum and splenic homogenate, and IFA of splenic homogenate. Bioassay results are written out as Positive or Negative. Samples are marked red when positive for infectious ASFV (or sequential samples are positive) and green when negative for infectious ASFV; grey boxes indicate no sample was generated. Key: CF, complete feed; RPMI, Roswell Park Memorial Institute; NT, not tested. Cargill feed additive applied at 0.9% inclusion, Purina feed additive applied at 1.0% inclusion, Kemin feed additive applied at 0.33% inclusion.

Discussion:

This study investigated three environmental storage conditions (cool storage, ambient storage, hot storage) for their effects on ASFV degradation in contaminated feed. Of the four matrices tested, time course of ASFV infectivity from longest to shortest was found in soybean meal, RPMI media, complete feed, and corncob particles. Considering the highest risk, it is recommended that contaminated feed be stored for >112 days at 40°F, >21 days at 68°F, and >7 days at 95°F to reduce the risk of ASFV transmission to pigs. As end point inactivation times were not determined for ASFV in soybean meal at any of the three temperatures, minimum storage times can be incorporated into risk-benefit analyses for feed biosecurity plans.

Soybean meal provided a stabilizing matrix for ASFV at an equal or greater ability than the stabilizing matrix of RPMI media, which contains amino acids, vitamins, inorganic salts, glucose, glutathione, fetal bovine serum, antibiotics and antimycotics. Soybean meal has previously been shown to provide a highly stabilizing matrix for a diverse range of viruses experimentally (Stoian et al., 2020) and is thought to have a protein and moisture content which promotes virus integrity and survival. The stability of ASFV in soybean meal raises significant concern due to the volume of soybean products which are imported each year into the US from ASFV-positive countries (Patterson et al., 2021, Patterson, 2022).

Other studies have also identified soybean meal as capable of increasing virus stability and prolonging virus viability. For example, Stenfeldt et al. (2022) investigated the stability of two foot and mouth disease virus (FMDV) strains (A24 Cruzeiro, O/SKR/2010) in soybean meal at 39°F and 68°F. Results showed that soybean meal increased virus stability at each temperature for both FMDV strains compared to media (Dulbecco's minimal essential media) and pelleted complete feed (Stenfeldt et al., 2022). Second, Caserta et al. (2022) characterized the rate of decay for Senecavirus A (SVA) in feeds stored at 39°F, 59°F and 86°F. Data demonstrated soybean meal increased SVA viability over time at all three temperatures compared to media (minimum essential media), vitamin D and lysine (Caserta et al., 2022).

Ground corncob particles did not increase the stability of ASFV compared to media and were the only untreated sample type with notable degradation of ASFV DNA resulting in negative PCR results during our study. Specifically, ASFV DNA was undetectable by 210 dpi at 68°F and by 60 dpi at 95°F in corncobs. Furthermore, evidence of ASFV infectivity was only detected for a maximum of 5 days at even the lowest temperature (40°F). In another study, PEDV was also less stable in corncob carriers than other feed ingredients such as soybean meal (Dee et al., 2016). This data suggests that corncob particles used as a carrier material for other diet components may be a lower risk for maintaining viable ASFV and serving as a transmission source to naïve pigs if contaminated.

Previous studies have investigated the stability of ASFV in feeds at storage temperatures with varying results. Fischer et al. (2020) found no evidence of ASFV infectivity in contaminated field crops (unprocessed wheat, barley, rye, triticale, corn, peas) after 2 hours of storage at 68°F (Fischer et al., 2020). In contrast, our study detected infectious ASFV in contaminated processed feed for at least 24 hours after storage at 68°F. Sindryakova et al. (2016) found no evidence of ASFV infectivity in contaminated compound feed after 40 days of storage at 40°F (Sindryakova et al., 2016). This is comparable to our study, in which ASFV was undetectable in contaminated complete feed after 60 days of storage at 40°F. It should be noted that both cited studies used cell culture alone for detection of infectious ASFV, a technique known to be less sensitive than swine bioassay. Moreover, the detection limit for ASFV on cell culture were highlighted in the current study as several samples negative on PAMs were subsequently positive on swine bioassay.

Our previous work had demonstrated MCFA (C6, C8, C10) and formaldehyde-based feed additives were effective in reducing ASFV Georgia 2007 infectivity in feed exposed to fluctuating temperature and humidity shipment conditions over 30 days (Niederwerder et al., 2021). Work by others with MCFA (C8, C10, C12) were mixed, showing an ability to inhibit ASFV BA71V infectivity in cell culture but not *in vitro* contaminated feed (Jackman et al., 2020). In the current study, MCFA and formaldehyde-based mitigants reduced infectivity of ASFV Georgia 2007 in feed maintained at stable temperatures. Specifically, mitigant-treated complete feed samples were negative for infectious ASFV between 57 – 59 days earlier than untreated complete feed samples at 40°F. The additional 2 days required for the Purina MCFA product efficacy at 40°F may have been due to increased virus stability at low temperatures. Taken together, use of feed additives may be particularly beneficial when feed is in cold storage whereby ASFV viability will be prolonged.

Minimum storage times recommended from the data generated in our study can be compared to previously reported minimum storage parameters, such as a minimum of 30 days at 75°F (Dee et al., 2022) and a minimum of 100 days at 50°F (CFIA, 2022), to build a database in which feed mills and producers can determine best practices for feed storage. This study underscores the longevity of ASFV survival in contaminated soybean meal and supports the concept of feed quarantine for targeted high-risk ingredients as part of a swine biosecurity program.

References

Alonso, C., M. Borca, L. Dixon, Y. Revilla, F. Rodriguez, J. M. Escribano and C. Ictv Report, 2018: ICTV Virus Taxonomy Profile: Asfarviridae. *The Journal of general virology,* 99, 613-614.

Calvin, S., A. Snow and E. Brockhoff, 2022: African swine fever risk and plant-based feed ingredients: Canada's approach to risk management of imported feed products. *Transboundary and emerging diseases,* 69, 176-181.

Carriquiry, M., A. Elobeid, D. Swenson and D. Hayes, 2020: Impacts of African Swine Fever in Iowa and the United States. Iowa State University Digital Repository.

Caserta, C., G. N. JC, A. Singrey, M. C. Niederwerder, S. Dee, E. A. Nelson and D. G. Diel, 2022: Stability of Senecavirus A in animal feed ingredients and infection following consumption of contaminated feed. *Transboundary and emerging diseases,* 69, 88-96.

CFIA, 2022: Import requirements for plant-based feed ingredients imported for use in livestock feed. In: Health of Animals Act Health of Animals Regulations, https://www.inspection.gc.ca/animal-health/terrestrial-animals/diseases/reportable/african-swine-fever/plant-based-feed-ingredients/eng/1553706365334/1553706365585#a51.

Dee, N., K. Havas, A. Shah, A. Singrey, G. Spronk, M. Niederwerder, E. Nelson and S. Dee, 2022: Evaluating the effect of temperature on viral survival in plant-based feed during storage. *Transboundary and emerging diseases*.

Dee, S., C. Neill, A. Singrey, T. Clement, R. Cochrane, C. Jones, G. Patterson, G. Spronk, J. Christopher-Hennings and E. Nelson, 2016: Modeling the transboundary risk of feed ingredients contaminated with porcine epidemic diarrhea virus. *BMC veterinary research,* 12, 51.

Dee, S. A., F. V. Bauermann, M. C. Niederwerder, A. Singrey, T. Clement, M. de Lima, C. Long, G. Patterson, M. A. Sheahan, A. M. M. Stoian, V. Petrovan, C. K. Jones, J. De Jong, J. Ji, G. D. Spronk, L. Minion, J. Christopher-Hennings, J. J. Zimmerman, R. R. R. Rowland, E. Nelson, P. Sundberg and D. G. Diel, 2018: Survival of viral pathogens in animal feed ingredients under transboundary shipping models. *PloS one,* 13, e0194509.

Finney, D. J., 1964: The Spearman-Karber method. In: D. J. Finney (ed), *Statistical method in biological assay*, 2nd edn., pp. 524-530. Charles Griffin, London.

Fischer, M., M. Mohnke, C. Probst, J. Pikalo, F. J. Conraths, M. Beer and S. Blome, 2020: Stability of African swine fever virus on heat-treated field crops. *Transboundary and emerging diseases,* 67, 2318-2323.

Gonzales, W., C. Moreno, U. Duran, N. Henao, M. Bencosme, P. Lora, R. Reyes, R. Núñez, A. De Gracia and A. M. Perez, 2021: African swine fever in the Dominican Republic. *Transboundary and emerging diseases,* 68, 3018-3019.

Jackman, J. A., A. Hakobyan, H. Zakaryan and C. C. Elrod, 2020: Inhibition of African swine fever virus in liquid and feed by medium-chain fatty acids and glycerol monolaurate. *Journal of animal science and biotechnology,* 11, 114.

Khanal, P., M. Olcha and M. C. Niederwerder, 2021: Detection of African swine fever virus in feed dust collected from experimentally inoculated complete feed using quantitative PCR and virus titration assays. *Transboundary and emerging diseases*.

King, D. P., S. M. Reid, G. H. Hutchings, S. S. Grierson, P. J. Wilkinson, L. K. Dixon, A. D. Bastos and T. W. Drew, 2003: Development of a TaqMan PCR assay with internal amplification control for the detection of African swine fever virus. *Journal of virological methods,* 107, 53-61.

Niederwerder, M. C., 2021: Risk and Mitigation of African Swine Fever Virus in Feed. *Animals : an open access journal from MDPI,* 11, 792.

Niederwerder, M. C., S. Dee, D. G. Diel, A. M. M. Stoian, L. A. Constance, M. Olcha, V. Petrovan, G. Patterson, A. G. Cino-Ozuna and R. R. R. Rowland, 2021: Mitigating the risk of African swine fever virus in feed with anti-viral chemical additives. *Transboundary and emerging diseases,* 68, 477-486.

Niederwerder, M. C. and R. A. Hesse, 2018: Swine enteric coronavirus disease: A review of 4 years with porcine epidemic diarrhoea virus and porcine deltacoronavirus in the United States and Canada. *Transboundary and emerging diseases,* 65, 660-675.

Niederwerder, M. C. and R. R. Rowland, 2017: Is There a Risk for Introducing Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) Through the Legal Importation of Pork? *Food and environmental virology,* 9, 1-13.

Niederwerder, M. C., A. M. M. Stoian, R. R. R. Rowland, S. S. Dritz, V. Petrovan, L. A. Constance, J. T. Gebhardt, M. Olcha, C. K. Jones, J. C. Woodworth, Y. Fang, J. Liang and T. J. Hefley, 2019: Infectious Dose of African Swine Fever Virus When Consumed Naturally in Liquid or Feed. *Emerging infectious diseases,* 25, 891-897.

Nielsen, S. S., J. Alvarez, D. J. Bicout, P. Calistri, E. Canali, J. A. Drewe, B. Garin-Bastuji, J. L. Gonzales Rojas, C. Gortázar Schmidt, M. Herskin, M. Miranda Chueca, V. Michel, B. Padalino, P. Pasquali, L. H. Sihvonen, H. Spoolder, K. Stahl, A. Velarde, A. Viltrop, C. Winckler, A. Boklund, A. Botner, A.

Gervelmeyer, O. Mosbach-Schulz and H. C. Roberts, 2021: Ability of different matrices to transmit African swine fever virus. *EFSA journal. European Food Safety Authority,* 19, e06558.

Patterson, G., 2022: An analysis of select swine feed ingredients and pork products imported into the United States from African swine fever virus affected countries. *Transboundary and emerging diseases,* 69, 128-136.

Patterson, G., M. C. Niederwerder and S. A. Dee, 2019: Risks to animal health associated with imported feed ingredients. *J Am Vet Med Assoc,* 254, 790-791.

Patterson, G., M. C. Niederwerder, G. Spronk and S. A. Dee, 2021: Quantification of soya-based feed ingredient entry from ASFV-positive countries to the United States by ocean freight shipping and associated seaports. *Transboundary and emerging diseases,* 68, 2603-2609.

Rowlands, R. J., V. Michaud, L. Heath, G. Hutchings, C. Oura, W. Vosloo, R. Dwarka, T. Onashvili, E. Albina and L. K. Dixon, 2008: African swine fever virus isolate, Georgia, 2007. *Emerging infectious diseases,* 14, 1870-1874.

Sauter-Louis, C., J. H. Forth, C. Probst, C. Staubach, A. Hlinak, A. Rudovsky, D. Holland, P. Schlieben, M. Göldner, J. Schatz, S. Bock, M. Fischer, K. Schulz, T. Homeier-Bachmann, R. Plagemann, U. Klaaß, R. Marquart, T. C. Mettenleiter, M. Beer, F. J. Conraths and S. Blome, 2020: Joining the club: First detection of African swine fever in wild boar in Germany. *Transboundary and emerging diseases*.

Sindryakova, I. P., Y. P. Morgunov, A. Y. Chichikin, I. K. Gazaev, D. A. Kudryashov and S. Z. Tsybanov, 2016: THE INFLUENCE OF TEMPERATURE ON THE RUSSIAN ISOLATE OF AFRICAN SWINE FEVER VIRUS IN PORK PRODUCTS AND FEED WITH EXTRAPOLATION TO NATURAL CONDITIONS. *Sel'skokhozyaistvennaya Biologiya,* 51, 467-474.

Stenfeldt, C., M. R. Bertram, H. C. Meek, E. J. Hartwig, G. R. Smoliga, M. C. Niederwerder, D. G. Diel, S. A. Dee and J. Arzt, 2022: The risk and mitigation of foot-and-mouth disease virus infection of pigs through consumption of contaminated feed. *Transboundary and emerging diseases,* 69, 72-87.

Stoian, A. M. M., V. Petrovan, L. A. Constance, M. Olcha, S. Dee, D. G. Diel, M. A. Sheahan, R. R. R. Rowland, G. Patterson and M. C. Niederwerder, 2020: Stability of classical swine fever virus and pseudorabies virus in animal feed ingredients exposed to transpacific shipping conditions. *Transboundary and emerging diseases,* 67, 1623-1632.

Stoian, A. M. M., J. Zimmerman, J. Ji, T. J. Hefley, S. Dee, D. G. Diel, R. R. R. Rowland and M. C. Niederwerder, 2019: Half-Life of African Swine Fever Virus in Shipped Feed. *Emerging infectious diseases,* 25, 2261-2263.

Zhou, X., N. Li, Y. Luo, Y. Liu, F. Miao, T. Chen, S. Zhang, P. Cao, X. Li, K. Tian, H. J. Qiu and R. Hu, 2018: Emergence of African Swine Fever in China, 2018. *Transboundary and emerging diseases,* 65, 1482-1484.

Zsak, L., M. V. Borca, G. R. Risatti, A. Zsak, R. A. French, Z. Lu, G. F. Kutish, J. G. Neilan, J. D. Callahan, W. M. Nelson and D. L. Rock, 2005: Preclinical diagnosis of African swine fever in contact-exposed swine by a real-time PCR assay. *Journal of clinical microbiology,* 43, 112-119.