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Stability of classical swine fever virus and pseudorabies virus in animal feed ingredients exposed to transpacific shipping conditions

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Abstract

Classical swine fever virus (CSFV) and pseudorabies virus (PRV) are two of the most significant trade-limiting pathogens affecting swine worldwide. Both viruses are endemic to China where millions of kilograms of feed ingredients are manufactured and subsequently imported into the United States. Although stability and oral transmission of both viruses through contaminated pork products has been demonstrated as a risk factor for transboundary spread, stability in animal feed ingredients had yet to be investigated. The objective of this study was to determine the survival of CSFV and variant PRV in 12 animal feeds and ingredients exposed to environmental conditions simulating a 37-day transpacific shipment. Virus was detected by PCR, virus isolation and nursery pig bioassay. CSFV and PRV nucleic acids were stable throughout the 37-day period in all feed matrices. Infectious CSFV was detected in two ingredients (conventional soybean meal and pork sausage casings) at 37 days post-contamination, whereas infectious PRV was detected in nine ingredients (conventional and organic soybean meal, lysine, choline, vitamin D, moist cat and dog food, dry dog food and pork sausage casings). This study demonstrates the relative stability of CSFV and PRV in different feed ingredients under shipment conditions and provides evidence that feed ingredients may represent important risk factors for the transboundary spread of these viruses.

KEYWORDS

animal feed, classical swine fever, pseudorabies, ships, survival, swine diseases

1 | INTRODUCTION

Classical swine fever virus (CSFV) and pseudorabies virus (PRV) are transboundary animal diseases and a significant threat to animal agriculture in the United States (U.S.). Both viruses are highly contagious and can cause high morbidity and mortality in pigs. CSFV is an enveloped positive-sense RNA virus in the family *Flaviviridae* (Paton & Greiser-Wilke, 2003) and PRV is an

enveloped double-stranded DNA virus in the family *Herpesviridae* (Mettenleiter, 2000). CSFV was officially eradicated from the United States in 1978; however, the continued persistence of CSFV as an endemic pathogen in many countries throughout the world, including in South and Central America, maintains this virus as a constant threat for re-introduction into U.S. swine herds (Brown & Bevins, 2018). Importantly, CSFV has recently been detected in countries and regions which have remained free of the virus for

many years, demonstrating the potential for re-emergence of this important swine pathogen. For example, CSFV was reported in Japan during September 2018 for the first time in 26 years (Postel et al., 2019). Shortly thereafter in October 2018, Brazil reported the detection of CSFV in backyard pigs in remote areas of a state which had remained negative for the disease for almost a decade (Feedstuffs, 2018).

Pseudorabies virus (PRV) was officially eradicated from U.S. commercial swine in 2004, but remains a constant threat to commercial pork production due to the maintenance of the virus in U.S. feral swine (Brown et al., 2019; Pedersen et al., 2013) and the emergence of highly virulent strains in other countries. In 2011, a variant Chinese pseudorabies virus emerged on pig farms in China, characterized as having increased virulence in older grow-finish pigs and causing disease in swine previously vaccinated with Bartha-K61 (An et al., 2013; Wu, Bai, Sun, Chang, & Zhang, 2013). Importantly, distribution of the variant Chinese PRV strain appears to be increasing, initially being reported in nine provinces in 2013 (Wu et al., 2013) and more recently being reported in 23 regions throughout China in 2016 (Sun et al., 2016). In October 2019, PRV re-emerged in domestic swine in Mexico after being negative for the virus since 2015 (OIE, 2019).

The introduction of porcine epidemic diarrhoea virus (PEDV) into the United States in April 2013 increased global awareness of the risk of contaminated feed as a transmission vehicle for transboundary animal disease spread (Aubry, Thompson, Pasma, Furness, & Tataryn, 2017; Bowman, Krogwold, Price, Davis, & Moeller, 2015; Dee et al., 2014, 2016; Niederwerder & Hesse, 2018; Schumacher et al., 2016). Furthermore, field reports have included contaminated feed as a potential contributor to African swine fever virus (ASFV) spread throughout China (Wen et al., 2019; Zhai, Wei, Sun, Lv, & Xu, 2019) and recent experimental studies have confirmed ASFV stability and transmission through feed (Niederwerder et al., 2019; Stoian et al., 2019). The lessons learned from PEDV and ASFV underscore the need to quantitate the risk that feed may play in the introduction of other high-consequence transboundary animal diseases, such as CSFV and PRV. Although both viruses are known to be transmitted orally in contaminated pork or swill (Edwards, 2000; Wittmann, 1991), little is known about the stability of the viruses in plant-based feed ingredients. However, animal feed has been suggested as a potential route for re-introduction of CSFV into the United States (Brown & Bevins, 2018), whole corn has been shown to support stability of PRV (Schoenbaum, Freund, & Beran, 1991), and swine feed contaminated with a CSFV modified live virus vaccine has been implicated as the cause of transboundary spread of the virus in Korea (Kim et al., 2008).

In our recent work (Dee et al., 2018), evidence of diverse viral stability in feed ingredients exposed to models of transoceanic shipment was demonstrated. Survival was dependent on virus and feed ingredient, and allowed the identification of high-risk combinations of virus and ingredient. The objective of the current study was to investigate the stability of CSFV Brescia and variant PRV (HeN1) in feed ingredients when subjected to temperature and humidity conditions simulating transpacific shipment.

2 | MATERIALS AND METHODS

2.1 | Environment and feed ingredients

A transpacific shipment model was adapted from work previously described (Dee et al., 2018, 2016) to assess the stability of CSFV and PRV in animal feed ingredients. Briefly, a 37-day timeframe was utilized to simulate a representative transoceanic shipment from Beijing, China to Des Moines, Iowa between the dates of 23 December 2012 and 28 January 2013. This timetable was selected due to the availability of historical data during both the land and sea portions of the simulated shipment. Environmental conditions (relative humidity and temperature) were programmed into an environmental chamber (Model 3911, Thermo Scientific Forma). Relative humidity conditions fluctuated every 4 hr between 8 a.m. to 4 p.m., followed by a constant relative humidity for 14 hr between 4 p.m. and 8 a.m. the subsequent day. Temperature conditions fluctuated every 6 hr daily.

Twelve feed or feed ingredients were included in the study based on previous work (Dee et al., 2018) and the volume of ingredients imported between 2012 and 2018 from China to the port of San Francisco, CA (Table 1). The relevance of these imports is demonstrated by comparing the volume of imported ingredients in the years 2012–2014 (mean ± *SD*: 16,674.9 ± 4,035.5 metric tons) to almost double this volume in the years 2015–2017 (mean ± *SD*: 33,228.4 ± 6,951.5 metric tons). In 2018, this trend continued with a total of 31,842.3 metric tons being imported from China into San Francisco. The 12 feed or feed ingredients tested in the model included conventional soybean meal, organic soybean meal, soy oil cake, dried distillers grains with solubles (DDGS), lysine, choline, vitamin D, moist cat food, moist dog food, dry dog food, pork sausage casings and complete feed in meal form. Controls included virus in minimum essential media (MEM; positive control) and sterile PBS in complete feed (negative control). Samples were organized into one of four identical batches, each representing a specific segment of the 37-day transpacific shipping period as previously described (Dee et al., 2018). Batch 1 was collected at 1 day post-contamination (dpc), batch 2 was collected at 8 dpc, batch 3 was collected at 25 dpc, and batch 4 was collected at 37 dpc. On designated days post-contamination, duplicate samples of each ingredient and controls were removed from the environmental chamber, processed and stored at −80°C until diagnostic testing was performed.

2.2 | Viruses

All use of viruses and animals were performed in accordance with the Federation of Animal Science Societies Guide for the Care

TABLE 1 Volume of animal feeds, feed ingredients and products of animal origin imported from China to San Francisco, CA, each year between 2012 and 2018[a]

Imported Product	HTS Code	2012	2013	2014	2015	2016	2017	2018
Soybean oilcake and other solid residues resulting from the extraction of soy bean oil, whether or not ground or in the form of pellets	2304.00.0000	15,126.6	7,977.6	13,575.9	24,201.4	36,962.3	31,787.9	29,606.3
Dog and cat food, put up for retail sale	2309.10.0010 2309.10.0090	3,841.1	2,897.7	574.2	7.0	1,395.3	2,317.4	1,748.4
Flours and meals of soybeans	1208.10.0000 1208.10.0090	1,832.6	1,816.1	1,346.3	979.6	185.4	333.0	0.0
Hog guts, bladders & stomachs, whole & pieces thereof, fresh, chilled, frozen, salted, in brine, dried or smoked, prepared for use as sausage casings	0504.00.0020	129.4	216.8	457.4	359.7	530.1	536.0	416.5
Lysine and its esters; salts thereof	2922.41.0010 2922.41.0090	33.0	95.0	19.8	0.0	0.3	1.6	49.1
Vitamins D and their derivatives	2936.29.5020	26.0	21.0	14.0	0.0	17.0	34.5	13.5
Choline and its salts	2923.10.0000	19.0	0.4	0.0	0.0	0.0	31.9	5.2
Brewing or distilling dregs and waste, whether or not in the form of pellets	2303.30.0000	1.4	1.3	2.0	1.3	1.0	2.6	3.3
Grand Total		21,009.1	13,025.9	15,989.6	25,549.0	39,091.4	35,044.9	31,842.3

[a]Volume shown in metric tons. Data based on the International Trade Commission Harmonized Tariff Schedule (HTS) found at https://dataweb.usitc.gov/. Table adapted from previous work (Dee et al., 2018) and updated by Patterson on 3 December 2019 to include years 2017 and 2018 and compare trends over the last 7 years.

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 Committee and the Institutional Biosafety Committee. CSFV Brescia strain and PRV HeN1 strain were propagated and titred using PK15 cells. First, 10-fold serial dilutions were created in MEM and dilutions in quadruplicate were added to confluent monolayers of PK15 cells in 96-well plates. After incubation for 1 hr at 37°C, plates were washed and MEM replaced. Following 3 days at 37°C, infected cells were visualized using either the PrioMab CSFV V3 Monoclonal Antibody (Thermo Fisher Scientific) or a polyvalent PRV anti-serum from a gnotobiotic pig (APHIS National Veterinary Services Laboratories). Briefly, cells were washed three times with PBS and fixed using 80% acetone. Primary antibodies were added at a dilution of 1:500 and allowed to incubate at 37°C for 1 hr. Plates were then washed three times with PBS, and either goat anti-mouse antibody (AlexaFluor 488, Life Technologies) or goat anti-swine FITC antibody (NOVUS) was added at a dilution of 1:400. Following a 1-hr incubation at 37°C, cells were observed under the inverted florescence microscope (Evos FL, Life Technologies). The 50% tissue culture infectious dose ($TCID_{50}$/ml) was calculated according to the method of Spearman and Karber (Finney, 1964).

2.3 | Feed inoculation and processing

Feed and feed ingredients were gamma-irradiated (minimum absorbed dose of 25 kilograys) prior to use in this study. Five grams of each ingredient was added to 50 ml conical tubes in duplicate for each batch (2 tubes × 4 batches = 8 tubes/ingredient/virus). Each feed or feed ingredient was inoculated with 100 µl of either CSFV Brescia or PRV HeN1 for a final concentration of approximately 10^5 $TCID_{50}$/ingredient. For the positive control, 100 µl of virus was added to 5 ml of MEM. For the negative control, 100 µl of sterile PBS was added to 5 grams of complete feed. Following inoculation or mock inoculation, samples were vortexed for 10 s at maximum speed. Finally, solid caps were replaced with vented caps to allow temperature and humidity exchange during incubation in the environmental chamber.

Feed and feed ingredient samples were removed in duplicate from the environmental chamber at the corresponding incubation times for each batch, including 1, 8, 25 and 37 dpc for batches 1, 2, 3, and 4, respectively. For processing, 15 ml of sterile PBS with antibiotics and anti-mycotics were added to each sample. Vented caps were replaced with solid caps prior to the samples being vortexed for 10 s. Following centrifugation at 10,000 *g* for 5 min at 4°C, the supernatant for each sample was aliquoted into cryovials and stored at −80°C.

2.4 | PCR

Nucleic acid was extracted using the MagMAX™ Total Nucleic Acid Isolation Kit (Thermo Fisher Scientific). Briefly, 50 µl of supernatant was combined with 20 µl of Bead mix (containing Lysis/Binding Solution, Carrier RNA and 100% isopropanol) on a U-bottom 96-well plate provided by the kit. After the plate was shaken for 1 min on an orbital shaker, cells were lysed using 130 µl Lysis/Binding solution and the plate was shaken for 5 min. The beads were captured on a magnetic stand and washed twice using 150 µl wash solution 1 and 2. The final elution volume was 50 µl. Extracted samples and controls were tested immediately by quantitative PCR.

For detection of CSFV, primers and probe along with the thermocycler set-up were based on previously published work (Risatti, Callahan, Nelson, & Borca, 2003). Primers/probe mixture was commercially synthesized using PrimeTime® Mini qPCR Assay (IDT Technologies): probe (FAM-CCTGAGTACAGGACAGTCGTCAGTA), sense primer (5′-CCCTGGGTGGTCTAAG-3′) and anti-sense primer (5′-CATGCCCTCGTCCAC-3′). The PCR mixture (20 µl volume reaction) consisted of 12.5 µl 2× RT-PCR Buffer (AgPath-IDss One-Step RT-PCR kit, Thermo Fisher), 2.5 µl 1× PrimeTime Mini (500 nM primers and 250 nM probe), 1 µl of 25× RT-PCR Enzyme Mix (AgPath-ID™ One-Step RT-PCR, Thermo Fisher) and 4 µl nuclease-free water. The mastermix was dispensed into a Hard-Shell® optical 96-well reaction plate (Bio-Rad). Test samples (5 µl of RNA) were added and the plate was spun to remove air bubbles. For each reaction, positive and negative controls were included. The CFX96™ Real-Time System (Bio-Rad) was used with the following cycling conditions: 45°C for 10 min reverse transcription, followed by reverse transcription inactivation and initial denaturation at 95°C for 10 min, and amplification followed by 40 cycles of 95°C for 15 s and 60°C for 45 s. Data analysis was performed using CFX96 software, and results were reported as the number of $TCID_{50}$ equivalents per 25 µl PCR reaction.

For detection of PRV, primers/probe mixture was commercially synthesized using PrimeTime® Midi qPCR Assay (IDT Technologies): probe (5′-FAM-CAA GAA CGT CAT CGT CAC GAC CG-BHQ-1-3′), sense primer (5′-AAG TTC AAG GCC CAC ATC TA-3′) and anti-sense primer (5′-TGA AGC GGT TCG TGA TGG-3′) as previously described (Sayler et al., 2017). The PCR mixture (15 µl volume reaction) consisted of 10 µl 2× iTaq Universal Probes supermix (Bio-Rad), 1 µl 1× PrimeTime Midi (500 nM primers and 250 nM probe) and 4 µl nuclease-free water. The master mix was dispensed into a Hard-Shell® optical 96-well reaction plate (Bio-Rad). Test samples (5 µl of DNA) were added, and the plate was spun to remove air bubbles. For each reaction, positive and negative controls were included. The CFX96™ Real-Time System (Bio-Rad) was used with the following cycling conditions: 95°C for 2 min denaturation and activation followed by 45 cycles of 94°C for 30 s, 58°C for 1 min and 60°C for 30 s. Data analysis was performed using CFX96 software and results were reported as the number of $TCID_{50}$ equivalents per 20 µl PCR reaction.

2.5 | Virus isolation and titration

Supernatant samples were tested for the presence of viable virus using PK15 cells. Briefly, serial dilutions of each sample were performed in MEM in triplicate. Diluted samples were added to washed confluent monolayers of PK15 cells in 96-well plates and incubated for 1 hr at 37°C. Following incubation, plates were washed and MEM replaced. After 3 days at 37°C, the cells were fixed with 80% acetone and stained using primary and secondary antibodies as described above. Staining was observed under the inverted microscope and the $TCID_{50}$/ml was calculated according to the method of Spearman and Karber (Finney, 1964).

2.6 | Swine bioassay

Swine bioassays were performed to detect infectious CSFV or PRV in batch 4 samples (37 dpc) that had detectable viral genome on PCR but were negative on virus isolation. A total of 14 nursery age barrow pigs (CSFV; mean age ± SD, 22.1 ± 1.1 days) and 10 nursery age barrow pigs (PRV; mean age ± SD, 22.2 ± 1.7 days) were obtained from high-health commercial herds for use in bioassays. All pigs were housed in individual 1.9 m^2 pens in one of three identical 66 m^2 rooms at the Kansas State University Biosecurity Research Institute and maintained under biosafety level 3 agriculture (BSL-3Ag) containment conditions. Each raised stainless-steel pen contained slotted fibreglass flooring and pens were separated by at least 1.5 m within the room. Three sides of the pen were solid with a 4th side consisting of bars and a gate. Rooms were environmentally controlled and complete exchange of air occurred 14.5 times/hr within each room. Up to six individually housed pigs were held in a room at any given time with one pig being maintained as a negative control to confirm the lack of cross-contamination and aerosol transmission between pens.

The inoculum was prepared for testing CSFV and PRV samples by first centrifuging the feed ingredient supernatant collected at 37 dpi. Supernatant from duplicate samples was pooled, and the inoculum was delivered as a 1 ml intramuscular injection. Each pig received a single sample type, with the exception of 1 pig which received a pooled inoculum containing 3 feed supernatant samples (soy oil cake, choline and vitamin D) to test for the presence of CSFV.

After 3 days of acclimation post-arrival to the BSL-3 facility, all pigs were inoculated or mock-inoculated (supernatant from negative control complete feed) with a 1 ml suspension intramuscularly. Pigs were monitored daily by a veterinarian or veterinary assistant for clinical signs associated with classical swine fever or pseudorabies, including depression, neurologic abnormalities, lameness, respiratory distress, pyrexia, reduced body condition, diarrhoea, and hyperaemia or haemorrhage. Pigs with significant clinical disease were treated or humanely euthanized as directed by the attending veterinarian. Serum was collected on 0 and 6 dpi via jugular venipuncture. At 6 dpi, pigs were humanely euthanized by intravenous pentobarbital sodium solution. Complete necropsies were performed by a veterinarian and tissue

samples were collected and stored at −80°C until testing for CSFV (tonsils) or PRV (tonsil and cerebrum) was performed.

Tissue lysates and serum were tested for the presence of CSFV or PRV by quantitative PCR as described above. Prior to nucleic acid extraction, frozen tissues were thawed, minced and passed through a cell strainer after adding PBS with antibiotics and anti-mycotics. The cell suspension was centrifuged at 4,000 × g for 30 min, and the supernatant transferred into a 50 ml conical tube stored at 4°C. The cell pellet was resuspended in sterile PBS with antibiotics and anti-mycotics followed by 3 freeze-thaw cycles. Finally, the cell suspension was centrifuged again at 4,000 × g for 30 min and the supernatant was pooled for testing.

3 | RESULTS

3.1 | Environmental conditions

Environmental conditions were programmed into the environmental chamber to fluctuate every 4 to 14 hr based on the historical data available (Figure 1). Overall, conditions during the 37-day model were moderate with a mean ± SD relative humidity of 64.0 ± 16.0% and a range of 25%–96%. Mean ± SD temperature was 6.1 ± 2.5°C with a range of 3.9–15.6°C.

3.2 | PCR

Nucleic acid was stable in all inoculated feed ingredients and media for both CSFV and PRV over the 37-day model (Figure 2a,b). All inoculated samples had detectable viral genome on 1, 8, 25 and 37 days post-contamination. All mock-inoculated complete feed samples were negative on qPCR. Overall, the quantity of CSFV $TCID_{50}$ equivalents detected on PCR was less than PRV at all time points. Across all feed ingredients, mean CSFV quantity was 2.7, 2.1, 2.2 and 2.1

log_{10} $TCID_{50}$ equivalents/PCR reaction on days 1, 8, 25 and 37, respectively. For PRV, mean quantity on days 1, 8, 25 and 37 in feed ingredients was 3.7, 4.0, 4.1 and 3.8 log_{10} $TCID_{50}$ equivalents/PCR reaction, respectively. Variation in genome quantity between samples and viruses may depend on the type and consistency of feed matrix (moisture, protein and fat content), extraction efficiency and/or genome stability.

3.3 | Virus titration

Virus titrations on PK15 cells were performed on duplicate samples from each inoculated ingredient on all four collection days. Figure 2c,d show viral decay over time, as measured by samples collected at 1, 8, 25 and 37 dpc. Negative control complete feed samples remained negative for CSFV and PRV on cell culture. At 1 dpc, infectious CSFV and PRV were detected in all samples with the exception of CSFV in lysine, which was negative on CSFV IFA throughout the study. Although initial inoculation dose (10^5 $TCID_{50}$/ ingredient) was similar for both viruses, titration results starting at 1 dpc demonstrated CSFV titres that were consistently lower than PRV titres. This may have been due to slight concentration differences between viral inocula, variations in virus stability during the first 24 hr post-inoculation, or differences in extraction efficiency of the two viruses in feed. Mean titres in all feed ingredients positive on virus isolation at 1 dpc was 3.4 log_{10} $TCID_{50}$ and 5.0 log_{10} $TCID_{50}$ for CSFV (11 ingredients) and PRV (12 ingredients), respectively.

By 8 dpc, infectious CSFV was only detectable in five inoculated feed ingredients, including conventional soybean meal, organic soybean meal, DDGS, dry dog food and pork sausage casings. Mean titre across the five feed ingredients was 3.1 log_{10} $TCID_{50}$. By 25 dpc, only two ingredients remained positive for CSFV on IFA, including conventional soybean meal (3.2 log_{10} $TCID_{50}$) and pork sausage casings (2.9 log_{10} $TCID_{50}$). By 37 dpi, CSFV was not detected on PK15 cells in any feed ingredients (Table 2).

FIGURE 1 Environmental conditions throughout the 37-day transpacific shipment model. Data are shown as the temperature (°C; black circles) and relative humidity (%; grey bars) during the model simulating transoceanic shipment. An environmental chamber was programmed to allow these conditions to fluctuate as often as every 4 hr daily
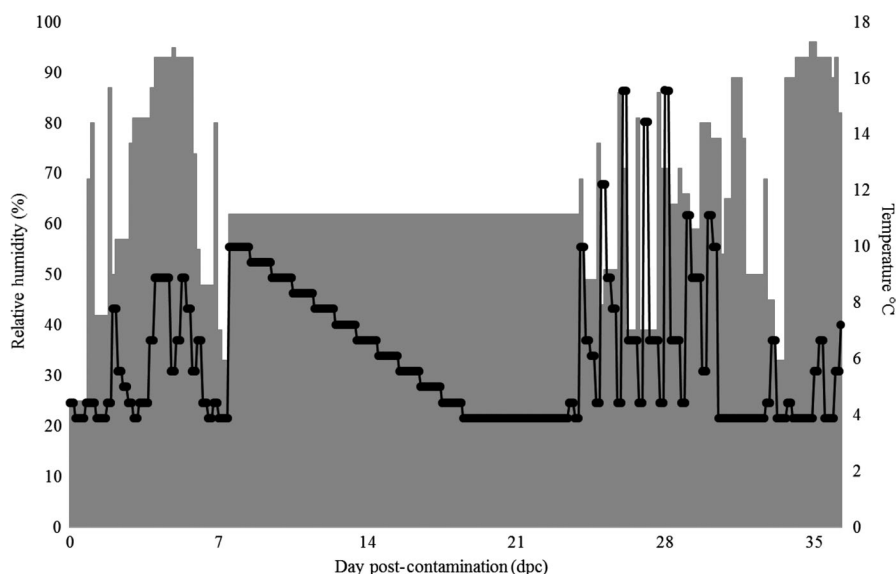
For PRV, all inoculated feed ingredients remained positive on virus isolation except for soy oil cake on 8 dpc. The remaining ingredients (*n* = 11) had a mean titre of 4.5 $\log_{10}$ TCID$_{50}$. By 25 dpc, the majority of ingredients (*n* = 10) still had detectable PRV on cell culture, with a mean titre of 3.7 $\log_{10}$ TCID$_{50}$; moist cat food and soy oil cake were negative for PRV on 25 dpc. At the conclusion of the study (37 dpc), infectious titres were calculated for four ingredients, including conventional soybean meal, organic soybean meal, vitamin D and moist dog food (Table 2; mean titre 3.9 $\log_{10}$ TCID$_{50}$). Additionally, PRV remained detectable in laboratory media at a titre of 3.0 $\log_{10}$ TCID$_{50}$ at 37 dpc. The remaining

eight feed ingredients had no detectable PRV on PK15 cells at the conclusion of the model.

3.4 | Swine bioassays

All samples negative on IFA in cell culture were tested in a nursery pig bioassay. For CSFV, samples tested included all 12 inoculated feed ingredients, the positive control in media and the negative control in complete feed. Clinical signs of CSF were observed in two pigs inoculated with feed supernatant from samples of conventional
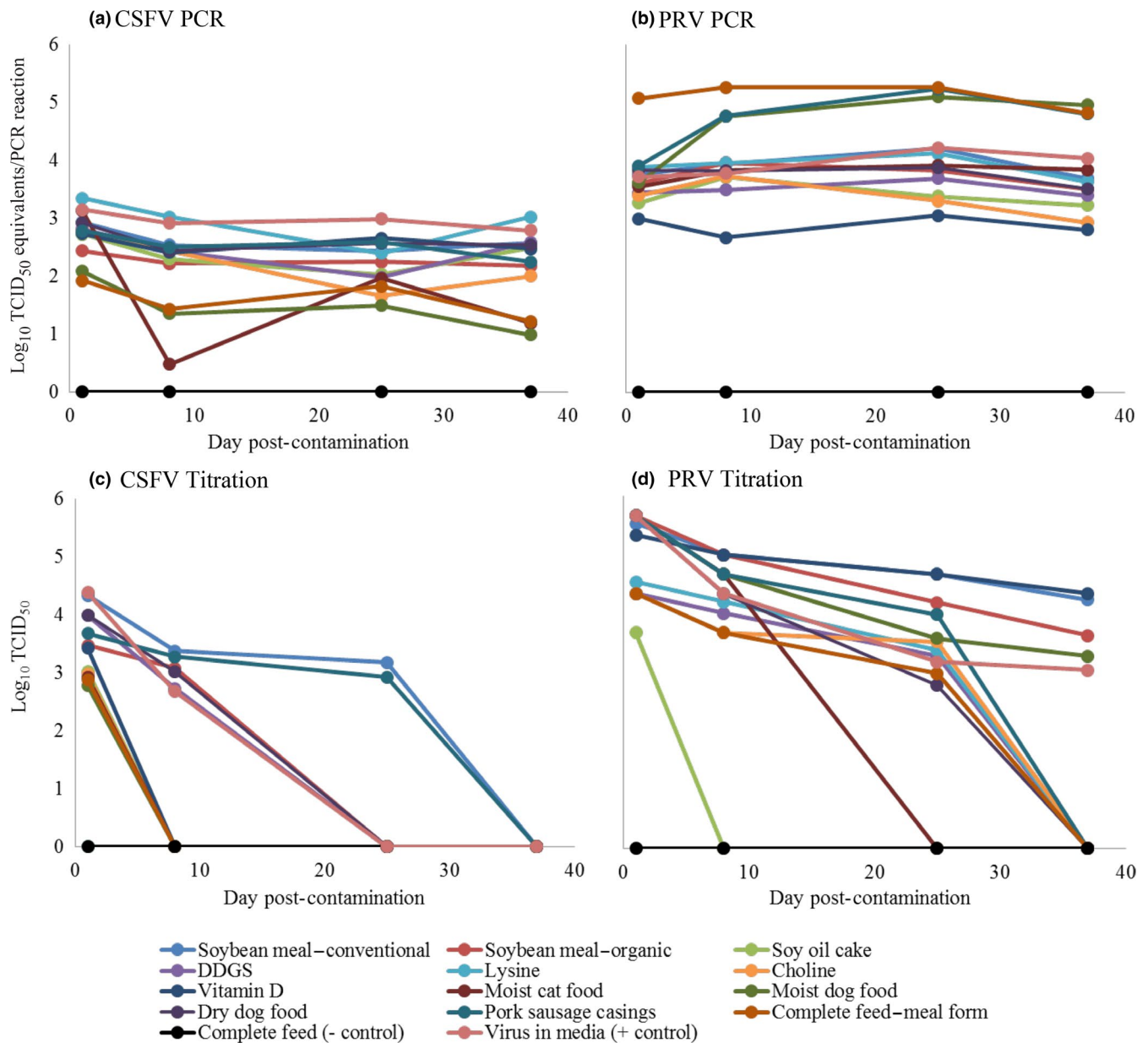
FIGURE 2 Stability of CSFV and PRV in feed ingredients as detected by quantitative PCR and virus titration over the course of a 37-day transpacific shipment model. Data are shown as the mean $\log_{10}$ TCID$_{50}$ equivalents/PCR reaction for duplicate samples tested by qPCR (a, b). All inoculated samples (*n* = 104/virus) had detectable viral genome on days 1, 8, 25 and 37 days post-contamination. Data are also shown as the mean $\log_{10}$ 50% tissue culture infectious dose (TCID$_{50}$) for duplicate samples tested by indirect florescent antibody titration on PK15 cells (c, d). Samples below the level of virus detection on cell culture are shown as 0 $\log_{10}$ TCID$_{50}$. The negative complete feed control remained negative on PCR and cell culture across all time points

soybean meal and pork sausage casings. In the pig inoculated with pork sausage casings supernatant, clinical signs were severe and required humane euthanasia at 5 dpi, including watery mucoid diarrhoea, depression, rough hair coat, pyrexia followed by hypothermia, dehydration and reduced appetite. One inoculated pig (lysine supernatant) died due to disease unassociated with CSFV at 3 dpi. All remaining pigs were humanely euthanized at 6 dpi. Serum and tonsils were tested by PCR to assess for the presence of CSFV infection in each pig. Serum and tonsils collected from pigs inoculated with conventional soybean meal and pork sausage casings were positive for CSFV on PCR. All other serum and tonsil samples collected from pigs inoculated with feed supernatant samples and from the negative control mock-inoculated pigs ($n = 3$) had no detectable CSFV on qPCR. (Table 2).

For PRV, samples tested in swine bioassay included eight feed ingredients (soy oil cake, DDGS, lysine, choline, moist cat food, dry dog food, pork sausage casings and complete feed) and the negative control in complete feed. No significant clinical signs were noted in pigs inoculated with PRV feed supernatant. All pigs were humanely euthanized at 6 dpi. Serum, tonsil and cerebrum samples were tested

by PCR to assess for the presence of PRV infection in each pig. Serum and tissue samples from pigs inoculated with five of the eight feed ingredients tested had detectable PRV, including lysine, choline, moist cat food, dry dog food and pork sausage casings. All other serum and tissue samples collected from inoculated or mock-inoculated pigs ($n = 2$) had no detectable PRV on qPCR (Table 2).

Overall, CSFV survived the 37-day model in two ingredients (conventional soybean meal and pork sausage casings) and PRV survived the 37-day model in nine ingredients (conventional and organic soybean meal, lysine, choline, vitamin D, moist cat and dog food, dry dog food and pork sausage casings). Additionally, viable PRV, but not CSFV, was detected in laboratory media after the 37-day shipping conditions (Table 3). Experimental design characteristics for swine bioassays limited the use of animals by only testing each sample in a single pig and reduced the likelihood of aerosol transmission between pigs in the same room by euthanizing pigs at 6 dpi for diagnostic testing. However, these methodological aspects of the study, combined with a lower CSFV titre detected at 1 dpc across feed matrices, may have contributed to an underestimation of risk for CSFV and PRV in feed and ingredients.

TABLE 2 Diagnostic testing of feed ingredients for CSFV and PRV in cell culture and nursery pig bioassay after exposure to 37 days of environment conditions simulating transpacific shipment[a]

| | CSFV | | | PRV | | | |
| | Cell Culture | Bioassay PCR | | Cell Culture | Bioassay PCR | | |
Ingredient	Mean Titre	Serum	Tonsil	Mean Titre	Serum	Tonsil	Cerebrum
Soybean meal—conventional	-	3.0	4.5	$10^{4.2}$	NT	NT	NT
Soybean meal—organic	-	-	-	$10^{3.6}$	NT	NT	NT
Soy oil cake[b]	-	-	-	-	-	-	-
DDGS	-	-	-	-	-	-	-
Lysine[c]	-	-	-	-	1.5	4.5	0.1
Choline[b]	-	-	-	-	0.4	2.0	-
Vitamin D[b]	-	-	-	$10^{4.3}$	NT	NT	NT
Moist cat food	-	-	-	-	1.1	2.2	-
Moist dog food	-	-	-	$10^{3.3}$	NT	NT	NT
Dry dog food	-	-	-	-	2.9	5.5	1.8
Pork sausage casings[d]	-	2.7	3.6	-	1.3	0.3	-
Complete feed—meal form	-	-	-	-	-	-	-
Complete feed (– control)[e]	-	-	-	-	-	-	-
Virus in media (+ control)	-	-	-	$10^{3.0}$	NT	NT	NT

Abbreviations: NT, not tested; -, not detected.

[a]Feed ingredient supernatant was tested on PK15 cells for the presence of infectious CSFV or PRV. If virus was detected on cell culture, data are shown as the mean titre ($TCID_{50}$) of duplicate samples collected at 37 dpi. If virus was not detected in cell culture, feed supernatant was tested in a nursery pig bioassay through intramuscular injection. One ingredient was tested per pig unless otherwise noted. Bioassay results are shown as log_{10} $TCID_{50}$ equivalents/PCR reaction in representative tissues collected at 3–6 dpi.

[b]Feed supernatants from duplicate samples of soy oil cake, choline and vitamin D inoculated with CSFV were pooled and tested in 1 nursery pig as a 1 ml suspension administered intramuscularly.

[c]Pig died at 3 dpi due to unrelated illness and samples collected for CSFV testing.

[d]Pig was euthanized due to severe clinical signs at 5 dpi and samples collected for CSFV testing.

[e]A total of five pigs were tested through intramuscular injection of feed supernatant samples from negative control complete feed. All pigs were negative for CSFV ($n = 3$) or PRV ($n = 2$) at 6 dpi.

TABLE 3 Virus viability in animal feeds and feed ingredients after exposure to 30–37 days of environmental conditions simulating transoceanic shipment[a]

Ingredient	SVA (FMDV)	ASFV	PRV	PSV (SVDV)	PEDV	FCV (VESV)	PCV2	BHV–1 (PRV)	PRRSV 174	CSFV	BVDV (CSFV)	VSV	CDV (NiV)	IAV-S
Soybean meal—conventional	(+)	(+)	(+)	(+)	(+)	(+)	(−)	(+)	(+)	(+)	(−)	(−)	(−)	(−)
Soybean meal—organic	(−)	(+)	(+)	(+)	(+)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)
Soy oil cake	(+)	(+)	(−)	(+)	NT	(−)	(−)	(+)	(−)	(−)	(−)	(−)	(−)	(−)
DDGS	(+)	(−)	(−)	(−)	NT	(−)	(−)	(−)	(+)	(−)	(−)	(−)	(−)	(−)
Lysine	(+)	(−)	(+)	(+)	(+)	(+)	(+)	(−)	(−)	(−)	(−)	(−)	(−)	(−)
Choline	(+)	(+)	(+)	(−)	(+)	(−)	(+)	(−)	(−)	(−)	(−)	(−)	(−)	(−)
Vitamin D	(+)	(−)	(+)	(+)	(+)	(−)	(+)	(−)	(−)	(−)	(−)	(−)	(−)	(−)
Moist cat food	(+)	(+)	(+)	(+)	NT	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)
Moist dog food	(+)	(+)	(+)	(+)	NT	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)
Dry dog food	(+)	(+)	(+)	(+)	NT	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)
Pork sausage casings	(+)	(+)	(+)	(+)	NT	(+)	(−)	(−)	(−)	(+)	(−)	(−)	(−)	(−)
Complete feed—meal form	(+)	(+)	(−)	(+)	NT	(+)	(+)	(−)	(−)	(−)	(−)	(−)	(−)	(−)
Complete feed (− control)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)
Virus in media (+ control)	(−)	(+)	(+)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)

Abbreviations: ASFV, African swine fever virus; BHV-1, bovine herpesvirus 1; BVDV, bovine viral diarrhoea virus; CDV, canine distemper virus; CSFV, classical swine fever virus; FCV, feline calicivirus; FMDV, foot-and-mouth disease virus; IAV-S, influenza A virus; NiV, Nipah virus; NT, not tested; PCV2, porcine circovirus type 2; PEDV, porcine epidemic diarrhoea virus; PRRSV, porcine reproductive and respiratory syndrome virus; PRV, pseudorabies virus; PSV, porcine sapelovirus; SVA, Seneca virus A; SVDV, swine vesicular disease virus; VESV, vesicular exanthema of swine virus; VSV, vesicular stomatitis virus.

[a]Table adapted to include CSFV and PRV from previous publication (Dee et al., 2018). A red box (+) indicates infectious virus was detected at the conclusion of the 30- to 37-day transoceanic shipment models. A green box (−) indicates no infectious virus was detected at the conclusion of the 30- to 37-day transoceanic shipment model. Results in column do not represent testing of viruses in parentheses.

4 | DISCUSSION

Classical swine fever and pseudorabies are two of the most significant infectious diseases to swine production worldwide. Both CSFV and PRV have long histories of being endemic to United States swine herds prior to lengthy and costly eradication programmes. Both diseases, along with African swine fever, are currently circulating in China, where millions of kilograms of feed ingredients are exported to the United States every year. The study described herein determined that CSFV and PRV are capable of surviving in feed ingredients subjected to a simulated transpacific shipment from Asia to North America, with PRV having increased stability across a broader range of ingredients. The current study adds CSFV and PRV to the 12 other transboundary animal viruses that have been tested for stability in feed ingredients subjected to transoceanic shipment conditions (Table 3) and builds on the growing body of evidence supporting the concept that feed ingredients should be considered as risk factors in the global spread of transboundary diseases.

Importantly, stability appears to depend on both the virus and feed ingredient. Conventional soybean meal and pork sausage casings were two ingredients which supported both CSFV and PRV stability throughout the 37-day model. Although these two ingredients were the only ones in which infectious CSFV was detected, infectious PRV was detected in an additional seven feed ingredients and viral media at the conclusion of the trial. The relative reduction in environmental stability of CSFV compared with DNA viruses of swine has been previously reported (Botner & Belsham, 2012; Krug, Lee, Eslami, Larson, & Rodriguez, 2011; Niederwerder & Rowland, 2017; Turner, Williams, & Cumby, 2000).

Interestingly, when compared to the results from the surrogate viruses previously tested in a biosafety level-2 laboratory for CSFV (bovine viral diarrhoea virus or BVDV) and PRV (bovine herpesvirus 1 or BHV-1; Dee et al., 2018), the actual target viruses demonstrated increased stability compared with the surrogate viruses utilized from the same viral family (Table 3). However, it should be noted that BVDV and BHV-1 could not be tested in a pig bioassay as they are primary

bovine viral diseases; thus, there was reduced diagnostic sensitivity by which infectious virus could be detected at the conclusion of the transoceanic model. Additionally, BHV-1 survived the 37-day model in soy oil cake, whereas this was one of the few ingredients in which PRV was undetectable. These are important considerations for the limitations in using surrogate viruses that are not primary porcine viruses for assessing risk of transboundary swine diseases.

Feed ingredients which promoted virus degradation or inactivation varied between CSFV and PRV. For CSFV, the ingredient in which this was most notable was lysine, in which no virus was detected as early as 1 dpc. For PRV, soy oil cake was the ingredient in which the most viral instability was noted, with no detectable virus as early as 8 dpc. It is possible that lysine or soy oil cake have some anti-viral properties that may reduce titres or viral stability of CSFV and PRV, respectively. It is important to note, however, that CSFV and PRV genomes were stable and consistently detected in all inoculated feed ingredients (including lysine and soy oil cake) as well as media throughout the 37-day model (n = 104 inoculated samples/virus; 100% PCR positive). Figure 2 highlights the important distinction between PCR detection of genome and cell culture detection of viable virus.

It is interesting that both CSFV and PRV had evidence of increased stability in two or more of the feed matrices when compared to the virus in media used for laboratory cell culture. For CSFV, this was demonstrated by stability of the virus in conventional soybean meal and pork sausage casings until 37 dpc and a lack of infectious virus detected in media at the conclusion of the model. For PRV, this was demonstrated by the titres detected in four feed ingredients (conventional and organic soybean meal, vitamin D and moist dog food) being higher than that detected in media at 37 dpc. This is consistent with our previous work on ASFV half-life in feed ingredients, where the calculated viral half-life in media was shorter than all feed ingredients tested (Stoian et al., 2019). These studies provide evidence that components of the feed ingredient matrix provide a protective environment which promotes viral stability compared with laboratory media.

The Swine Health Information Center Swine Disease Matrix ranks viral diseases of swine based on the likelihood of introduction into the United States swine herd and the financial losses to production and trade (SHIC, 2018). Due to the resulting production and market impacts of CSFV and PRV introduction, these viruses are ranked as the third and fourth highest priority diseases. Foot and mouth disease virus (FMDV) and ASFV are ranked first and second on the Matrix. Although FMDV has yet to be directly tested in the transboundary environmental model, Seneca virus A (SVA) has been tested as a surrogate and demonstrated high stability across a broad range of animal feed ingredients. It is worth noting that three of the top four viruses in the Swine Disease Matrix were also three of the viruses which had the greatest stability in feed ingredients subjected to transoceanic shipping conditions (Table 3).

Taken together, these results provide novel evidence that contaminated feed ingredients can support CSFV and PRV stability and represent a risk for the transport of swine pathogens globally. This study has identified specific high-risk ingredients which promote CSFV and/or PRV survival and will provide valuable data for quantitative risk assessments and focusing mitigation strategies. Due to the severe economic and animal welfare consequences of CSFV or PRV introduction combined with the access of feed ingredients to commercial pigs in high biosecurity herds, mitigating the risk of feed ingredients as a route for disease introduction and transmission should be considered a high priority.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICAL STATEMENT

The authors confirm that the ethical policies of the journal have been adhered to and the appropriate ethical review committee approval has been received.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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