The emergence, distribution, and persistence of viral pathogens in swine production systems

by

Grace E. Houston

B.S., Kansas State University, 2016
D.V.M., Kansas State University, 2020

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine/Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2023

Abstract

As swine industries continue to emphasize biosecurity throughout all aspects of swine production, an understanding of viral characteristics is pivotal. Therefore this work aimed to evaluate characteristics of various viruses through different scenarios of swine production. Chapter 1 describes a clinical case where a farrow-to-finish production site was diagnosed with atypical porcine pestivirus (APPV) and an investigation was conducted to understand potential routes of introduction and dissemination within a population of previously-naïve pigs. It was found that litters from both gilts and sows had clinical signs suggestive of APPV, which reduced over time, and that semen doses appeared to be the most likely route of introduction of APPV. Chapter 2 focused on understanding viral pathogen dissemination through production sites and swine production feed mills. Within this investigation, two feed mills and three breed-to-wean facilities were evaluated after suspicion of an epidemiological link for dissemination of porcine deltacoronavirus through feed delivery. The feed mill surfaces that contained detectable porcine enteric viral RNA were associated with feed delivery trucks. Given this knowledge of transportation vehicles contributing to the spread of viral pathogens, chapter 3 was conducted to evaluate the detection and infectivity of viruses within truck cabs after disinfectant application methods. For detection of viral RNA, there was a disinfectant treatment by surface type interaction ($P < 0.0001$) indicating that how the disinfectant is applied and the surface that the disinfectant is applied to impacts the amount of viral RNA found in environmental samples. When considering the infectivity of these samples from disinfectant application, environmental samples failed to produce infectivity in bioassay analysis. Research using environmental sampling techniques for viral pathogens is still evolving given they are a novel diagnostic tool. The research included in chapter 4 was conducted to evaluate sample processing methods and the

presence of organic matter on detection of porcine epidemic diarrhea virus (PEDV) from environmental samples. There was a surface inoculation type by processing method interaction ($P < 0.001$) indicating material present in environmental samples and sample processing prior to laboratory analysis impacted the sample cycle threshold (Ct) value. Lastly, chapters 5-7 were conducted to understand the potential implications if African swine fever virus (ASFV) were to be introduced into a feed manufacturing facility. Research was conducted using a pilot scale feed mill within BSL3-Ag containment to manufacture ASFV inoculated feed. For environmental samples, sampling zone impacted the $\log_{10}$ p72 genomic copy number/mL ($P < 0.0001$). When holding environmental samples at room temperature up to 180 days after feed manufacturing, there was no evidence of ASFV degradation across holding times ($P = 0.433$). For feed samples, the batch of feed when manufactured influenced the $\log_{10}$ genomic copy number/g ($P < 0.0001$) content of feed samples with greater amounts of ASFV in early batches that decreased with each subsequent batch. When held at room temperature up to 180 days after feed manufacturing, the days held at room temperature and the batch of feed in which feed samples were collected from impacted the amount of ASFV DNA in feed samples ($P = 0.023$). Thus, viral pathogenesis continues to be a challenge within swine production systems, highlighting the importance of applied research to fully understand the epidemiological implications of viral pathogen emergence, distribution, and persistence.

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Approved by:

Co-Major Professor
Jordan T. Gebhardt

Approved by:

Co-Major Professor
Cassandra K. Jones

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Acknowledgements

This research would not be possible without my personal "village" of support. First, thank you to my committee members, Drs. Jordan Gebhardt, Cassie Jones, Jason Woodworth, Chad Paulk, and Steve Dritz, for constructive criticism, encouragement, and direction. I enjoyed learning and working with you guys; you took this diamond in the rough and made her sparkle. Thank you to the graduate students associated with Animal Sciences and Industry, Grain Science and Industry, and Diagnostic Medicine/Pathobiology. When I found myself up a creek with no paddle, at least one of you were willing to lend an extra paddle, trade boats, or float with me down the creek. To the fellow graduate students who became friends, this isn't a goodbye, this is a see you later. Thank you to the students I've met either through my role as a co-advisor with the swine veterinarian club, through my role as a graduate teaching assistant, or through my role as an instructor for livestock evaluation; you reminded me of a younger version of myself and helped me realize that my cynicism should come down about 5%. Next, thank you to my friends and family. Not everyone knows someone that can't seem to leave school; it's been an honor to be that friend or family member for you. Thank you to my parents, you both were always encouraging when it came to my long term goals. Lastly, thank you to my husband, Zachary Houston, who takes everything in stride. Life is fun with you.

Thank you to our collaborators and sponsors who made all this work possible. Thank you to Dr. Jurgen Richt Laboratory and Center of Excellence for Emerging and Zoonotic Animal Disease collaborators, Dr. Jianfai Bai collaborators, Grain Science and Industry Collaborators, Dr. Rachel Palinski, and Iowa State University Veterinary Diagnostic Laboratory and Bioassay analysis collaborators. Thank you to all the organizations that provided funding for the work like Swine Health Information Center, National Pork Board, NBAF Transition Funds from the state

of Kansas, the Department of Homeland Security Center of Excellence for Emerging and Zoonotic Animal Diseases, and the AMP Core of the NIGMS COBRE Center on Emerging and Zoonotic Infectious Diseases (CEZID).

Dedication

To Zachary Houston, my husband.

Preface

The dissertation is original work complete by the author, G. E. Houston; some works under her maiden name of C. G. Elijah. Chapter 1 was published in Frontiers in Veterinary Science (doi:10.3389/fvets.2022.998344). Chapters 2 (doi:10.54846/jshap/1250) and 4 (doi:10.54846/jshap/1311) were published in Journal of Swine Health and Production. Chapter 5 (doi:10.1371/journal.pone.0256138) was published in PLoS ONE. Chapter 6 (doi:10.1111/tbed,14177) was published in Transboundary and Emerging Diseases. Each of the chapters were formatted according to the required standards of the corresponding journal.

Chapter 1 - Detection and investigation of atypical porcine pestivirus in a swine production system[1]

Abstract

A commercial farrow-to-finish farm was suspicious of atypical porcine pestivirus (APPV) after observing clinical signs of congenital tremors (CT) and splay leg (SL) of newborn pigs. If introduced onto the farrow-to-finish, the two potential routes of introduction could be through replacement gilts or incoming semen doses. Therefore, this study aimed to determine the prevalence of clinical APPV within the sampled population, identify the route of APPV introduction to this system, and determine prevalence of detectable APPV RNA within a population of gilt multiplication farm offspring through an isolation nursery and finisher barn. Farrowing records were analyzed for the presence of CT or SL and corresponding parity of the dam. Overall, prevalence of clinically affected litters within batch farrowing groups ranged from 0 to 31%. Phylogenetic analysis was conducted on a serum sample from a gilt at the isolation nursery, semen dose for the farrow-to-finish farm, and serum of a CT piglet. Results indicated that the virus circulating in clinically affected piglets was most similar to an incoming semen dose (98.9% nucleotide identity). Blood samples were collected at four time points and revealed APPV clinical prevalence was 37.5 to 77.5% during the nursery phase and 0 to 26% during the finisher phase. Oral fluids were also collected during the finisher phase and APPV clinical prevalence was 100% for all sampling time points. In summary, introduction of APPV into naïve herds is associated with increased clinical CT and SL cases and is detectable in asymptomatic

[1] This work has been published through *Frontiers in Veterinary Science.* Houston GE, Jones CK, Woodworth JC, Palinski R, Paulk CB, Petznick T and Gebhardt JT. (2022) Detection and investigation of atypical porcine pestivirus in a swine production system. *Front. Vet. Sci.* 9:998344. doi:10.3389/fvets.2022.998344

pigs during the nursery and finisher production phases. This study found that potential screening tests for APPV could include oral fluids or qRT-PCR analysis of semen doses especially when trying to identify prevalence levels on naïve farm.

Keywords: atypical porcine pestivirus (APPV), investigation, persistence, production system, swine

Introduction

Atypical porcine pestivirus (APPV) was first identified in the United States in 2015. It is a Flavivirus linked to congenital tremors (CT) and splay-leg (SL) in pigs (1, 2, 3). The CT is characterized by muscle spasms in the head and body that can persist for weeks to months, typically diminishing by marketing (4, 5). While SL is a temporary dysfunction of the posterior legs following birth (2, 3, 4). Preweaning mortality associated with CT and SL is most often associated with inadequate feeding or difficulty standing or moving rather than the condition itself (5).

Atypical porcine pestivirus targets the cerebellum and lymph nodes, but has also been detected in feces, boar preputial swabs, preputial fluid, and semen (6, 7, 8). In 2018, APPV molecular prevalence was 28.7% in the Midwestern United States (US) and notably higher in states with the greatest swine production - IA, 30.6%; IL, 32.9%; MN, 37.4% (9). Atypical porcine pestivirus is associated with transient or persistent infections in asymptomatic pigs promoting global dissemination (10). Furthermore, APPV is highly mutable producing many genetically divergent strains (9). For this report, a commercial farrow to finish production system observed an increase in CT and SL. Serum was collected from clinically affected piglets, submitted to the Kansas State Veterinary Diagnostic Laboratory (KSVDL), and was found to have detectable APPV RNA via qRT-PCR analysis. The objectives of this study were to

determine the prevalence of clinical APPV on the farrow-to-finish farm, determine the route of introduction onto the farm, and determine if APPV persisted within asymptomatic pigs co-housed with gilts intended for the farrow-to-finish farm and their environment.

Materials and methods

All blood sampling and oral fluid collection were approved by Kansas State University institutional animal care and use committee (IACUC) protocol #4457.

Case history

A commercial farrow-to-finish farm located in Central Kansas was used in this experiment. The farm batch farrows 30 sows every 35 days. Gilts and sows are stalled until confirmed pregnant, then moved to group housing. From December 2019 to June 2020, 1 to 2 litters per batch exhibited CT or SL. The prevalence rates of CT and SL increased in July 2020, which led to suspicion of APPV. Serum samples from CT piglets and two semen samples were submitted to KSVDL for APPV qRT-PCR testing. The APPV qRT-PCR assay utilized and made commercially available is based on the assay described by Yuan et al. (11). For this assay, a Ct less than 37 is considered positive for APPV RNA, suspect between 37 and 39, and negative for APPV RNA with a Ct above 39. Serum samples from CT piglets had a cycle threshold (Ct) value of 26.3-28.5 while one of the two semen samples had a Ct value of 30.5. Atypical porcine pestivirus was a likely diagnosis given the clinical picture and APPV qRT-PCR results. However, to confirm a diagnosis of APPV given the absence of confirmation through histopathology of tissue, one of the CT piglet serum samples was submitted for metagenomics. Metagenomics analysis indicated a low number of influenza and porcine reproductive and respiratory syndrome virus reads, significant porcine endogenous retrovirus reads, and a diversity of bacteria, including Proteobacteria, Clostridium, Bacillus, and Mycoplasma. As none

of the identified organisms fully explained the clinical signs, APPV was deemed the likely cause of CT and SL in newborn pigs at this facility.

The commercial farrow-to-finish farm receives gilts from an isolation nursery located off site. This isolation nursery receives weaned gilts and barrows from a gilt multiplication facility every 2 months (Figure 1). The barrows in the isolation nursery, at the end of the production turn, are shipped to a separate finisher facility while the gilts are transported to the commercial farrow-to-finish farm. Prior to transport, all gilts are tested for porcine reproductive and respiratory syndrome virus RNA and antibodies and negative gilts are introduced onto the commercial farrow-to-finish farm. In August 2020, following the CT/SL outbreak on the commercial farrow-to-finish farm, 5 randomly selected barrows were bled and serum submitted for APPV qRT-PCR 24-hours after placement into the isolation nursery. Two of the five barrows had detectable APPV RNA (Ct values of 27.5 and 30.4). Given that there was detectable APPV RNA in the isolation nursery and the connection to the commercial farrow-to-finish facility, the isolation nursery was deemed as a potential source of APPV introduction. During the entirety of the study, no clinical signs of CT or SL were noted at the isolation nursery.

Farrowing data record analysis

Farrowing records from January 10, 2019 to March 2, 2021 were analyzed for the prevalence of CT or SL litters. If a comment of "shakers" or "splays" was on the farrowing card, the litter was included in litter prevalence and divided by the total number of females that farrowed during the specified batch. Parity information was also analyzed from the same farrowing cards. If the farrowing card indicated the parity of the female as "1", these females were designated as gilts, while all other parities were designated as sows.

Environmental sampling

Environmental sampling using 10 × 10 cm cotton gauze was conducted similar to Elijah et al. (12). Briefly, environmental swabs were taken after the isolation nursery was sanitized but before new pigs were placed at the location. A total of 27 sample sites were identified at the farm, including entryways into barns, changing areas for employees, boots utilized by workers, and floors of selected pens. It was chosen to focus on areas of direct pig contact and potential fomites given evidence to suggest that fomites play an important role in the transmission of this disease (4). If a site was selected for environmental swabbing, a clean pair of gloves was donned and a 10 × 10 cm cotton surgical gauze square pre-moistened with 5 mL of phosphate-buffered solution (PBS) was used to sample the area. Environmental samples were stored in an -80°C freezer prior to submission. For submission, five of 27 environmental samples were chosen at random, defrosted, 20 mL of PBS added to the environmental sample, manually agitated for 5-10 s, and analyzed for APPV RNA. For submission, all five selected environmental samples were from pen floors that had direct pig contact.

Blood and oral fluids collection

After the initial confirmation of APPV, serum from the barrow population was collected twice in isolation nursery and twice in the finisher barn. For the first isolation nursery sampling date, 200 barrows were individually bled. Forty barrows were removed from the facility as part of a concurrent research trial after this point, so 160 barrows were individually bled in the second sampling date. For each collection date in the isolation nursery, serum samples were pooled and submitted for APPV testing (5 pigs/pen/pool). When a positive APPV qRT-PCR was identified in the pooled samples, a subset of individual pig serum were submitted. In the finishing barn, there were 2 large pens that can accommodate 150 animals per pen. Upon arrival to the finishing

barn, all barrows from the isolation nursery are allocated between the two large pens. For serum sampling during the finisher phase, 15 pigs were randomly selected from each pen. For oral fluid collection, five ropes were hung in each pen and evenly dispersed throughout the pen so that all pigs within the pen could chew on oral fluid ropes. Oral fluid and serum samples collected from the finisher barn were submitted for APPV qRT-PCR.

Sequencing and phylogenetic analysis

Atypical porcine pesitvirus qRT-PCR positive samples were submitted for APPV E2 sequencing at the KSVDL. Submitted samples included one piglet serum sample from the farrow-to-finish farm (Ct=26.3), one semen dose intended for artificial insemination of farrow-to-finish gilts or sows (Ct=30.5), and one replacement gilt serum sample from the isolation nursery (Ct= 27.5). All three sequences were submitted to Genbank prior to publication; piglet sample, semen dose, and gilt sample can be accessed by their respective Genbank accession number as follows: ON651441, ON651443, and ON651442.

Viral RNA was extracted with the MagMax viral RNA Isolation kit (Thermo Fisher) on a Kingfisher platform. Amplicons were generated from viral RNA using Superscript III One-Step RT-PCR System with Platinum Taq and specific primers, using a 56°C annealing temperature and 30s extension time. Amplicons were purified using the HighPrep PCR clean-up System, library prepped by Nextera XT v2 DNA Library prep kit and sequenced on an Illumina Iseq (300-cycle cartridge), as specified by the manufacturer. Raw reads were trimmed for quality and mapped to the closest reference (Genbank #MK728876). Consensus sequences were extracted from the mapping and used for subsequent analysis. All bioinformatics was performed in CLC workbench v20 using default parameters. The resulting testing generated a partial E2 sequence form the semen dose but complete E2 sequences from the replacement gilt and piglet samples.

Consensus sequence alignment was performed using MUSCLE (13) in MEGA-X v10.0. The evolutionary history was inferred by using the maximum likelihood method with the Tamura-Nei model (14) and evaluated with 1,000 bootstrap replicates (15). Cut off value for the consensus tree was set to 70%. Pairwise differences were computed in Mega X using the pairwise differences function (16).

Results

Prevalence of clinical APPV cases was indistinguishable between sows (prevalence of affected litters within batch farrowing group ranging from 4 to 17%; average of 2.96% of litters affected over sampling period; CT CI = 1.03 – 4.88%; SL CI = 0.04-0.94%) and gilts (prevalence of affected litters within batch farrowing group ranging from 4 to 14%; average of 3.94% of litters affected over sampling period; CT CI = 2.42 – 5.46%; Figure 2). In this study, APPV RNA was detected at both introduction points for the commercial farrow-to-finish farm. The boar semen and replacement gilt serum contained APPV RNA, however, the clinical piglet APPV E2 sequence was most similar to that of the boar semen (98.9% versus 95.9% at the nucleotide level; Table 1). In addition, phylogenetic analyses clustered the gilt sequence in a separate clade than the semen and piglet sequences, further supporting the hypothesis that the boar semen and clinical piglet were more similar when compared to the gilt (Figure 3).

Atypical porcine pestivirus pen-level prevalence in the isolation nursery increased from 15 of 40 pens (37.5%) to 31 of 40 pens (77.5%) during the study period (Figure 4). Randomly selected pig prevalence increased from 4 of 20 pigs (20%) to 6 of 13 pigs (46%; Table 2). Of the five pen swab samples submitted, one, had detectable APPV RNA (Ct = 35.70) and another was suspect for APPV RNA (Ct =37.64). During the finishing stage, eight of the 30 (27%) randomly selected pigs had detectable serum APPV RNA for the first sampling date but none were positive

at the second sampling date (Table 3). During the finisher phase, on both sampling dates, all oral fluid samples had detectable APPV RNA.

Discussion

Atypical porcine pestivirus was identified, unintentionally, in the US in 2015 when samples were submitted for whole genomic sequencing for porcine respiratory and reproductive syndrome virus (1). The implications of this virus for swine production systems was uncertain until research established that inoculation of dams with APPV could results in clinical cases of CT and SL in piglets (2, 3, 6). However, even with considerable amounts of research-based evidence to suggest that CT and SL reside by weaning, there are inconsistent management strategies existing in commercial swine production systems for these piglets (17). For example, some production systems elect humane euthanasia of all affected piglets while other production systems elect to let these piglets mature to weaning and re-evaluate proper management at that time. There is not a single best solution as to what works best for a production system but as understanding of this virus continues to grow, it's pivotal to ensure that swine production systems receive the most science-based support to avoid unnecessary piglet mortality (17). Additionally, CT/SL piglets that look seemingly normal can also be a source of virus to other naïve pigs resulting in continuous exposure of APPV within a production system (10). This is incredibly important to consider, especially in livestock intended for breeding purposes. However, there are gaps in understanding as to which samples to take, when to take them, and the availability of diagnostic tests. Some research work has looked at viral presence of APPV RNA in conjunction with APPV antibodies but this was a relatively small sample size and tests utilized are only available in research settings (18). Therefore, when a commercial farrow-to-finish production site observed an increased prevalence of APPV, there was an opportunity to

understand the introduction of APPV onto the farm but also learn if diagnostics tests are available to evaluate long term APPV prevalence within a production flow of asymptomatic pigs.

One reason for increased appearance of CT/SL in litters from gilts is decreased prior exposure to pathogens (2, 3, 6). For this study, the similar clinical prevalence in sows and gilts indicated both groups had no previous exposure to APPV. However, after two months, gilt litters were primarily affected with clinical signs suggestive of APPV but by the end of the research period, clinical signs suggestive of APPV were not observed for any gilt or sow during lactation. These findings coupled with previous studies suggest an appropriate acclimation period for naïve females would decrease the likelihood of CT/SL appearance in their litters. Research has found that APPV RNA can be detected in boar semen and persist in boar reproductive tissues for long periods of time (2, 5, 19). The findings from this study in conjunction with other research indicate biosecurity measures such as screening of incoming semen would help to minimize the risk of APPV introduction and identify boars that are shedding APPV thereby serving as a potential source of APPV to other animals.

Of the randomly selected pigs for this study, no individual was positive for APPV at both sampling time-points potentially indicating transient infections. Pig identification numbers were obtained from finisher pigs to retrospectively analyze if they had been housed in pens that had detectable APPV RNA in the isolation nursery. Of the 30 randomly selected finisher pigs, only 15 of these pigs were bled in the isolation nursery, and these pigs were all housed in pens that had no detectable APPV RNA in the isolation nursery. These findings suggest the animals, within this population, housed in APPV negative pens were infected at a later age and maintained an asymptomatic infection for some time. These findings are similar to those in previous studies (2, 10). This study also tested oral fluids for detection of APPV and found they

were an adequate means of APPV detection. Others have found the pig's saliva to be a source of APPV shedding implicating the potential for oral fluids to serve as a potential diagnostic sample (4, 10). In the current investigation, population-based oral fluid samples contained detectable RNA through the end of the finishing phase even after RNAemia was no longer detected in the subset of the population which was individually sampled. Thus, this data supports that oral fluids may be a useful diagnostic technique to detect APPV in a population of pigs. Further research should focus on understanding the duration of RNAemia in relation to shedding of virus through oral fluids to understand the potential utility of the oral fluid sampling diagnostic technique and the potential application of this technique for clinical decision making.

This study also found detectable APPV RNA in the environment after the isolation nursery was cleaned and disinfected. While this finding does not suggest this APPV may infect the subsequently-housed animals, it suggests the disinfection procedures used in this facility may not be sufficient to properly rid the virus from the environment. Others have reported that disinfection is an important part of control and prevention programs for APPV (4). Our findings suggest enhanced cleaning and disinfection procedures are required to eliminate all APPV RNA from the environment.

The data for this study are limited to one production site. Ideally more production sites would have been used to increase the sample size but the objective of this study was to identify introduction of APPV onto a naïve farm and understand the prevalence of APPV within the asymptomatic pigs. Furthermore, the diagnosis of APPV was made based on presence of RNAemia in a clinically affected piglet, case history and clinical presentation consistent with this diagnosis, and through ruling out other pathogens through metagenomic sequencing. Given this was a production facility which elected not to euthanize any clinically affected pigs for the

purpose of histopathological analysis, the primary diagnosis was established based on molecular diagnostic techniques. Future research describing increased incidence of APPV cases in commercial swine facilities similar to that described in the current investigation should describe histopathological and serological findings as well as diagnostic capabilities improve over time. This work hopes to serve as a guide for other swine production systems when faced with a recent onset of CT or SL with unexplainable cause and elaborate on diagnostic tests available to aid in the investigative efforts.

In summary, APPV detected in a piglet with CT was similar to virus found in incoming semen, indicating that semen doses could serve as a route of APPV introduction onto the commercial farrow-to-finish farm. Further sample analysis and characterization would be needed to definitively conclude the origin of the APPV introduction within this farm. This study also found that APPV was detectable in populations of pigs without clinical signs of APPV indicating that these pigs could be a potential route of introduction for other naïve pigs. More evidence is needed to fully understand viral dynamics of transiently and persistently infected pigs and how this contributes to susceptible breeding livestock in swine production systems.

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Figure 1.1 Depiction of the relationship between the isolation nursery barn, the farrow-to-finish site, and off-site finisher barn and timeline of events during this investigation of atypical porcine pestivirus persistence (APPV) in pigs. Arrows indicate movement of pigs while lines indicate order of events of sampling which corresponds to the shading of blue in the graphic.

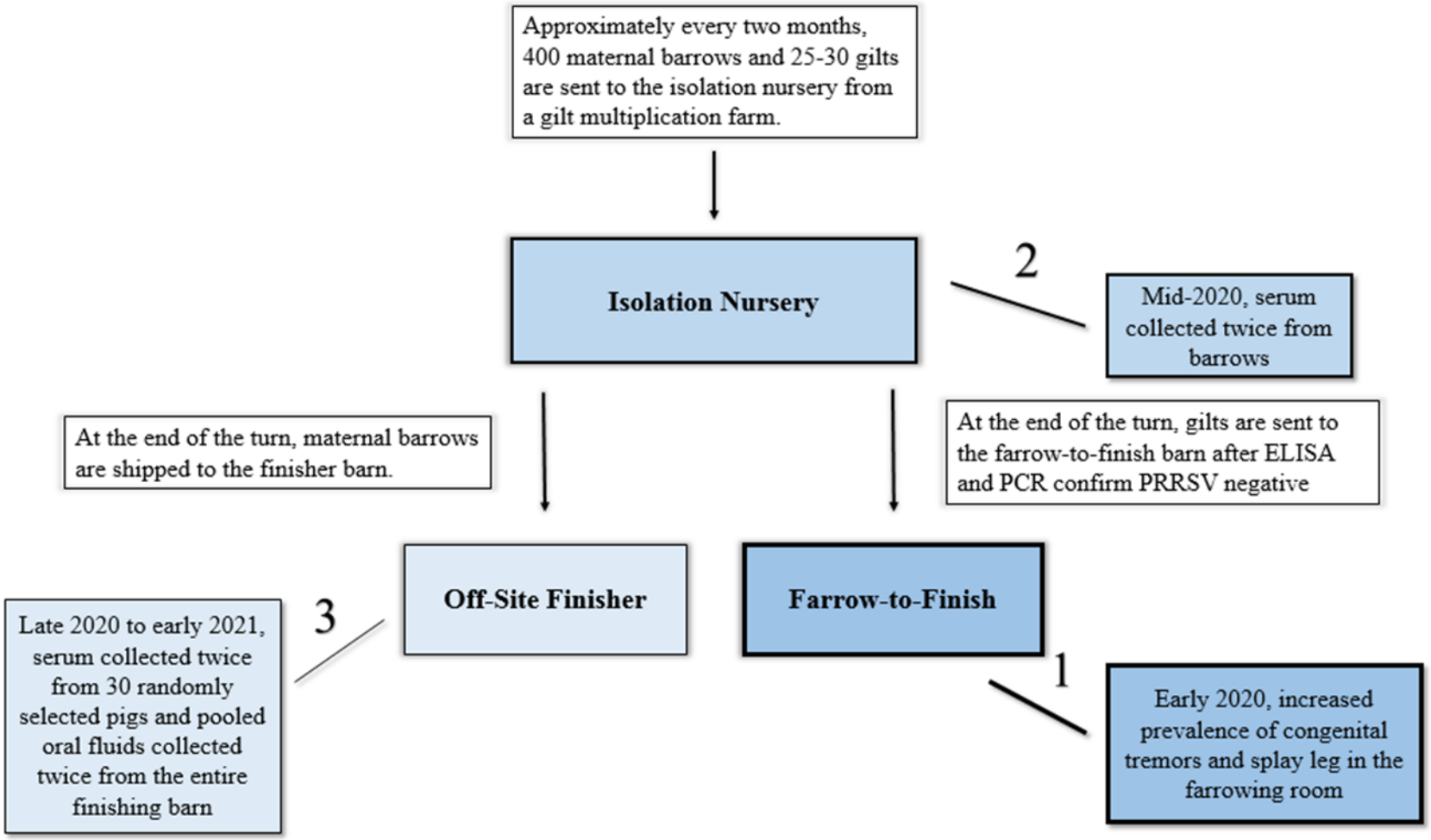

Figure 1.2 Percentage of litters from a farrowing batch group with clinical signs of congenital tremors (CT) or splay leg (SL) then break down of the litters with clinical cases based on parity (females considered a gilt if this was their first litter farrowed, all other females considered sows). Date is designated as month-year.

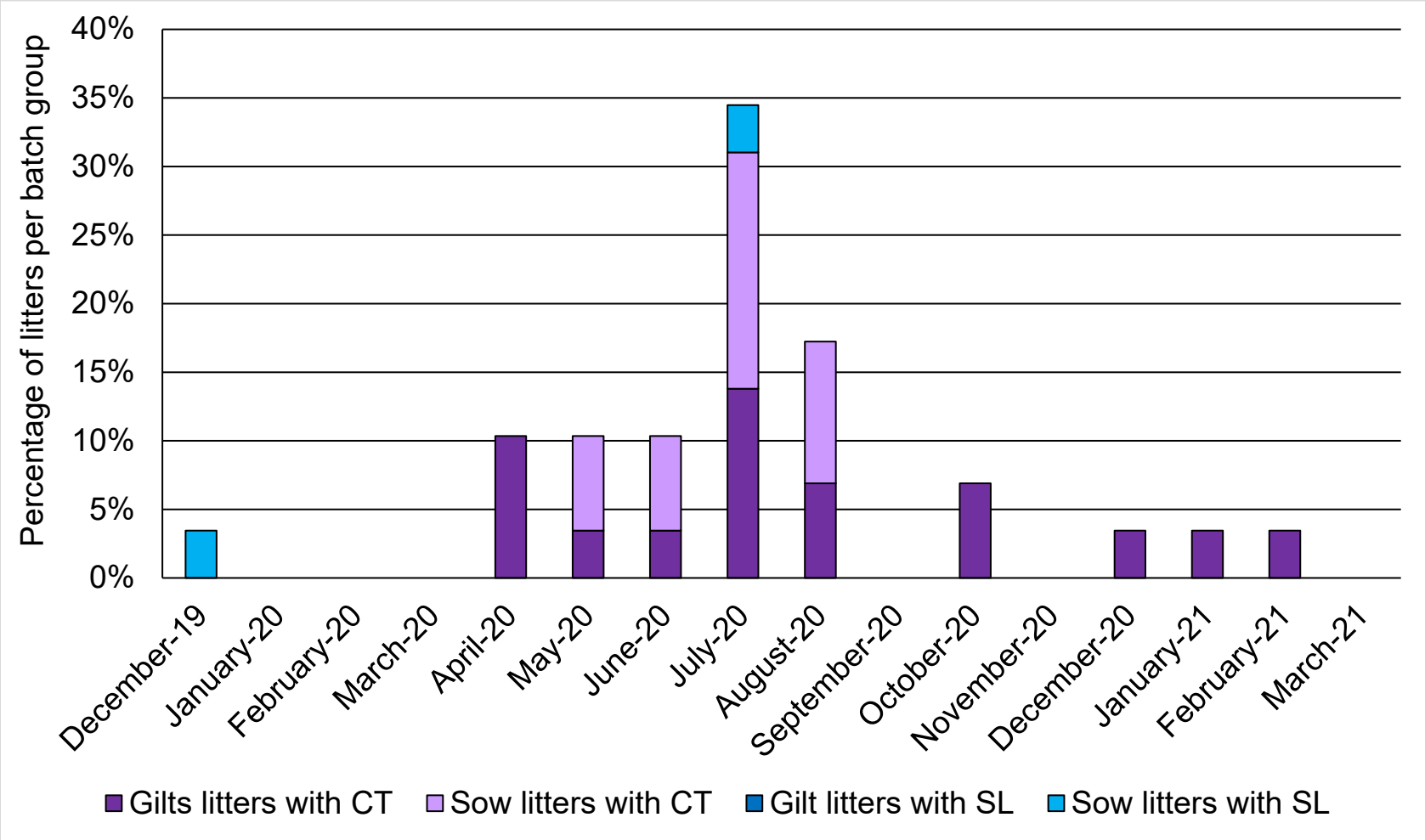

Table 1.1 Amino acid similarities (%) among atypical porcine pestivirus (APPV) E2 regions of 3 newly generated strains in this study.

Strain Name	APPV-semen-dose	APPV-piglet-clinical	APPV-Gilt-Sample
APPV-semen-dose	--	--	--
APPV-piglet-clinical	98.77	--	--
APPV-Gilt-Sample	94.73	95.55	--

Figure 1.3 Maximum likelihood phylogenetic tree generated with 1,000 bootstrap replicates (MEGA-X) for atypical porcine pestivirus E2; evolutionary history was inferred by using the maximum likelihood method and Tamura-Nei model. Sequences from this study, designated with a red diamond, were obtained from a semen dose, serum from a clinically affected piglet, and serum from an incoming gilt. Reference sequences are named by isolate name and Genbank reference number listed after the black asterisk. Frequencies for a branch that are below 70% are not displayed.

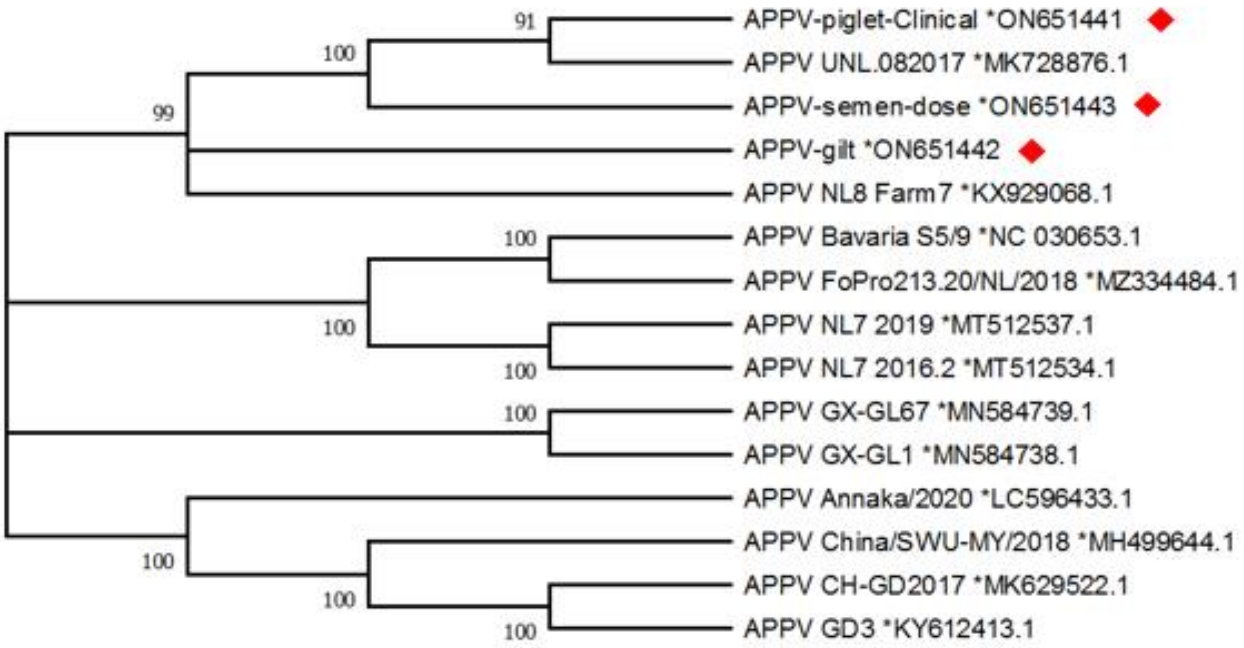

Figure 1.4 Prevalence of Atypical porcine pestivirus (APPV) RNAemia in serum samples pooled by pen in an isolation nursery facility. Numbers within figure illustrate pen location within each of two identical nursery facilities located on the same premise. The diagram on the left (A) is from sampling on 8/20/2020, while the diagram on the right (B) is from sampling on 9/30/2020. Grey indicates a suspected detectable APPV RNA, red pen indicates pooled serum with detectable APPV RNA, and white pen indicates no detectable APPV RNA.

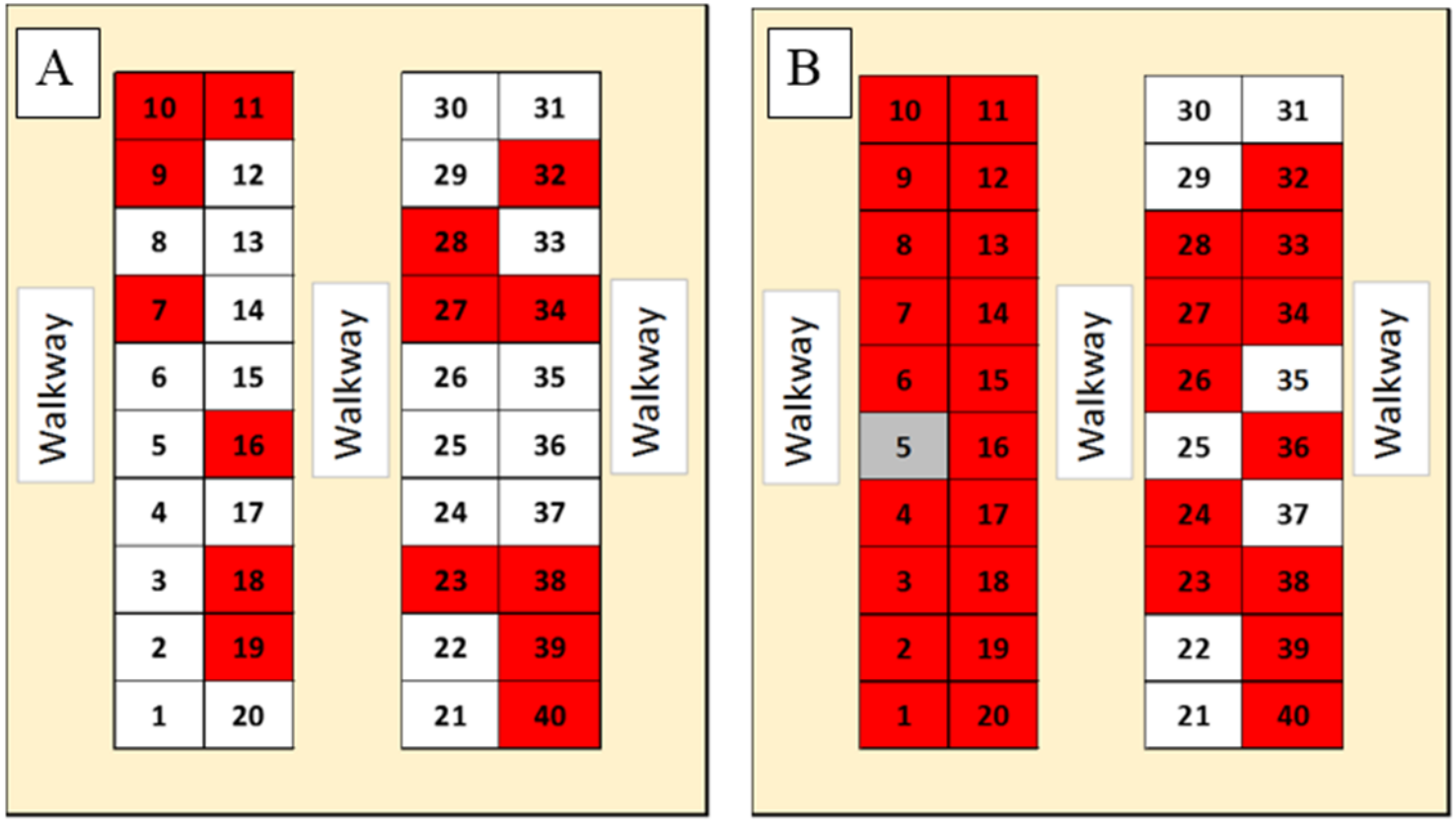

Table 1.2 Detectable atypical porcine pestivirus (APPV) RNAemia status for 4 pens in an isolation nursery barn on two separate sampling dates.

	Sampling day	
Item	8/20/2020	9/30/2020
Pen 11		
Pig 173	Positive - 24.38	-
Pig 188	ND	ND
Pig 47	ND	-
Pig 104	ND	Positive - 26.99
Pig 37	ND	-
Pen 16		
Pig 70	ND	-
Pig 110	Positive - 24.66	-
Pig 36	ND	ND
Pig 54	ND	ND
Pig 137	ND	Positive - 26.32
Pen 28		
Pig 96	ND	Positive - 30.46
Pig 123	ND	Positive - 31.78
Pig 105	ND	-
Pig 168	Positive - 30.08	ND
Pig 29	ND	Positive - 35.33
Pen 38		
Pig 181	ND	Positive - 33.14
Pig 184	ND	ND
Pig 182	ND	ND
Pig 69	Positive - 33.01	-
Pig 12	ND	ND

If the individual pig's results had detectable APPV RNA, the cycle threshold value is given after the hyphen. ND = non-detectable APPV RNA. A dash indicates the individual pig was not present for the second sampling date. Pigs were part of a concurrent research trial and one pig pen from every pen were randomly selected to be euthanized.

Table 1.3 Detectable atypical porcine pestivirus (APPV) RNA for 30 individual pig serum on different sampling dates.

	Sampling day	
Item	12/3/2020	1/16/2021
Serum		
1	Positive – 36.02	ND
2	Positive – 30.89	ND
3	ND	ND
4	ND	ND
5	ND	ND
6	ND	ND
7	ND	ND
8	ND	ND
9	ND	ND
10	Positive – 35.67	ND
11	ND	ND
12	ND	ND
13	ND	ND
14	ND	ND
15	ND	ND
16	ND	ND
17	ND	ND
18	ND	ND
19	ND	ND
20	ND	ND
21	ND	ND
22	Positive – 34.81	ND
23	ND	ND
24	ND	ND
25	Positive – 36.29	ND
26	Positive – 32.19	ND

27	Positive – 37.24	ND
28	Positive – 35.24	ND
29	ND	ND
30	ND	ND
Oral fluid		
1	Positive – 25.62	Positive – 23.36
2	Positive – 21.90	Positive – 25.44
3	Positive – 24.78	Positive – 23.42
4	Positive – 28.06	Positive – 22.06
5	Positive – 25.21	Positive – 28.67
6	Positive – 20.35	Positive – 25.52
7	Positive – 19.80	Positive – 27.66
8	Positive – 20.71	Positive – 24.14
9	Positive – 21.80	Positive – 24.36
10	Positive – 21.68	Positive – 21.40

If the individual pig's results had detectable APPV RNA, the cycle threshold value is given after the hyphen. ND = non-detectable APPV RNA.

Chapter 2 - Understanding the role of feed manufacturing and delivery within a series of porcine deltacoronavirus investigations[2]

Abstract

Two feed mills and three breed-to-wean facilities were investigated after being diagnosed with porcine deltacoronavirus (PDCoV) with initial suspicion that feed manufacture and delivery processes were involved in disease transmission. Both feed mills were audited and environmental samples collected in areas that were deemed high risk for virus contamination. All breed-to-wean facilities had PDCoV detected as would be expected, while the only positive samples for enteric coronaviruses associated with feed mills were feed delivery trucks. These results indicate that feed delivery surfaces can help spread virus during an ongoing disease outbreak and must be considered when determining the outbreak origin.

Keywords: swine, epidemiology, feed safety, porcine deltacoronavirus

Introduction

The swine industry has made advancements in biosecurity practices since the introduction of porcine epidemic diarrhea virus (PEDV) and porcine deltacoronavirus (PDCoV) in 2013 and 2014. Both diseases spread quickly through US swine production systems due to naïve herd status and fomites playing a large role in disseminating these viruses. Both PEDV and PDCoV rely on fecal-oral transmission; therefore, these viruses can be prevented if fecal contamination is limited.[1] The US swine industry quickly applied this concept to our animal

[2] This work was published in *Journal of Swine Health and Production.* Elijah CG, Harrison OL, Blomme AK, Woodworth JC, Jones CK, Paulk CB, Gebhardt JT. Understanding the role of feed manufacturing and delivery within a series of porcine deltacoronavirus investigations. *J Swine Health Prod.* 2022;30(1):17-23. doi:10.54846/jshap/1250

transportation system and how workers and veterinarians enter and exit facilities. Practices adopted during this time, such as truck washing, disinfection, and heat treating or the usage of shoe covers, are now considered normal day-to-day practices for swine production settings.

Within the last decade, feed safety became heavily emphasized once it was hypothesized that a contaminated batch of feed ingredients imported from Asia was responsible for bringing PEDV and PDCoV to the US.[2] Prior to the realization that feed can serve as a vector for virus transmission, feed safety concerns primarily focused on controlling Salmonella, other bacteria, and mycotoxins in feed mills. Since then, scientists have proven that PEDV-contaminated feed can cause clinical disease and once in the feed mill environment, impractical methods such as wet cleaning and disinfection are required to successfully remove PEDV from the feed mill.[3,4] Most feed safety research has focused on PEDV, but this research opened the door to the idea that a feed mill could serve as a transmission source of any virus. Currently, feed safety has a focus on bioexclusion of endemic pathogens as well as prevention of potential foreign animal disease introduction through feed and feed ingredients. The industry has also begun to further understand the epidemiological role the feed delivery supply chain has on feed mills and production sites. Taking what is known about fomites, such as people and trucks, feed safety research is working to understand the interaction between the feed mill and these moving pieces. Therefore, the authors conducted an investigation where multiple isolated facilities were diagnosed with PDCoV. The goals were to 1) understand if the feed mill was the origin of disease, and 2) determine if trucks or people, either coming from the infected farms or coming from the feed mills, served as vectors to spread this virus.

Case description

Herd histories

Three swine breed-to-wean herds, designated as sites A, B, and C, were diagnosed with PDCoV within one week in November 2020, with reports of initial clinical signs in the gestation area of the respective facilities (Figure 1). All 3 sites were located in the Midwestern United States and operate in accordance with Pork Quality Assurance Plus guidelines. All diagnostic samples confirming clinical disease within the production sites were collected under standard veterinary oversight procedures. All environmental swabs were collected from surfaces with no animal contact and environmental sampling personnel did not enter the production facilities. Site A and B were operated by the same production system, whereas site C did not share any management oversight with the other two sites. Workers for site A reported clinical signs of PDCoV in the gestation barn on November 9, 2020 and the diagnosis of PDCoV was confirmed that afternoon via polymerase chain reaction (qPCR) from samples sent to Kansas State University Veterinary Diagnostic Laboratory (KSU VDL). Workers from site B reported clinical signs on November 9, 2020 and with the diagnosis confirmed by Iowa State University Veterinary Diagnostic Laboratory (ISU VDL) on November 11, 2020. Veterinarians from site B instructed workers to collect 1 feed sample from the gestation barn after confirmation of clinical signs of PDCoV. The sample was placed in the freezer, and submitted it to ISU VDL on November 30, 2020. Workers from site C reported 60 animals with scours in the gestation barn on November 11, 2020. Site C receives gilts from sites A and B, but gilts are raised in off-site gilt development units (GDU) and the timeline of animal deliveries did not indicate an epidemiological link between site C and sites A and B. A clinical diagnosis of PDCoV for site C

was confirmed by laboratory evaluation the evening of November 11, 2020. Once PDCoV was diagnosed, all sites conducted controlled oral exposure with infected fecal material.

Feed mill histories

Feed mill 1 supplies site C and 12 to 15 other sow farms and only makes swine diets. Prior to the outbreak on site C, this feed mill monitored high risk areas such as boot soles, foot pedals, reclaim trucks, and office space every week. When clinical signs were first observed in gestation, the company reviewed their diets and determined that wheat middlings was the only ingredient unique to the gestation diet. Environmental samples were collected from all major ingredient bins, as it was believed that samples of accumulated dust would be more representative over a longer period compared to subsamples of feed or feed ingredients. The mill investigated the transport and handling of the wheat middlings and determined that the trucks used for transportation were not used for any other purpose, such as transporting ingredients other than wheat coproducts.

Feed mill 2 supplied feed to sites A and B and also supplied the same gestation feed to three other sites that also were infected with PDCoV but were not part of this investigation. Our investigation was focused on understanding the potential link between feed manufacture and delivery with acute outbreaks, so these additional three sites were excluded from this investigation because a significant amount of time had elapsed since clinical signs were noted at the farms. Feed delivery records reported that feed mill 2 delivered diets to site A and B from November 9-12, 2020, but what type of diet, how much, and what bin diets were delivered to are not recorded. Previous to this investigation, this feed mill had collected and submitted 7 environmental samples to the KSU VDL following initial clinical signs at a farm and suspicion

of a potential link to the feed mill. All 7 samples were free of detectable PDCoV RNA and a link between the feed mill and farm outbreak was not found.

Feed mill and production site investigations

Investigations of the production sites and feed mill locations took place on November 14, 2020; approximately one week after observing clinical signs and confirming clinical diagnosis within production sites. Samples from sites A, B, and C focused on feed contact and nonfeed contact surfaces outside of the barn. Environmental sampling was limited to feed bins of gestation, lactation, and GDU unit barns and areas of high foot traffic or potential for high viral load. No feed samples or environmental samples were collected interior to the entry shower because all sites conducted controlled oral exposure once confirming PDCoV on site, so environmental samples would knowingly test positive for PDCoV. Site A had 12 sampled locations including feed bins, entry benches, and barn exhaust fans. Site B had 22 sampled locations including feed bins, spilled feed under feed bins, and areas of high foot traffic like barn entries, visitor log sign in, and areas around the crossover benches before the entry shower. Site C had 13 sampled locations including feed bins, netting surrounding exhaust fans near feed bins, and fan exhaust shrouds. Feed mill sampling locations included high-risk ingredients like porcine derived ingredients, areas of high foot or vehicle traffic (receiving and load out bay and warehouse floor), feed trucks going from farm to feed mill, and bulk feed bins. Feed delivery surfaces were those within the feed delivery trucks including dashboards, foot mats, truck steps, and driver seats. Feed mill 1 had 42 samples and feed mill 2 had 44 samples.

In addition to sampling the feed mills, audits were conducted using the Kansas State University Swine Feed Mill Biosecurity Audit template (https://www.asi.k-state.edu/research-and-extension/swine/biosecurity%20audit.doc). The audit evaluated the biosecurity practices

within the feed mill and the feed delivery system and was completed by one member of the research team by systematically proceeding through the audit document. Feed mill 1 was well kept and clean. Employees had a good understanding of biosecurity and good feed mill practices. Feed delivery trucks were required one night down time between sites and washed once deliveries were finished. However, to prepare for the upcoming holiday season, the warehouse was more crowded than normal resulting in occasional spillage and bag ripping. If spillage occurred, these ingredients are swept up and discarded in the garbage. Feed mill 2 was generally clean and well kept; the receiving pit was covered, warehouse was swept and well maintained, and the mill only manufactured swine diets. When talking with the feed delivery driver, washing trucks and sanitizing wheels and wheel wells were done as biosecurity practices when delivering to various phases of swine production systems. However, there was a porcine-based ingredient on location (choice-white grease) and this facility only had one mixer so all diets went through the same equipment. Truck drivers were allowed to walk through the warehouse without shoe covers and feed trucks were allowed to haul diet ingredients and complete diets in the same trailer. Both the choice-white grease and no clear standard operating procedures (SOPs) for truck drivers had the potential to introduce PDCoV, PEDV, or other diseases within the feed mill and unintentionally contaminate other production sites and animals.

Environmental sampling was performed using one of two methods depending upon accessibility of sampling locations. The first method utilized a premoistened 10-cm square cotton gauze surgical sponge as previously described.[5] This method was utilized when sample areas were easily accessible and the selected area could be swabbed by hand. The second method utilized premoistened paint roller covers (Marathon 22.9 cm × 0.95 cm nylon/polyester paint roller cover, Purdy North America) and a paint roller extension set (152 cm fiberglass paint roller

frame utility pole, Mr. LongArm, Inc) as previously described.[3] The second method was used when sampling was particularly challenging, for example, inside of feed bins. Samples were placed on ice and transported back to Manhattan, Kansas. Before submitting to the lab, surgical gauze environmental swabs had 20 mL of phosphate buffered solution (PBS) added to the conical tube and manually agitated while paint rollers were squeezed inside the transportation plastic bag (Ziploc one-gallon size freezer bags; S.C. Johnson & Son, Inc) and the liquid was poured into a conical tube. If 20 mL could not be extracted from the roller, approximately 20 mL of PBS was added onto the roller and wrung out a second time. Samples were stored at -20°C until shipped to the ISU VDL. All samples were processed at ISU VDL for triplex qPCR for PEDV, PDCoV, and transmissible gastroenteritis virus (TGEV). Extractions from all samples were amplified using two amplification procedures. One amplification sequence used the standard ISU VDL cycle threshold (Ct) cutoff value of 36, and retained sample extractions were amplified using a Ct cutoff value of 45.

For the first round of qPCR analysis, 17 of 133 samples (12.8%) had detectable PEDV or PDCoV RNA with a Ct cutoff value of 36 (Table 1). Site A had 4 environmental swabs with detectable PDCoV RNA taken from the fans outside the gestation and farrowing barns and on the clean and dirty side of the entrance bench (Table 2). Site B had 6 environmental swabs with detectable PDCoV RNA taken from a feed bin outside the GDU, spilled feed outside the bin, footpath to the barn entrance, beneath shoes on the entrance floor, clean side of the entrance bench, and outside the barn entrance. Site C had 5 environmental swabs with detectable PDCoV RNA taken from exhaust fan netting around 4 different feed bins and a gestation barn fan shroud. Feed mill 2 had 2 environmental swabs with detectable PEDV RNA taken from the feed truck

pedals and floor and feed truck steering wheel and dashboard. Feed mill 1 had no samples with detectable PEDV, PDCoV, or TGEV RNA.

For the second round of qPCR analysis, 3026 of 133 samples (22.519.5%) had detectable PEDV or PDCoV RNA with a Ct cutoff value of 45. Site A had no additional environmental swabs with detectable PDCoV RNA. Site B had 9 additional environmental swabs with detectable PDCoV RNA taken from 4 GDU feed bins, spilled feed by another GDU bin, spilled feed under a lactation feed bin, nursery piglet feed bin, and the floor by the visitor entry and showers. Site C had 2 additional environmental swabs with detectable PDCoV RNA taken from 2 more gestation bin fan shrouds. Feed mill 1 had 2 environmental swabs with detectable PDCoV RNA taken from the feed truck steps and inside the feed truck cab. Feed mill 2 had no additional environmental swabs with detectable PEDV RNA. The site B feed sample submitted on November 30, 2020 was confirmed nondetectable for PEDV, TGEV, and PDCoV on December 2, 2020 at both cutoff values.

Discussion

For this investigation, nonfeed contact surfaces were the majority of surfaces contaminated with PDCoV and PEDV. Since sites A, B, and C conducted controlled oral exposure once clinical signs appeared, PDCoV quickly dispersed through the environment and could be found on all surfaces including exhaust fans, exhaust fanfeed bin netting, and fan shrouds. Research done with PEDV has found that once introduced, nucleic acids for the virus can be found throughout the environment.[6] Investigations like this should take into account whether locations have used controlled oral exposure as a disease management strategy because environmental sampling will be of lesser value due to the nature of controlled oral exposure. Interestingly, the only surfaces associated with the feed mill that had detectable RNA for porcine

enteric viruses were from the feed delivery system. These surfaces are freely movable, or transient in nature, and able to travel from one farm to the next which is probably how these surfaces became contaminated with virus. Others have found that surfaces associated with the feed supply chain contributed to the spread of African swine fever virus (ASFV) while feed contact surfaces were negative for ASFV.[7] Another study found that contaminated personal protective equipment and people can contribute to the spread of PEDV.[8] These findings highlight the importance of preventing pathogen introduction into the feed mill and the feed in order to eliminate potential transmission. An important, but not unexpected, takeaway message from the current investigation was that contamination with PDCoV can be found outside of clinically affected farms and that this contamination can be detected in high traffic areas for personnel and trucks. This highlights the need to implement or revisit biosecurity protocols for employees and truck drivers. While these protocols may be labor or cost intensive, it is pivotal that all people and vehicles moving in and out of the supply chain understand the importance of following and maintaining good biosecurity to control the spread of disease.

Another finding of this investigation is that neither feed mill had detectable quantities of enteric coronaviruses in environmental samples. When conducting disease outbreak investigations, particularly those incorporating environmental sampling, collection of appropriate samples in a timely manner is critical to allow for the greatest epidemiological value. Sample collection in the current investigation took place within 48 hours of notification of the desire to conduct sampling by the involved parties. When using environmental sampling to aid in a diagnostic investigation, the sooner the samples can be collected the lower likelihood of secondary epidemiological links causing confounding. A list of sampling locations was generated based on previous feed investigation experience to maximize the likelihood of

detecting contamination if present. In this investigation, authors felt our response was timely to collect meaningful diagnostic information. When conducting investigations such as the one described in this manuscript, it is very important that personnel collecting samples are appropriately trained and collect samples in an aseptic manner.

Even though no swine enteric viruses were detected in either feed mill, there are multiple preventative strategies both feed mills could implement to mitigate the risk of feed delivery trucks potentially serving as vectors for disease that should remain out of the feed mill. Feed mitigants, like commercially available formaldehyde or medium chain fatty acids, can be expensive but reduce viral contamination in the feed.[9,10] Another solution to help reduce introduction of pathogens into a mill would be to implement truck and visitor SOPs to improve biosecurity within the feed mill. These moving pieces within the feed mill will always be present, but additional training will help to reduce the likelihood of introducing a health hazard into the feed mill.[11] During this investigation, authors would have liked more detailed record keeping and hence recommend all feed deliveries to have detailed records. Feed delivery records were obtained from feed mill 2 to further investigate the presence of PDCoV inside the feed bins at site B but there were not sufficient details within the records to make a definitive link between the feed and outbreak of PDCoV. The records showed supply date and trip location but did not provide details on type of diet transported or what bin was filled. Since there were not enough details present in the delivery records, a link between the PDCoV outbreak and presence of PDCoV RNA in the feed bin can only be speculated. The records did show that feed was unloaded into the bins during a time when PDCoV was intentionally spread through a farm. It is possible these bins were in front of exhaust fans and the bins were unintentionally contaminated with PDCoV from exhaust air. Because the feed sample and feed mill surfaces from feed mill 2

had no detectable RNA for PEDV, PDCoV, or TGEV, a link could not be made between the feed mill and PDCoV farm outbreak. Had there been more information available from the feed records, a possible link between the outbreak and feed mill could have been identified.

Lastly, site B had the largest portion of environmental samples testing positive for PDCoV using a Ct value of 36 and 45. When the Ct cutoff was 36, only 6 of 22 samples were positive but 9 additional samples were positive when the Ct cutoff value was increased to 45. The laboratory performing the analysis, matrix of the sample, and viral load of the sample must all be considered when interpreting diagnostic sample results.[12] There are differences between diagnostic laboratories regarding primers and threshold limit values. Current molecular based diagnostic techniques are not validated for environmental swabs or feed/ingredient samples and consequently care has to be taken when interpreting diagnostic results. In this investigation, using a Ct limit of 45 cycles resulted in a greater number of positive samples. Given where these samples were collected, it was logical there would be virus present, albeit at a low level. Thus, increasing the Ct limit from 36 to 45 within this investigation likely increased the sensitivity of detecting environmental contamination with PDCoV. While increasing the Ct cutoff value to 45 increased the sensitivity of the test results, this practice also may increase the rate of false-positive results. The purpose of this investigation was to identify areas of contamination and make biosecurity recommendations based on results. When interpreted appropriately, having a greater diagnostic sensitivity can help identify areas of concern and the consequences of false positives are outweighed by the value of increased sensitivity in this situation. Individuals must be cautious when interpreting results near the limit of detection for diagnostic assays, but if used appropriately, increasing the Ct limit as demonstrated in the current report can add value to diagnostic investigations using environmental swabs and feed/ingredient matrices.

To further understand the possible connection between the farms with clinical disease, genetic comparison of viruses through sequencing could be a useful tool. However, this was not possible in the current investigation. Additionally, a limitation of the qPCR assay used in the current experiment is that no information is provided regarding the ability for the identified genetic material to be infectious. The assay simply detects a specific sequence of RNA and provides no information regarding potential infectivity. Additional work is necessary to further understand the infectivity characteristics of environmental swabs in diagnostic investigations, but when results are interpreted appropriately qPCR can serve as a rapid, cost-effective diagnostic tool that can provide useful information.

In conclusion, this diagnostic investigation did not find evidence within the feed supply chain indicating feed or feed delivery was associated with outbreaks of PDCoV. Due to the nature of timing, it is believed that the contamination identified at the infected sites was due to the intentional exposure through controlled oral exposure. Furthermore, it is not known what the specific mechanism of transmission was to these farms, although other routes must be considered such as personnel and other possible fomites such as incoming supplies. The goal of this investigation was to evaluate the likelihood of a link between feed manufacturing and delivery with the outbreak of clinical disease, so greater investigation into potential routes of entry were not explored. This investigation highlights the importance of biosecurity during controlled oral exposure because viral contamination can be detected outside of the farm perimeter and common events such as feed delivery may serve as a mechanism for transfer of viral contamination back to the feed mill or to other farms. The current investigation emphasizes the importance of biosecurity in the feed supply chain at both the feed manufacturing and delivery stages, with particular focus needing to be directed towards personnel movement.

Implications

Under the conditions of this report:

- People and transportation vehicles can serve as fomites for pathogens.
- No evidence of contamination within the feed mills for PDCoV was detected.

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Figure 2.1 Timeline of events for feed mill investigation. Sites A, B, and C are three breed-wean facilities located in the Midwest. PDCoV = porcine deltacoronavirus; KSU VDL = Kansas State University Veterinary Diagnostic Laboratory; ISU VDL = Iowa State University Veterinary Diagnostic Laboratory; PEDV = porcine epidemic diarrhea virus; TGEV = transmissible gastroenteritis virus.

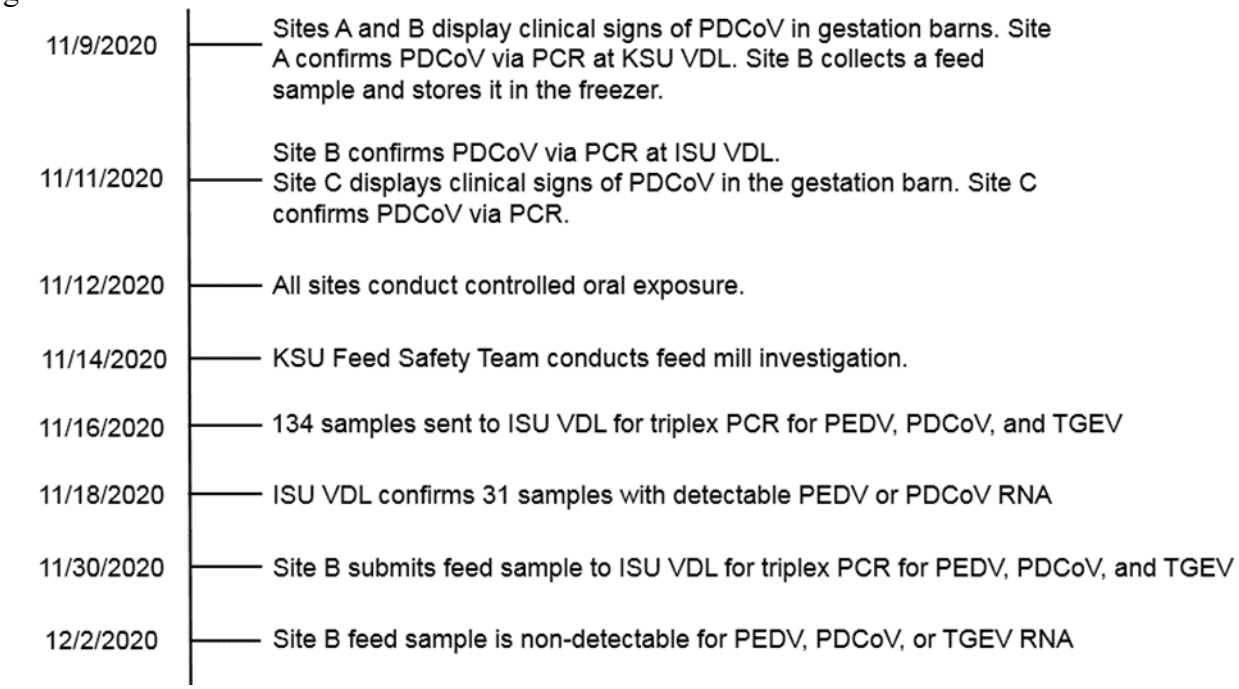

Table 2.1 Number of environmental swabs positive for viral RNA collected from live animal production sites and feed mills

		qPCR Ct limit					
		PDCoV		PEDV		TGEV	
Location	Zone	36	45	36	45	36	45
Site A	Feed bin - feed contact (n = 8)	0	0	0	0	0	0
	Surfaces exterior facility (n = 2)	2	2	0	0	0	0
	Personnel entry (n = 2)	2	2	0	0	0	0
Site B	Feed bin - feed contact (n =13)	1	6	0	0	0	0
	Feed sample (n = 1)	0	0	0	0	0	0
	Feed spills exterior facility (n = 3)	1	3	0	0	0	0
	Personnel entry (n = 6)	4	6	0	0	0	0
Site C	Feed bin - feed contact (n = 6)	0	0	0	0	0	0
	Surfaces exterior facility (n = 7)	5	7	0	0	0	0
Mill 1	Feed contact surface (n = 26)	0	0	0	0	0	0
	Non-feed contact surface (n = 10)	0	0	0	0	0	0
	Transient surface (n = 6)	0	2	0	0	0	0
Mill 2	Feed contact surface (n = 29)	0	0	0	0	0	0
	Non-feed contact surface (n = 8)	0	0	0	0	0	0
	Transient surface (n =7)	0	0	2	2	0	0

qPCR = polymerase chain reaction; Ct = cycle threshold; PDCoV = porcine deltacoronavirus; PEDV = porcine epidemic diarrhea virus; TGEV = transmissible gastroenteritis virus.

Table 2.2 Summary of qPCR Ct values for positive samples from live animal production sites and feed mills

		qPCR Ct limit					
		PDCoV		PEDV		TGEV	
Site	Sampling location	36	45	36	45	36	45
A	Farrowing exhaust fan	31.7	31.1	ND	ND	ND	ND
	Gestation exhaust fan	29.3	28.6	ND	ND	ND	ND
	Dirty side of entrance bench	29.5	29.1	ND	ND	ND	ND
	Clean side of entrance bench	35.5	36.0	ND	ND	ND	ND
B	GDU Bin 1	ND	38.8	ND	ND	ND	ND
	Spilled feed under GDU bins	35.7	36.2	ND	ND	ND	ND
	GDU Bin 2	33.0	32.6	ND	ND	ND	ND
	GDU Bin 3	ND	38.0	ND	ND	ND	ND
	GDU Bin 4	ND	36.9	ND	ND	ND	ND
	GDU Bin 5	ND	37.8	ND	ND	ND	ND
	Spilled feed under gestation bins	ND	38.7	ND	ND	ND	ND
	Spilled feed under lactation bins	ND	38.9	ND	ND	ND	ND
	Nursery holding room feed bin	ND	36.4	ND	ND	ND	ND
	Foot path exterior to facility	33.4	33.0	ND	ND	ND	ND
	Beneath shoe on floor	29.1	28.7	ND	ND	ND	ND
	Clean side of bench	35.2	34.7	ND	ND	ND	ND
	Floor by visitor log	ND	39.1	ND	ND	ND	ND
	Floor by showers	ND	39.0	ND	ND	ND	ND
	Outside near entry door	30.5	30.3	ND	ND	ND	ND
C	Netting by gestation bin 1	34.7	34.3	ND	ND	ND	ND
	Netting by gestation bin 2	30.9	30.2	ND	ND	ND	ND
	Netting by gestation bin 3	32.0	31.5	ND	ND	ND	ND
	Netting by gestation bin 4	34.7	33.6	ND	ND	ND	ND
	Fan shroud 1	ND	37.5	ND	ND	ND	ND
	Fan shroud 2	29.9	29.3	ND	ND	ND	ND
	Fan shroud 3	ND	35.7	ND	ND	ND	ND
Mill 1	Feed truck - steps	ND	37.3	ND	ND	ND	ND
	Feed truck - steering wheel, pedals, floor mat	ND	37.1	ND	ND	ND	ND
Mill 2	Feed truck - floor and pedals	ND	ND	33.4	33.2	ND	ND
	Feed truck - steering wheel and dashboard	ND	ND	35.6	35.0	ND	ND

qPCR = polymerase chain reaction; Ct = cycle threshold; PDCoV = porcine deltacoronavirus; PEDV = porcine epidemic diarrhea virus; TGEV = transmissible gastroenteritis virus; ND = no genetic material detected.

Chapter 3 - Evaluation of truck cab decontamination procedures following inoculation with porcine epidemic diarrhea virus and porcine reproductive and respiratory syndrome virus

Abstract

This experiment aimed to evaluate commercially available disinfectants and their application methods against porcine epidemic diarrhea virus (PEDV) and porcine reproductive and respiratory syndrome virus (PRRSV) on truck cab surfaces. Plastic, fabric, and rubber surfaces inoculated with PEDV or PRRSV were placed in a full-scale truck cab and treated with randomly assigned disinfectants. Treatments were: 1) no disinfectant; 1:256 dilution of Synergize (Neogen Corp, Lexington, KY) applied through 2) misting fumigation (Hurricane Ultra II Portable Electric Fogger, Curtis Dyna-Fog Ltd., Westfield, IN) or 3) pump sprayer (Chapin Sure Spray 1 Gallon Tank Sprayer, Menards, Eau Claire, WI); 1:64 dilution of Intervention (Virox, Oakville, ON) applied through 4) misting fumigation or 5) pump sprayer; 6) 10% bleach (7.55% sodium hypochlorite germicidal bleach; Clorox, Oakland, CA) solution applied through pump sprayer; 7) no chemical treatment for 10 hr; or 8) gaseous fumigation over 10 hr with chlorine dioxide (ProKure G; ProKure Solutions, Phoenix, AZ). After application, surfaces were environmentally sampled with cotton gauze and submitted for PEDV and PRRSV qPCR duplex analysis. There was a disinfectant × surface interaction ($P < 0.0001$), indicating a detectable amount of PEDV or PRRSV RNA was impacted by disinfectant treatment and surface material. For rubber surfaces, 10% bleach application had lower detectable amounts of RNA compared to all other treatments ($P < 0.05$) except Intervention via misting fumigation, which was intermediate. In both fabric and plastic surfaces, there was no evidence ($P > 0.05$) of a

difference in detectable RNA between disinfectant treatments. For disinfectant treatments, fabric surfaces with no chemical treatment had less detectable viral RNA compared to the corresponding plastic and rubber ($P < 0.05$);Intervention via pump sprayer applied to fabric surfaces had less detectable viral RNA than plastic ($P < 0.05$); 10% bleach via pump sprayer applied to fabric and rubber surfaces had less detectable viral RNA than plastic ($P < 0.05$); 10 hr downtime with no chemical application or gaseous fumigation for 10 hr applied to fabric surfaces had less detectable viral RNA than other surfaces ($P < 0.05$). Sixteen treatments were evaluated via swine bioassay, but all samples failed to produce infectivity. Applying 10% bleach via pump sprayer had the greatest number of PCR negative samples (9/18) compared to other disinfectant treatments (ranging from 0-2 negative samples), indicating it might be the best disinfectant application if wanting to completely eliminate viral RNA from surfaces. In summary, commercially available disinfectants successfully reduced detectable viral RNA on surfaces but did not eliminate viral genetic material, highlighting the importance of bioexclusion of pathogens of interest.

Keywords: disinfectants, porcine epidemic diarrhea virus, porcine reproductive and respiratory syndrome virus, truck cabs, swine

Lay summary

There is evidence to suggest that truck cabs within swine production systems can contain detectable viral RNA like porcine epidemic diarrhea virus (PEDV) and porcine reproductive and respiratory syndrome virus (PRRSV). Therefore, this study evaluated commercially available disinfectants and application methods to determine their ability to reduce virus within truck cabs. Plastic, fabric, and rubber surfaces were inoculated with PEDV or PRRSV, placed in a full-scale truck cab, and treated with a randomly assigned order of disinfectant treatments. After

application, surfaces were swabbed with cotton gauze and submitted for analysis to determine the amount of PEDV or PRRSV RNA on surfaces after disinfectant treatment. Results from this study found that disinfectant treatment and the surface material impacted detectable amounts of virus within samples. All disinfectant treatments were able to reduce the amount of detectable viral RNA on surfaces but did not get rid of the virus entirely on the surfaces. These findings highlight that if concerned about pathogen transmission within truck cabs, the best practice would be to exclude virus from the cab altogether.

Teaser text

There is evidence to suggest that cabs of transportation vehicles can be fomites for viral transmission to swine production sites. This research sought to evaluate commercially available disinfectants on the reduction of viral RNA and found that disinfectants reduce the amount of viral genetic material but do not eliminate viral genetic material from surfaces.

Introduction

Research suggests that transportation vehicles can play a role in the transmission of bacterial (Fedorka-Cray et al., 1997) and viral pathogens (Dee et al., 2004c; Lowe et al., 2014; Elijah et al., 2022; Gebhardt et al., 2022). Cleaning and disinfecting metal surfaces has been shown to reduce detectable porcine reproductive and respiratory syndrome virus (PRRSV) and porcine epidemic diarrhea virus (PEDV) RNA (Dee et al., 2004a,b; Dee et al., 2005; Baker et al., 2017; Hotlkamp et al., 2017; Baker et al., 2018). These methods were so successful at reducing detectable viral RNA that power washing, disinfecting, and heating semi-truck trailers responsible for live animal transportation is commonplace in the current United States swine industry. However, when considering the role of feed delivery in pathogen introduction, the likelihood of detecting PEDV or porcine deltacoronavirus RNA was highest within the truck cab of the feed delivery truck as opposed to the trailer (Greiner, 2016). A challenge associated with the cab of feed delivery trucks is that multiple surface types are present within a single space. When considering the efficacy of disinfectants and surface types, previous work has found that surface type can influence the detection of viral RNA after disinfectant treatment (Muckey et al., 2021). However, most disinfection protocols are developed by extrapolations from laboratory settings or are otherwise lacking in applying lab bench data to real-world settings. Therefore, the objective of this study was to evaluate commercially available disinfectants, and application methods on different surfaces present within truck cabs for efficacy in reducing detectable PEDV and PRRSV RNA and subsequence infectivity of surface environmental samples.

Materials and methods

The surface inoculation and sample collection portion of this study were conducted at the Cargill Feed Science Research Center (FSRC) at the O.H. Kruse Feed Mill of Kansas State

University (KSU) in Manhattan, KS, with approval by the KSU Institutional Biosafety Committee (Project Approval #1511). The inoculation of surfaces was done within a biosafety cabinet (BSC) housed within the BSL-2 space of the FSRC. This study was set up in an 8 × 3 × 2 factorial with eight disinfectant methods, three different surfaces, and two viruses.

Preparation of inoculum

To make the viral inoculation for this study, 25 mL of PEDV (USA/Co/2013 isolate with a titer of 1.33 × 106 TCID50/mL; GenBank accession number: KF272920) and 25 mL of PRRSV (1-7-4 isolate with a titer of 1.33×10^6 $TCID_{50}$/mL) was placed into separate containers. In each container, 225 mL of phosphate buffered saline (PBS) was added to achieve a final concentration of 10^5 $TCID_{50}$/mL. Each virus was divided into five containers containing approximately 45 mL, sealed and stored at -80°C for two weeks. When virus was needed for the study, containers of each virus were defrosted in lukewarm water bath (approximately 36°C to 40°C) and used immediately. Inoculum samples were taken during the study to evaluate Ct values over time. When evaluating the Ct values of pure virus stock, the PRRSV stock was always one to two Ct values higher (Ct values ranging from 23.3 – 24.0) than the virus stock of PEDV (Ct values ranging from 22.1 – 22.3).

Preparation of surfaces and disinfectant

Plastic (0.32 × 10.16 × 20.32 cm white high-density polyethylene panel; Menards, Eau Claire, WI), rubber (Heavy-Duty 45.72 × 45.72 × 0.64 cm Rubber Gym Tiles; BCG, Boston, MA), and fabric surfaces (upholstery fabric; Joann's Fabrics, Hudson, OH) were cut into 10 cm × 10 cm squares for the creation of surface coupons. Velcro strips were applied to the back of the surface coupons prior to inoculation, and surface coupons were placed into a transportation container (Promoze Food Storage Containers, Seattle, WA) and remained in these storage

containers until ready for placement into the truck cabs. Three truck cabs of similar make and model were sourced from a local salvage yard (Beloit, KS). The truck cabs were removed from their frame at the salvage yard, transported to the FSRC for the study, and placed on wheels for easy mobility. Truck cabs were visually and physically inspected for any holes or inadequate seals and sealed as necessary using either silicone sealant (DAP; Menards, Eau Claire, WI) or super glue (Gorilla Glue; Gorilla Glue Co, Sharonville, OH). At the start of the study, truck cabs were wheeled into the BSL-2 space, wheels locked, and remained in the same location for the duration of the study. Each truck had eight disinfectant treatments applied in random order and there were three replications of each disinfectant treatment. Disinfectant treatments include: 1) no disinfectant; 2) 1:256 dilution of Synergize (Neogen Corp, Lexington, KY) applied through misting fumigation (Hurricane Ultra II Portable Electric Fogger, Curtis Dyna-Fog Ltd., Westfield, IN); 3) 1:256 dilution of Synergize applied through pump sprayer (Chapin Sure Spray 1 Gallon Tank Sprayer, Menards, Eau Claire, WI); 4) 1:64 dilution of Intervention (Virox, Oakville, ON) applied through misting fumigation; 5) 1:64 dilution of Intervention applied through pump sprayer; 6) 10% bleach (7.55% sodium hypochlorite germicidal bleach; Clorox, Oakland, CA) solution applied through pump sprayer; 7) no chemical treatment for 10 hr; or 8) gaseous fumigation over 10 hr with chlorine dioxide (ProKure G; ProKure Solutions, Phoenix, AZ).

The designated concentration of wet disinfectants was prepared daily. Once prepared, solutions were poured into their respective application method tool: pump sprayer or misting fumigation. The water-based chlorine dioxide pouch was utilized following manufacturer-labeled instructions for the gaseous fumigation application.

Surface inoculation and disinfectant application

Surfaces were inoculated with either 1 mL of PRRSV (titer of 10^5 $TCID_{50}$/mL) or PEDV (titer of 10^5 $TCID_{50}$/mL). Surfaces were allowed to dry for 1 hr prior to placement within an individual truck cab. When surfaces were ready for placement, surface coupons were placed within the truck cab with nitrile gloves changed in-between new surface coupons. Coupon placement was predetermined prior to the start of the study to ensure consistent placement of surface coupons with each treatment and corresponded to the location within truck cabs from which the surfaces would naturally be located. Plastic coupon surfaces were placed on the dashboard, rubber surface coupons were placed on the floorboard, and fabric surface coupons were placed on the driver's seat.

After surfaces were placed in the truck cab, a randomly assigned disinfectant treatment application was conducted. For the pump sprayer application, the applicator was positioned outside the truck cab on the driver's side, and the liquid was applied in a snake-like application going from the front to the back of the cab resulting in 50-90 g of disinfectant solution being used per treatment application. For the misting fogger application, the head of the fogger was angled and secured at 90° (parallel with the ground), placed in the passenger side seat, and aimed for the driver side of the truck cab. Once set in location, the flow rate was set to 2, turned on, the passenger door was closed, and the fogger was allowed to run for 5 min. The amount of disinfection used for this application ranged from 220-340 g. Once the application of the pump sprayer and misting fogger was completed, wet application methods were allowed to dry for 15 min prior to environmental sample collection.

For the gaseous fumigation treatment, the plastic container was placed in the passenger's seat, doors were closed, and fumigation was allowed to occur for 10 hr after which the driver and

passenger doors were opened, and the truck cab was allowed to dissipate for 1 hr before environmental samples were collected. For the no-chemical treatment and 10 hr downtime treatment, surface coupons were inoculated with virus, placed in truck cabs as previously mentioned, and remained in the truck cab for approximately 15 min or 10 hr, respectively, before sampling.

Environment sampling

Environmental samples were collected with cotton gauze as previously described (Elijah et al., 2021). Briefly, a 10 cm × 10 cm cotton gauze, pre-moistened with 5 mL PBS, was utilized to swab the surface coupon after treatment application. Environmental samples were transferred to the BSC, 20 mL of PBS added to them, inverted for 5-10 sec, and allowed to sit at room temperature for 1 hr. The samples were then vortexed for 15 sec, and the supernatant was transferred into 1.75 mL cryovials and 15 mL conical tubes. Samples were stored at -80°C until PCR analysis. Once all samples were collected, surface coupons were discarded, and cabs were cleaned, sprayed with 1:256 glutaraldehyde, and allowed to dry for 20 min to prevent virus accumulation within the truck cabs.

Reverse transcription real-time PCR analysis

Reverse transcription real time PCR (qRT-PCR) was conducted at the Molecular Research and Development Laboratory within the Kansas State Veterinary Diagnostic Laboratory. Fifty µL of supernatant from each sample was loaded into a deep-well plate and extracted using a Kingfisher Flex magnetic particle processor (Fisher Scientific, Pittsburg, PA) and the MagMAX-96 Viral RNA Isolation kit (Life Technologies, Grand Island, NY) according to manufacturer's instructions with one modification, reducing the final elution volume to 60 µL. One negative extraction control consisting of all reagents except the sample was included in each

extraction. Positive controls of each stock virus were also included with each extraction. Extracted RNA was frozen at -80°C until assayed by qRT-PCR. Analyzed values represent cycle threshold (Ct) at which virus was detected. If a sample had no detectable PRRSV or PEDV RNA, a sample was assigned a value of 45 as a total of 45 cycles were run for each sample. For result interpretation, greater Ct values indicate less detectable viral RNA.

Bioassay analysis

A total of 16 treatments were selected for pig bioassay to assess the infectivity of virus present in them (Table 1). The bioassay procedure was conducted 13 months after surface inoculation and environmental sample collection. The project was approved through Iowa State University's institutional animal care and use committee (Project #IACUC-22-082) and institutional biosafety committee (Project #22-045). A total of 48 crossbred, 10-day-old pigs of mixed sex were sourced from a single commercial, crossbred farrow-to-wean herd with no prior exposure to PEDV and PRRSV. The Iowa State University Veterinary Diagnostic Laboratory confirmed all pigs negative for PRRSV by blood samples and PEDV, porcine deltacoronavirus, and transmissible gastroenteritis virus by fecal swabs via the respective real-time RT-PCR. Blood serum testing further confirmed that pigs were negative for PRRSV antibody by nucleocapsid protein-based ELISA and negative for PEDV antibody by an indirect fluorescent antibody assay and ELISA, respectively. Pigs were allowed 3 days of acclimation prior to beginning the bioassay. Three pigs were housed in a room, and each pig was challenged via oral gavage of PEDV inoculum, intramuscular (IM) injection, and intranasal application of PRRSV. The oral gavage method was modeled similarly to Thomas et al. (2015) utilizing a 10 French feed tube and 60 mL syringe (10 mL/pig). For the PRRSV inoculum, 3 mL was given in the muscle of the cervical region. For PRRSV intranasal inoculation, the tip of a 3 mL Luer slip

syringe was inserted inside one of the pig's nostrils, and 1 ml of inoculum was allowed to drip directly into the pig's nostril. Rectal swabs were collected on days -3, 0, 4, and 7 post inoculation (dpi) from all pigs and tested for PEDV RNA by qRT-PCR. Serum samples were collected on -3, 0, 4, and 7 dpi from all pigs and tested for PRRSV RNA by qRT-PCR. Following humane euthanasia at 7 dpi, small intestine, cecum, and an aliquot of cecal contents as described by Schumacher et al. (2018), and lung samples were collected at necropsy. A negative bioassay was concluded if all rectal swabs, serum samples, lung tissue, and cecum contents had non-detectable levels of PEDV or PRRSV. If any samples had detectable RNA, the result would be considered a positive bioassay.

Statistical analysis

Data were analyzed in a split-plot design with truck cab as the experimental unit for disinfectant treatment and surface coupons as the experimental unit for surface type (fabric, plastic, or rubber) and virus (PEDV or PRRSV). There were three replications per treatment. The Ct value of each sample was analyzed with ANOVA and F-test through the aov function in R programming language (R Foundation for Statistical Computing, Vienna, Austria). Fixed effects considered the disinfectant treatment, surface treatment, and virus type, while the random effect was truck cab defining it as the experimental unit for disinfectant treatment to account for the split-plot design. Results of Ct data are reported as least squares means ± standard error of the mean. All statistical models were evaluated using visual assessment of studentized residuals, and assumptions appeared to be reasonably met. A Tukey multiple comparison adjustment was utilized to control Type I error rate. Results were considered significant at $P \leq 0.05$ and marginally significant between $P > 0.05$ and $P \leq 0.10$.

Results and discussion

Both PEDV and PRRSV are considered endemic, or present at normal levels, within swine-dense regions of the United States. It has been documented that transportation vehicles can contribute to the spread of both pathogens in addition to the direct contact routes of transmission (Dee et al., 2002; Dee et al., 2003; Dee et al., 2004c; Lowe et al., 2014). Given this potential risk of viral transmission, there has been research documenting the benefits of power washing, disinfecting, and then heating livestock trailers to reduce the amount of potential infective material to limit the spread of disease to naïve pigs for both PEDV and PRRSV (Dee et al., 2004a,b; Dee et al., 2005; Baker et al., 2017; Holtkamp et al., 2017; Baker et al., 2018). However, most of such research has focused on how to reduce transmission in transportation with live animals and not on transportation vehicles that can frequently visit production sites without live animals, including feed delivery trucks. There is evidence to suggest that when sampling for viruses or bacteria within a feed mill, a majority of the samples containing detectable pathogens were from the cab of the delivery truck, most likely due to the driver getting in and out of the cab for deliveries at production sites and potentially "tracking" virus back into the cab (Fedorka-Cray et al., 1997; Greiner et al., 2016; Elijah et al., 2022; Gebhardt et al., 2022). There are ways to mitigate this risk, including the use of shoe covers for each delivery or the application of disinfectant to the truck cab since the application of disinfectant has been successful for PEDV and PRRSV reduction in terms of livestock trailers; however, challenges exist when trying to transfer these procedures to truck cabs. The problems to consider when applying disinfectant to the truck cab are how to apply the disinfectant, the type of disinfectant (in terms of wet, dry, or fumigation), and the implications of disinfectant on the surfaces since there are various surface types present throughout the truck cab, unlike livestock trailers which

are primarily made of metal. There is minimal research looking at different application methods and how these applications impact the amount of detectable virus within truck cabs on different surface types. Therefore, this study aimed to quantify the impact of commercially available disinfectants applied through different application methods on detectable viral RNA on fabric, plastic, and rubber surfaces inoculated with either PEDV or PRRSV and if detectable viral RNA could cause infection in susceptible pigs via swine bioassay.

There was no evidence of a disinfectant × surface × virus interaction ($P = 0.959$; Table 2), surface × virus interaction ($P = 0.926$), or disinfectant × virus interaction ($P = 0.508$). There was a significant disinfectant × surface interaction ($P < 0.0001$; Table 3), indicating that the quantity of PEDV or PRRSV RNA detected on surfaces differed based on disinfectant treatment. For rubber surfaces, environmental samples from the 10% bleach application had the least ($P < 0.05$) amount of PEDV or PRRSV RNA detected compared to the other disinfectant methods with Intervention via misting fumigation application as intermediate. For plastic and fabric surfaces, there was no evidence of a statistical difference between decontamination methods ($P > 0.05$). Furthermore, for the no-disinfectant treatment, environmental swabs from fabric surfaces detected less PEDV or PRRSV RNA than from plastic or rubber surfaces ($P < 0.05$). For the pump sprayer with Intervention disinfectant treatment, environmental swabs from fabric surfaces detected less ($P < 0.05$) PEDV or PRRSV RNA compared to rubber surfaces with plastic surfaces as intermediate. For the pump sprayer with 10% bleach disinfectant treatment, environmental swabs from fabric and rubber surfaces detected less PEDV or PRRSV RNA than plastic surfaces ($P < 0.05$). For the no chemical 10 hr downtime disinfectant treatment, environmental swabs from fabric surfaces detected less PEDV or PRRSV RNA when compared to plastic or rubber surfaces ($P < 0.05$). Finally, for the gaseous treatment 10 hr downtime

disinfectant application, environmental swabs from fabric surfaces detected less PEDV or PRRSV RNA when compared to plastic or rubber surfaces ($P < 0.05$). These results are similar to Muckey et al. (2021), where there was also a treatment × surface interaction indicating that when implementing disinfectant applications, consideration should be given to the surface since surface material has the potential to influence the amount of detectable viral RNA. Given this commonality between research studies, there is some suggestion that surface type can influence the amount of detectable viral RNA recovered within sampling techniques.

The main effect of disinfection treatment was statistically significant ($P = 0.016$; Table 4), with environmental swabs collected after 10% bleach treatment detecting less PEDV or PRRSV RNA ($P < 0.05$) when compared to environmental swabs from no-disinfectant, Intervention via pump sprayers, and no chemical application 10 hr downtime with all other disinfectant treatment applications being intermediate. Disinfectants have been shown to reduce the amount of detectable PRRSV or PEDV RNA (Dee et al., 2004a,b; Dee et al., 2005; Bowman et al., 2015). However, research suggests that application of sodium hypochlorite, or bleach, results in the greatest reduction of PEDV, PRRSV, and other enteric viruses or bacteria, sometimes producing non-detectable test results for pathogens compared to other disinfectants or soaps used based on the surface material, which is consistent with the findings from this study (Dee et al., 2004a,b; Dee et al., 2005; Gerba and Kennedy, 2007; Honisch et al., 2014; Bowman et al., 2015).

The main effect of the surface was statistically significant ($P < 0.0001$), with environmental swabs from plastic surfaces detecting greater amounts of PEDV or PRRSV RNA when compared to rubber and the samples from rubber detecting greater amounts of PEDV or PRRSV RNA when compared to fabric ($P < 0.05$). Rabuza et al. (2012) reported that when

sampling with cotton gauze, a methodology similar to that used in this experiment, the cotton gauze method extracted lower quantities of Klebsiella pneumoniaee and Staphylococcus aureus from inoculated fabric surfaces when compared to other methods like contact plating, destructive elution methodology, or nondestructive elution methodology. For contact plating, the fabric sample had RODAC agar poured over it so it could stick to the medium, while destructive or nondestructive elution methodologies are wash-off methods where microorganisms are eluted from the fabrics by shaking the fabrics for a specific time in an elution medium. Then, if the sample was assigned the nondestructive elution methodology, the sample was subjected to forced desorption by pressing the microorganism through the fabric without destroying the fabric. While contact plating, destructive elution methodology, or nondestructive elution methodology are employed to quantify bacteria on fabric surfaces, all methodologies require a fabric sample. These methodologies have their strengths, but there is minimal real-world application since pieces of a seat cover cannot be cut off a seat within a truck cab. The advantage of the sampling methodology used in the current experiment is the ease of application in a practical field setting. In addition, when consulting hospital procedures for disinfectant application, spraying disinfectants on various surfaces is a common solution for disinfection. However, excessive wetting of fabric surfaces is highly discouraged due to patient discomfort, and disinfectants will continuously leak out of fabric surfaces for extended periods (Creamer and Humphreys, 2008). Fabric surfaces have the potential to absorb and hold wet disinfectants for longer periods when compared to other non-absorptive surfaces like plastic or rubber, indicating that inherent properties of the surface could lend to longer disinfectant contact times. It is uncertain which factor may have potentially impacted the Ct results from this study but it highlights the need for more research to be done on disinfectant application on fabric surfaces and applicable sampling

methodology for fabric surfaces. The main effect of virus ($P < 0.0001$) was significant, with environmental swabs detecting greater amounts of PEDV RNA compared to the detection of PRRSV RNA ($P < 0.05$).

Supernatant from environmental samples of surfaces inoculated with PEDV and PRRSV after disinfectant treatment application were utilized in bioassay and failed to produce infectivity. To our knowledge, this is the first published work utilizing inoculum from environmental samples that the positive control treatment groups (with Ct values ranging 26.4 – 37.2) did not cause clinical infection in pigs via bioassay. A potential explanation for the failure to produce infectivity is the time from sample collection and processing to the time of bioassay, potentially impacting infectivity factors for PEDV and PRRSV. For example, the length of time from sample collection to bioassay for this study was 13 months, while for the dust sample study, the length of time from sample collection to bioassay was 11 months (Schumacher et al., 2017). More research is needed to understand the impacts of long-term storage on virus infectivity.

This study aimed to test disinfectant applications that could be implemented between feed deliveries or while feed trucks are loaded at the feed mill. Additionally, the data presented here provide value to a broader segment of the industry in comparing disinfectants through different applications that could be quickly implemented through a variety of truck cabs like those used in live-animal delivery, veterinary services, and other trucks associated with the swine and animal industries.

In conclusion, disinfection of truck cabs has its challenges given the variety of surfaces present. The disinfectant treatments tested in this trial could only reduce the detectable viral RNA and did not completely eliminate viral genetic material across surface types. Based on this study and methodology utilized for bioassay, this amount of detectable viral RNA was non-

infectious. Additional research is warranted to refine methods of detecting infectious viruses across varying surfaces.

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Table 3.1 Summary of the 16 treatments chosen for live pig bioassay[1]

Surface type	Disinfectant treatment
Rubber (n = 8)	No disinfectant No chemical, 10 hr downtime Gaseous fumigation, 10 hr downtime Misting fumigation, Intervention Misting fumigation, Synergize Pump sprayer, Intervention Pump sprayer, Synergize Pump sprayer, 10% bleach
Plastic (n = 4)	No disinfectant No chemical, 10 hr downtime Gaseous fumigation, 10 hr downtime Pump sprayer, 10% bleach
Fabric (n = 4)	No disinfectant No chemical, 10 hr downtime Gaseous fumigation, 10 hr downtime Pump sprayer, 10% bleach

[1]Supernatant from environmental samples taken after disinfectant application to a truck cab were used as the inoculum for the live pig bioassay. A total of 48 crossbred, 10-day-old pigs of mixed sex were given 10 mL oral gavage of porcine epidemic diarrhea virus (PEDV) and 3 mL intramuscular (IM) injection, and 1 mL/nostril intranasal application of porcine reproductive and respiratory syndrome virus (PRRSV) after a 3 day acclimation period.

Table 3.2 Effect of surface type, disinfectant, and virus on the detection of viral RNA during truck cab decontamination[1]

	Surface type					
	Fabric		Plastic		Rubber	
Item	PEDV	PRRSV	PEDV	PRRSV	PEDV	PRRSV
Proportion PCR positive						
No-disinfectant[2]	3/3	3/3	3/3	3/3	3/3	3/3
Misting fumigation[3]						
Intervention[4]	3/3	3/3	3/3	3/3	2/3	2/3
Synergize[5]	2/3	3/3	3/3	3/3	3/3	3/3
Pump sprayer[6]						
Intervention	3/3	3/3	3/3	3/3	3/3	3/3
Synergize	2/3	3/3	2/3	3/3	3/3	3/3
10% Bleach[7]	2/3	0/3	3/3	3/3	1/3	0/3
10 hr downtime[8]						
No chemical	3/3	2/3	3/3	3/3	3/3	3/3
Gaseous treatment[9]	3/3	1/3	3/3	3/3	3/3	3/3
Cycle threshold[10]						
No-disinfectant	34.6	37.2	26.7	30.6	26.7	31.4
Misting fumigation						
Intervention	33.4	38.2	28.1	31.6	34.2	36.7
Synergize	36.2	36.4	29.7	34.2	30.3	33.3
Pump sprayer						
Intervention	34.8	37.5	28.3	31.3	28.8	32.0
Synergize	37.3	38.7	33.0	32.6	30.6	33.5
10% Bleach	40.7	45.0	26.7	31.2	41.2	45.0
10 hr downtime						
No chemical	36.4	40.3	27.8	29.8	29.7	30.2
Gaseous treatment	36.8	44.4	28.3	31.9	28.6	33.2

[1]Surfaces were inoculated with 1 mL of porcine epidemic diarrhea virus (PEDV) or porcine reproductive and respiratory syndrome virus (PRRSV), randomly placed within the truck cab, and subjected to a randomly assigned disinfectant treatment. Samples with no detectable RNA were assigned a value of 45.

[2]Surfaces were inoculated with pure virus and allowed to sit within the truck cab for 15 min. These surfaces were not treated with a disinfectant application.

[3]Truck cabs had a hurricane fumigation system placed in the passenger seat and directed toward the driver's side. The hurricane fumigation system was filled with respective disinfectant and was allowed to run 5 min for each treatment.

[4]1:64 dilution of Intervention (Virox, Oakville, ON)

[5]1:256 dilution of Synergize (Neogen Corp, Lexington, KY)

[6]Truck cabs had disinfectant applied with a conventional pump sprayer with the designated disinfectant.

[7]Household bleach (10% dilution; The Clorox Company, Oakland, CA; 7.55% sodium hypochlorite).

[8]Surfaces were inoculated with pure virus and allowed to sit within the truck cab for 10 hr. These surfaces were not treated with a disinfectant application.
[9]Truck cabs had gaseous chlorine dioxide (ProKure G; ProKure Solutions, Phoenix, AZ) placed on the passenger side seat and allowed to fumigate the truck cab for 10 hr.
[10] Disinfectant × surface × virus interaction $P = 0.959$; SEM = 1.84

Table **3.3** Effect of surface type and disinfectant on the detection of viral RNA during truck cab decontamination[1]

	Proportion PCR positive			Ct value[2]		
Item	Fabric	Plastic	Rubber	Fabric	Plastic	Rubber
No-disinfectant[3]	6/6	6/6	6/6	35.9^{c,d,e,f,g,h}	28.6^{a,b}	29.0^{a,b}
Misting fumigation[4]						
Intervention[5]	6/6	6/6	5/6	35.8^{a,b,c,d,e,f,g,h}	29.8^{a,b,c,d}	35.4^{a,b,c,d,e,f,g,h}
Synergize[6]	5/6	6/6	6/6	36.6^{a,b,c,d,e,f,g,h}	31.9^{a,b,c,d,e,f}	31.8^{a,b,c,d,e,f}
Pump sprayer[7]						
Intervention	6/6	6/6	6/6	36.1^{b,d,e,f,g,h}	29.8^{a,c}	30.4^{a,b,c,d,e,f}
Synergize	5/6	5/6	6/6	38.0^{e,f,g,h}	32.8^{a,b,c,d,e,f,g}	32.0^{a,b,c,d,e,f}
10% Bleach[8]	2/6	6/6	1/6	42.9^{h}	29.0^{a,b,c,d}	43.1^{h}
10 hr downtime[9]						
No chemical	5/6	6/6	6/6	38.4^{f,g,h}	28.8^{a,b,c,d}	30.0^{a,b,c,d}
Gaseous treatment[10]	4/6	6/6	6/6	40.6^{g,h}	30.1^{a,b,c,d,e}	30.9^{a,b,c,d,e,f}

[1]Surfaces were inoculated with 1 mL of porcine epidemic diarrhea virus (PEDV) or porcine reproductive and respiratory syndrome virus (PRRSV), randomly placed within the truck cab, and subjected to a randomly assigned disinfectant treatment. Samples with no detectable RNA were assigned a value of 45.

[2]Disinfectant × surface, $P < 0.0001$; SEM = 1.45

[3]Surfaces were inoculated with pure virus and allowed to sit within the truck cab for 15 min. These surfaces were not treated with a disinfectant application.

[4]Truck cabs had a hurricane fumigation system placed in the passenger seat and directed toward the driver's side. The hurricane fumigation system was filled with respective disinfectant and was allowed to run 5 min for each treatment.

[5]1:64 dilution of Intervention (Virox, Oakville, ON)

[6]1:256 dilution of Synergize (Neogen Corp, Lexington, KY)

[7]Truck cabs had disinfectant applied with a conventional pump sprayer with the designated disinfectant.

[8]Household bleach (10% dilution; The Clorox Company, Oakland, CA; 7.55% sodium hypochlorite).

[9]Surfaces were inoculated with pure virus and allowed to sit within the truck cab for 10 hr. These surfaces were not treated with a disinfectant application.

[10]Truck cabs had gaseous chlorine dioxide (ProKure G; ProKure Solutions, Phoenix, AZ) placed on the passenger side seat and allowed to fumigate the truck cab for 10 hr.

[a,b,c…h]Means lacking common superscripts differ, $P < 0.05$.

Table 3.4 Main effects of disinfectant, surface type, and virus on the detection of viral RNA during truck cab decontamination[1]

Item	Proportion PCR positive	Ct value	SEM	*P* =
Disinfectant			1.11	0.016
No-disinfectant[2]	18/18	31.2[a]		
Hurricane fumigation[3]				
Intervention[4]	16/18	33.7[a,b]		
Synergize[5]	17/18	33.4[a,b]		
Pump sprayer[6]				
Intervention	18/18	32.1[a]		
Synergize	16/18	34.3[a,b]		
10% Bleach[7]	9/18	38.3[b]		
10 hr Downtime				
No chemical[8]	17/18	32.4[a]		
Gaseous treatment[9]	16/18	33.9[a,b]		
Surface Type			0.53	< 0.0001
Fabric	39/48	38.0[c]		
Plastic	47/48	30.1[a]		
Rubber	41/48	32.8[b]		
Virus			0.48	< 0.0001
PEDV	62/72	32.0[a]		
PRRSV	62/72	35.3[b]		

[1]Surfaces were inoculated with 1 mL of porcine epidemic diarrhea virus (PEDV) or porcine reproductive and respiratory syndrome virus (PRRSV), randomly placed within the truck cab, and subjected to a randomly assigned disinfectant treatment. Samples with no detectable RNA were assigned a value of 45.
[2]Surfaces were inoculated with pure virus and allowed to sit within the truck cab for 15 min. These surfaces were not treated with a disinfectant application.
[3]Truck cabs had a hurricane fumigation system placed in the passenger seat and directed toward the driver's side. The hurricane fumigation system was filled with respective disinfectant and was allowed to run 5 min for each treatment.
[4]1:64 dilution of Intervention (Virox, Oakville, ON)
[5]1:256 dilution of Synergize (Neogen Corp, Lexington, KY)
[6]Truck cabs had disinfectant applied with a conventional pump sprayer with the designated disinfectant.
[7]Household bleach (10% dilution; The Clorox Company, Oakland, CA; 7.55% sodium hypochlorite).
[8]Surfaces were inoculated with pure virus and allowed to sit within the truck cab for 10 hr. These surfaces were not treated with a disinfectant application.
[9]Truck cabs had gaseous chlorine dioxide (ProKure G; ProKure Solutions, Phoenix, AZ) placed on the passenger side seat and allowed to fumigate the truck cab for 10 hr.
[a,b,c]Means within main effect lacking common superscripts differ, $P < 0.05$.

Chapter 4 - Evaluating the impact of organic matter and sample processing techniques on RNA detection using environmental samples[3]

Abstract

This study evaluated sample processing methods and the presence of organic matter on detection of porcine epidemic diarrhea virus (PEDV) from environmental samples using real-time reverse transcriptase-polymerase chain reaction (qRT-PCR). Steel coupons were inoculated with PEDV and different types of organic material contamination. Surface samples were collected and processed in one of four ways: none, centrifugation, syringe filtration, or combination of centrifugation and syringe filtration, then submitted for PEDV qRT-PCR. There was a surface inoculation type by processing method interaction ($P < .001$) that impacted the sample cycle threshold value. Centrifugation resulted in the most consistent detection of PEDV RNA.

Keywords: swine, environmental samples, feed safety

Introduction

Swine veterinarians have come to rely heavily on polymerase chain reaction (PCR) assays for viral detection in samples like oral fluids, tissues, and environmental samples. The advantages of using PCR assays are that it is fast, sensitive, and can be used across multiple

[3] This work was published in *Journal of Swine Health and Production*. Houston GE, Blomme AK, Harrison OL, Bai J, Woodworth JC, Jones CK, Poulsen-Porter EG, Paulk CB, Gebhardt JT. Evaluating the impact of organic matter and sample processing techniques on RNA detection using environmental samples. *J Swine Health Prod.* 2023. doi:10.54846/jshap/1311

sample types.[1] Typically, oral fluids and tissue samples are used to diagnose clinical disease and help guide health decisions within populations of pigs. Environmental samples can help swine veterinarians detect pathogens on a variety of surfaces and address gaps in biosecurity practices for swine production systems or feed mills. Unfortunately, environmental samples can be heavily contaminated with dirt, feces, dust, feed, or a combination of these organic substances that naturally occur in the sample. This wide variety of contamination is an important factor when considering the accuracy of the PCR assay. The organic materials present in the environmental sample can inhibit the PCR reaction, resulting in decreased sensitivity or false-negative results.[1] There are multiple ways to approach sample handling to account for the potential of inhibitory substances depending upon which step of the PCR reaction is inhibited.[1,2] When considering veterinary diagnostic laboratories, most PCR assays are validated for blood, tissue, and other clinical samples but environmental samples have yet to be validated. This is due to the fact that environmental samples can often contain different types of substances or a combination of substance that could inhibit the PCR assay. Thus, if a validated and standardized protocol for environmental samples would be created, these protocols would have to account for all of the potential inhibitory substances but also be time efficient. Ideally, the protocol could also be done relatively quickly in a laboratory so samples would still have the same turnaround time for submission. Therefore, the objective of this project was to evaluate different surface contamination types commonly found in environmental samples and if different processing techniques conducted prior to real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis would impact sample porcine epidemic diarrhea virus (PEDV) detection.

Procedures

General

Dirt and finishing pig feces were collected before this experiment and aliquoted into 5 g samples. For the organic matter mixture, 10 g of the same dirt and 10 g of the same feces were mixed together with 3 mL of deionized water. Once the organic matter was thoroughly mixed, it was aliquoted into 5 g samples. Dirt, feces, and organic matter were confirmed to have no detectable PEDV or porcine deltacoronavirus (PDCoV) RNA via PCR prior to the start of the experiment. Once confirming dirt, feces, and organic matter mixture had no detectable PEDV or PDCoV RNA, all material was frozen at -80°C until the experiment was conducted. Virus used was PEDV isolate USA/Co/2013 with a titer of 1.33×10^5 median tissue culture infectious dose/mL.

Surface inoculation

Fifteen, autoclaved, steel, 10 × 10 cm coupons were placed within a biosafety level (BSL)-2 biosafety cabinet. A coupon was inoculated with one of the five surface inoculation types: 1 mL of PEDV; 1 mL of PEDV and 5 mL of phosphate buffered saline (PBS); 1 mL of PEDV and 5.0 g of dirt; 1 mL of PEDV and 5.0 g of feces; or 1 mL of PEDV and 5.0 g of organic matter mixture. Each treatment was replicated 3 times using 3 separate steel coupons.

Surface inoculation type sampling

After inoculation, the coupon sat for 15 minutes within the BSL-2 cabinet. After the 15 minute time limit, each steel coupon was environmentally swabbed as previously described.[3] Once the environmental sample was taken, 20 mL of PBS was added to the sample, it was inverted for 5 to 10 seconds, and then allowed to incubate at room temperature (24°C) for 1 hour.

At the end of incubation, the sample was vortexed for 15 seconds and then processed for qRT-PCR analysis.

Sample processing

For each environmental sample, 4 samples were taken directly from the conical tube after vortexing to achieve 4 different processing techniques. For sample A, 1 mL was taken from the environmental sample, placed in a cryovial, and submitted for qRT-PCR analysis without further processing. For sample B, 1 mL was taken from the environmental sample, placed into a new conical tube, and centrifuged for 10 minutes at 706g. Following centrifugation, the supernatant was pipetted into a cryovial then submitted for qRT-PCR analysis. For sample C, 1 mL was taken from the environmental sample, filtered through a 0.45-µm, 25-mm syringe filter into a cryovial, and then submitted for qRT-PCR analysis. For sample D, 1 mL was taken from the environmental sample, placed into a new conical tube, centrifuged as previously described, filtered through a 0.45-µm, 25-mm syringe filter into a cryovial, and then submitted for qRT-PCR analysis.

qRT-PCR analysis

The Molecular Research and Development Laboratory within the Kansas State University Veterinary Diagnostic Laboratory conducted the qRT-PCR analysis. Fifty µL of supernatant from each sample was loaded into a deep-well plate and extracted using a Kingfisher Flex magnetic particle processor (Fisher Scientific) with the MagMAX-96 Viral RNA Isolation kit (Life Technologies) according to manufacturer instructions with one modification, reducing the final elution volume to 60 µL. One negative extraction control consisting of all reagents and PBS in place of the sample was included in the extraction. Positive controls of each stock virus were also included with each extraction. Extracted RNA was frozen at -80°C until assayed by qRT-

PCR. Analyzed values represent cycle threshold (Ct) at which virus was detected. A total of 45 cycles were ran for each sample so if a sample had no detectable PEDV RNA for the qRT-PCR assay, the sample was assigned a value of 45.

Statistical analysis

Statistical analysis of variance for the sample Ct values was performed using the aov function utilizing R programming language (R Foundation for Statistical Computing; version 4.1.1). Fixed effects included the inoculation treatment, sample processing treatment, and the associated interaction. Results of Ct data are reported as least squares means (SEM). All statistical models were evaluated using visual assessment of studentized residuals and model assumptions appeared to be appropriate. A Tukey multiple comparison adjustment was incorporated when appropriate. Results were considered significant at $P \leq .05$ and marginally significant between $P > .05$ and $P \leq .10$.

Results

There was an inoculated surface contamination type by sample processing method ($P < .001$) interaction that impacted the sample Ct value (Table 1). For surfaces inoculated with pure virus and virus with PBS, there was no difference in the sample Ct values across the different types of sample processing methods ($P > .05$). For surfaces inoculated with virus and dirt, samples that were centrifuged had greater amounts of PEDV RNA detected (or lower Ct values) compared to samples that were not processed ($P < .05$). For surfaces inoculated with virus and feces, nonprocessed samples or centrifuged samples had greater amounts of PEDV RNA detected (or lower Ct values) compared to syringe filtered samples and centrifuged and syringe filtered samples ($P < .05$). For surfaces inoculated with virus and organic matter mixutre,

centrifuged samples had greater amounts of PEDV RNA detected (or lower Ct values) compared to all other types of sample processing ($P < .05$).

There were also statistically significant main effects of surface contamination type ($P < .001$) and sample processing ($P < .001$; Table 2). For surface contamination type, surfaces inoculated with pure virus and virus with PBS had greater amounts of PEDV RNA detected (lower Ct values) compared to surfaces inoculated with virus and dirt ($P < .05$), while surfaces inoculated with virus and feces and virus and organic matter mixture had lower levels of PEDV RNA detected (higher Ct values) compared to all other surfaces ($P < .05$). For sample processing type, centrifugation of samples resulted in a greater amount of PEDV RNA detected (lower Ct values) compared to all other treatments ($P < .05$). Furthermore, syringe filtration or centrifugation and syringe filtration resulted in the lowest amount of PEDV RNA detected (higher Ct values; $P < .05$).

Discussion

Nucleic acid (NA) extraction and the PCR reaction are the 2 major steps that can influence the test results. For NA extraction, most commercial extraction kits, like the one used in this study, are able to remove most PCR inhibitory materials from the sample and enrich NA content for PCR detections. For a PCR reaction, there are 3 general steps – denaturation (unwind the double helix pattern of DNA), primer annealing (specific primers to attach to the unwound DNA), and extension (polymerase binds to the primer and unwound strand complex to make complimentary strands); then those complimentary strands are amplified and the rate of amplification corresponds with a Ct value.[4] Since primers can be designed for a wide variety of microorganisms and the assay is completed in minutes, PCR is a commonly used diagnostic tool across medical professions.[2,5] For swine veterinarians, PCR assays are used for many disease

syndromes and can include many different sample types like oral fluids, tissues, and environmental samples. However, when considering the 3 steps of a PCR reaction, there are ways for the accuracy of this assay to become comprised and therefore, give inaccurate results. For example, several potential issues that can arise during the PCR analysis process that could lead to false-positive or false-negative results include substances that inhibit any step of the assay, potential contamination during sample collection prior to PCR, or potential laboratory contamination while conducting the PCR assay.[2] There are many sources on how to counteract the potential for problems pertaining to all 3 basic steps of PCR but for the sake of this paper, the rest of the discussion will focus on inhibitory substances.

In general, inhibitory substances can naturally occur in the sample or be introduced into the sample during sample processing.[1] For example, common inhibitor substances can include body fluids or reagents in clinical and forensic sciences like hemoglobin, urea, or heparin; food substances or particles like glycogen, fats, or calcium; and environmental compounds like humic acids, heavy metals, or phenolic compounds.[6] These substances have the potential to interfere with PCR amplification and influence the sensitivity thereby negatively effecting the performance of the PCR assay.[7] There are many potential inhibitory substances and what is present in one sample matrix may be completely different in another sample matrix.[1] When considering common samples submitted for PCR by swine veterinarians, most of those sample types have the potential to include dirt, feces, blood, dust, soil, or a combination of these materials which can potentially inhibit a portion of a PCR reaction. Given this information, it does not mean that veterinarians should stop using PCR for diagnostics, but further reiterates that veterinarians should understand the potential pitfalls associated with their samples. It is important for veterinarians and diagnosticians to consider how best to handle the sample

submission to maximize the PCR assay sensitivity. There are multiple methods that can be used to overcome potential inhibitory substances which can include biochemical methods, immunological methods, physical methods, or physiological methods; with the physical methods being the most user friendly.[7] Ideally the method used to process samples prior to PCR analysis would be cost effective, time efficient, and relatively easy to implement. Therefore, this study aimed to evaluate methods of sample processing, specifically physical methodologies, on different surface inoculation contamination types of environmental samples and how that impacted PEDV detection via PCR analysis.

For this study, there was an inoculated surface contamination by sample processing technique interaction indicating that the inoculation contamination type and how that environmental sample was processed prior to qRT-PCR analysis impacted the Ct value of the sample. As samples contained more inhibitory substances like dirt, feces, or a combination of both, how that sample was processed influenced the results of the PCR assay. No one single processing technique was beneficial across all surface inoculation types. However, when the inoculation type was virus with dirt, feces, or organic matter mixture, the centrifugation methodology consistently identified PEDV RNA across all inoculation types as shown by the lower Ct values and proportion of positive PCR results when compared to other processing methods. Hall et al[8] found similar results when evaluating inhibitor resistance methods for diagnostics in clinical and environmental samples. Specifically, they found that of the 9 possible methods for inhibitor resistant, not a single method performed the best for all the sample matrices, but one method, KAPA blood PCR kit, did produce the most consistent results across the different sample matrices.[8] The current study and Hall et al8 highlight that the best method

for overcoming a variety of inhibitory substances is the method that produces the most consistent results.

Another finding from this study was that the centrifugation processing technique of samples had the lowest Ct values compared to other sample processing techniques. Similarly, one study found that centrifugation of urine samples helped to maximize PCR sensitivity and was also the most time efficient method compared to the traditional dot-plot hybridization method.[9] When considering sample processing techniques, this study and the current study both highlight the importance that the technique should be relatively easy, cost effective, and time efficient. Another finding from the current study was that the more "pure" surface contamination types had lower Ct values when compared to surfaces inoculated with feces or organic matter mixture. There was no statistically significant difference in Ct values for the pure virus inoculation and virus inoculation after dilution with PBS, but the detection of PEDV RNA was generally reduced as dirt, feces, or the combination were included on the environmental surface. This conclusion is similar to another research study that detailed the different ways forensic samples are processed before PCR analysis in order to obtain the purest sample possible to allow for proper PCR amplification.[10] Syringe filtering of samples in the current study reduced the ability to detect RNA in samples, especially those with dirt, feces, or the combination of both. It was hypothesized that the syringe filtering might also be trapping the RNA and not just dirt and feces. To the authors' knowledge this is the first study to find these results associated with syringe filtration and processing samples prior to RT-PCR.

This study highlight that the best sample for RT-PCR is a sample free of substances that potentially interfere with PCR analysis like dirt, feces, and soil. However, when considering the environment most swine veterinarians acquire their sample from (barns with dirt, feces, and dust;

environmental samples containing dirt, dust, and other materials), these findings further highlight the importance of proper sample processing to prevent potential inhibitory substances prior to PCR analysis. Based on the results of the current study, centrifugation of environmental samples at 706g for 10 minutes resulted in the most consistent recovery of PEDV RNA across a range of environmental organic material loads.

Implications

Under the conditions of this study:

- Organic material in environmental samples can interfere with qRT-PCR analysis.
- Processing samples before qRT-PCR can improve diagnostic sensitivity.
- Centrifugation maximized qRT-PCR sensitivity for environmental samples.

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Table 4.1 Effect of inoculation type and environmental sample processing technique on detection of PEDV on steel surfaces*

Item	Sample processing technique†			
	No processing	Centrifuge	Syringe filter	Centrifuge + syringe filter
qRT-PCR proportion, No. positive/No. samples				
Pure virus	3/3	3/3	3/3	3/3
Virus and PBS	3/3	3/3	3/3	3/3
Virus and dirt	2/3	3/3	3/3	3/3
Virus and feces	3/3	3/3	0/3	0/3
Virus and organic matter	1/3	3/3	0/3	2/3
Ct value‡				
Pure virus	24.5[a]	24.6[a]	28.9[abcd]	27.5[abc]
Virus and PBS	24.8[ab]	24.7[ab]	28.0[abc]	28.4[abc]
Virus and dirt	35.9[de]	28.2[abc]	32.0[cd]	30.8[abcd]
Virus and feces	31.8[bcd]	32.5[cd]	45.0[f]	45.0[f]
Virus and organic matter	42.4[ef]	31.3[abcd]	45.0[f]	40.9[ef]

*Steel coupons, measuring 10 × 10 cm were inoculated with PEDV, isolate USA/Co/2013 with a titer of 1.33×10^5 TCID50/mL. Surfaces were inoculated with 1 mL of pure virus, 1 mL of virus diluted into 5 mL of PBS, 1 mL of virus inoculated with 5 g of dirt, 5 g of feces, or 5 g of organic matter mixture consisting of a 1:1 ratio of dirt and feces. After surfaces were allowed to sit for 15 min, the steel coupon was environmentally swabbed. Environmental samples were inverted for 5-10 s, incubated for 1 hr, vortexed for 10-15 s, and then processed according to designated sample processing technique.
†Sample processing techniques included no processing, centrifuged for 10 min at 706g (centrifuge), filtered with a 0.45-µm, 25-mm syringe filter (syringe filter), or centrifuged for 10 min at 706g then filtered through a 0.45-µm, 25-mm syringe filter (centrifuge + syringe filter). After processing, samples were submitted for PEDV qRT-PCR assay.
‡ If there was no detectable RNA in the sample, the sample was assigned a Ct value of 45.
[abcdef] Inoculation contamination type by sample processing interaction, $P < .001$; SEM = 1.41. Means lacking common superscripts differ, $P < .05$.
PEDV = porcine epidemic diarrhea virus; qRT-PCR = real-time reverse transcriptase-polymerase chain reaction; PBS = phosphate buffered saline; Ct = cycle threshold.

Table 4.2 Main effects of surface inoculation type and sample processing technique on detection of PEDV on steel surfaces*

Item	qRT-PCR proportion, No. positive/No. samples	Ct†
Surface inoculation		
Pure virus	12/12	26.4[a]
Virus and PBS	12/12	26.5[a]
Virus and dirt	11/12	31.7[b]
Virus and feces	6/12	38.6[c]
Virus and organic matter	6/12	39.9[c]
Sample processing		
No processing	12/15	31.9[e]
Centrifuge	15/15	28.2[d]
Syringe filter	9/15	35.8[f]
Centrifuge + syringe filter	11/15	34.5[f]

*Steel coupons, measuring 10 × 10 cm, were inoculated with PEDV, isolate USA/Co/2013 with a titer of 1.33×10^5 TCID50/mL. Surfaces were inoculated with 1 mL of pure virus, 1 mL of virus diluted into 5 mL of PBS, 1 mL of virus inoculated with 5 g of dirt, 5 g of feces, or 5 g of organic matter mixture consisting of a 1:1 ratio of dirt and feces. After surfaces were allowed to sit for 15 min, the steel coupon was environmentally swabbed. Environmental samples were inverted for 5-10 s, incubated for 1 hr, vortexed for 10-15 s, and then processed according to designated sample processing technique. Samples were processed as either no processing, centrifuged for 10 min at 706*g* (centrifuge), filtered with a 0.45-µm, 25-mm syringe filter (syringe filter), or centrifuged for 10 min at 706*g* then filtered through a 0.45-µm, 25-mm syringe filter (centrifuge + syringe filter). After processing, samples were submitted for PEDV qRT-PCR assay.

† If there was no detectable RNA in the sample, the sample was assigned a Ct value of 45.

[abc] Main effect of surface contamination type on Ct values, $P < .001$; SEM = 0.80.

Means lacking common superscripts differ, $P < .05$.

[def] Main effect of sample processing technique on Ct values, $P < .001$; SEM = 0.74. Means lacking common superscripts differ, $P < .05$.PEDV = porcine epidemic diarrhea virus; qRT-PCR = real-time reverse transcriptase-polymerase chain reaction; Ct = cycle threshold; PBS = phosphate buffered saline.

Chapter 5 - Evaluating the distribution of African swine fever virus within a feed mill environment following manufacture of inoculated feed[4]

Abstract

It is critical to understand the role feed manufacturing may have regarding potential African swine fever virus (ASFV) transmission, especially given the evidence that feed and/or ingredients may be potential vectors. The objective of the study was to evaluate the distribution of ASFV in a feed mill following manufacture of contaminated feed. To accomplish this, a pilot-scale feed mill consisting of a mixer, bucket elevator, and spouting was constructed in a BSL-3Ag facility. First, a batch of ASFV-free feed was manufactured, followed by a batch of feed that had an ASFV-contaminated ingredient added to feed, which was then mixed and discharged from the equipment. Subsequently, four additional ASFV-free batches of feed were manufactured using the same equipment. Environmental swabs from 18 locations within the BSL-3Ag room were collected after each batch of feed was discharged. The locations of the swabs were categorized into four zones: 1) feed contact surface, 2) non-feed contact surface < 1 meter away from feed, 3) non-feed contact surface > 1 meter from feed, and 4) transient surfaces. Environmental swabs were analyzed using a qPCR specific for the ASFV p72 gene and reported as genomic copy number (CN)/mL of environmental swab processing buffer. Genomic copies

[4] This work was published in *PLoS ONE.* Elijah CG, Trujillo JD, Jones CK, Gaudreault NN, Stark CR, Cool KR, Paulk CB, Kwon T, Woodworth JC, Morozov I, Gallardo C, Gebhardt JT, Richt JA. (2021). Evaluating the distribution of African swine fever virus within a feed mill environment following manufacture of inoculated feed. *PLoS ONE 16(8):*e0256138. doi:10.1371/journal.pone.0256138

were transformed with a log10 function for statistical analysis. There was no evidence of a zone × batch interaction for log10 genomic CN/mL ($P = 0.625$) or cycle threshold (Ct) value ($P = 0.608$). Sampling zone impacted the log10 p72 genomic CN/mL ($P < 0.0001$) and Ct values ($P < 0.0001$), with a greater amount of viral genome detected on transient surfaces compared to other surfaces ($P < 0.05$). This study illustrates that once ASFV enters the feed mill environment it becomes widespread and movement of people can significantly contribute to the spread of ASFV in a feed mill environment.

Introduction

Commercial swine feed serving as a fomite for transmission of viral pathogens was not deemed a significant concern until soon after diagnosing porcine epidemic diarrhea virus (PEDV) in the US in 2013. It was reported that contaminated feedstuffs or their packaging arriving from Asia may have been involved with the introduction and transmission of PEDV in North America [1]. Due to the US naïve status to PEDV at the time along with the movement of contaminated vehicles associated with feed and animal delivery, the virus became endemic in the US. Another contributing factor to the quick spread of PEDV in the US was the feed mill. Once introduced into the feed mill, PEDV became widely distributed [2], serving as a continuous source of disease to the workers and feed delivery vehicles. Decontamination methods were often unsuccessful to rid the environment of PEDV but were also expensive and time consuming [3] while sequencing of diets within the feed mill to dilute the virus within the feed were also unsuccessful at eliminating PEDV [4]. The outbreak of PEDV in the US was the first to suggest that the feed manufacturing and distribution system aided in the widespread transmission of disease.

African swine fever is a devastating virus endemic in Africa, Asia, and Europe with substantial impacts on swine production and economic implications [5]. Multiple routes of ASFV transmission to domestic swine have been characterized including domestic swine to domestic swine, wild suids to domestic swine, and via the soft tick of the Ornithodoros genus serving as a vector [6]. Within these, multiple mechanisms of transmission can occur whereby domesticated swine become infected with ASFV including direct animal contact, contact with contaminated fomites, or exposure to contaminated feedstuffs or water [7]. Extended stability of ASFV within pork products has been widely documented [8], detection of ASFV DNA within pork products crossing international borders has been documented [9, 10], and strong evidence of ASFV transmission via contaminated food products has been documented [11, 12].

Given the known fact that contaminated pork products can lead to ASFV infection in naïve animals, recent research has focused on further understanding of the risk of commercial swine feed serving as a vector for ASFV transmission. Recent research has shown that ASFV can survive in various feed ingredients during transboundary, transatlantic shipping and work has been conducted characterizing the infectious dose in water and feed [13, 14]. Field evidence suggests that ASFV can be distributed throughout the feed supply chain [15], but there are no controlled studies demonstrating the properties of ASFV in a feed mill environment, making it impossible to develop science-based recommendations or policy. Therefore, the objective of this study was to use an ASFV-contaminated ingredient in the feed manufacturing process to evaluate the cross-contamination to subsequent batches of feed and contamination of the feed mill environment.

Materials and methods

The study was conducted at the Biosecurity Research Institute (BRI) in Manhattan, KS, with approval by the Kansas State University Institutional Biosafety Committee (project approval #1427.1). The feed manufacturing process was done within a BSL-3Ag large animal room while laboratory work was done within a BSL-3+ laboratory space.

Preparation of the inoculum

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

Feed manufacturing

Feed was manufactured as described by Schumacher et al. [2]. Briefly, the feed manufacturing system was first primed with an ASFV-free batch of feed which was subsequently followed by a second batch of feed that was contaminated with ASFV. Four additional batches of ASFV-free feed were then mixed and discharged through the same equipment without any cleaning or disinfection occurring between batches. For this study, a corn and soybean-meal based diet with a composition normally fed to gestating sows was manufactured at the Kansas State University O.H. Kruse Food Technology Innovation Center (Manhattan, KS; Table 1) and transported to the BSL-3Ag facility.

Negative Control (Batch 1) – Priming the feed mill: To initiate the trial, a 25 kg batch of ASFV-free feed was mixed in a 50 kg capacity steel mixer with a 0.0.113 cm^3 electric paddle mixer (H.C Davis Sons Manufacturing, model # SS-L1; Bonner Springs, KS). The feed was mixed for five minutes then discharged at a rate of approximately 4.5 kg/min into the conveyor

(Universal Industries, Cedar Falls, IA) that carried 74 buckets (each 114 cm_3) of feed. The feed was conveyed and discharged through a downspout into double-lined bags.

Positive Control (Batch 2) - ASFV-contaminated feed: Upon completion of priming the system with the initial batch of ASFV-free feed, 530 mL of a genotype II (Armenia 2007) ASFVfrican swine fever virus (2.7×10^6 $TCID_{50}$/mL) was then mixed with 4.7 kg of diet in a 5 kg stainless steel mixer (Cabela's Inc., Sidney, NE) to make 5.23 kg of ASFV-contaminated feed. This was subsequently added to 20 kg of feed and then mixed, conveyed, and discharged using the same equipment and procedures as previously described for the negative control. The final concentration of the inoculated positive control batch of feed was 5.6×10^4 $TCID_{50}$/gram.

Sequences 1-4 (Batch 3, 4, 5, and 6) - Milling of subsequent batches of feed: Following discharge of the positive control batch of feed, the same process of mixing, conveying, and discharging 25 kg batches of feed was repeated four additional times using ASFV-free diet.

Environmental Sampling

Environmental sampling was conducted similar to Huss et al. [3] and Schumacher et al. [2]. Environmental swabs were taken for the negative control, positive control, and batch sequences 1-4. Negative control samples were taken after priming the feed mill, positive control samples were taken after the usage of ASFV- contaminated feed, and batch sequences 1-4 samples were taken after each subsequent batch. All environmental swabs collected on previously marked environmental surfaces prior to inoculation with ASFV had no detectable ASFV DNA.

After each batch of feed was manufactured, environmental surfaces were swabbed using 10 cm × 10 cm cotton surgical gauze squares pre-moistened with 5 mL of phosphate-buffered solution (PBS) and individually stored in a 50 mL conical tube prior to usage. Prior to sample

collection, a clean pair of outside gloves were donned and tubes aseptically opened by a sampling assistant. The previously chosen and marked location was swabbed, the environmental swab placed back in the conical tube, and outside gloves were changed. Once the experiment was concluded, samples were transferred to the BSL-3+ laboratory following appropriate procedures.

Locations for environmental sampling were chosen based off proximity to feed (Table 2). Feed contact surface locations were the mixer ribbon, mixer barrel, mixer discharge, bucket elevator bucket, bucket elevator belt, and bucket elevator discharge. Non-feed contact surfaces < 1 m from feed locations were wall less than 1 m to mixer, wall less than 1 m to bucket elevator, floor less than 1 m to mixer, floor less than 1 m to bucket elevator, and ceiling less than 1 m to mixer. Non-feed locations > 1 m from feed locations were wall greater than 1 m from mixer, floor greater than 1 m from mixer, floor greater than 1 m from bucket elevator, and ceiling greater than 1 m from mixer. Transient surface locations were the boot soles of researchers walking through all other zones.

DNA extraction and quantitative ASFV real-time PCR (qPCR)

Environmental swabs were tested at a BSL-3+ laboratory in the Biosecurity Research Institute in Manhattan, KS. Briefly, to each swab within a 50 mL conical tube, 20 mL of PBS was added, the tube was capped and inverted, and incubated overnight in 4ºC. Tubes were vortexed for about 30 seconds and held upright for 5 minutes. Approximately 10 mL of supernatant was recovered, aliquoted into 5 mL cryovials, and stored at -80°C until processed for qPCR. In preparation for magnetic bead-based DNA extraction, 500 µL of PBS eluent was combined with 500 µL of Buffer AL (Qiagen, Germantown, MD, USA), briefly vortexed, and incubated at 70°C for 10 minutes in an oscillating heat block. DNA extraction was carried out

using the GeneReach DNA/RNA extraction kit on a TacoTM mini automatic nucleic acid extraction system (GeneReach, Boston, MA, USA). The extraction was performed according to the manufacturer's instructions with modifications. Briefly, 200 µL of AL sample lysate was transferred to column A of the taco deep-well extraction plate which contained 500 µL of the GeneReach lysis buffer and 50 µL of magnetic beads, followed by addition of 200 µL of molecular grade isopropanol (ThermoFisher Scientific, Waltham, MA, USA). The extraction consisted of two washes with 750 µL of wash buffer A, one wash with 750 µL wash buffer B, and a final wash with 750 µL of 200 proof molecular grade ethanol (ThermoFisher Scientific). After a five-minute drying time, DNA was eluted with 100 µL elution buffer and subsequently transferred into 1.5 mL DNA/RNA-free centrifuge tubes (VWR) for storage. Positive and negative extraction controls were included in sample processing and consist of the positive extraction control, a partial sequence of the ASFV p72 gene cloned into plasmid Bluescript II and PCR-grade water.

Real-time quantitative PCR (qPCR) was carried out using primers and probes designed to detect the gene encoding for ASFV p72 [16] and PerfeCTa® FastMix II® (Quanta Biosciences, Gaithersburg, MD, USA) on the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). qPCR reactions were performed in duplicate with each well containing 5 µL of template DNA, 0.2 µL (200nM) of each primer (Integrated DNA Technology, Coralville, IA, USA), and 0.4 µL (200nM) of FAM probe (Thermo Fisher Scientific) in a total reaction volume of 20 µL. Thermocycling conditions were 95°C for 5 minutes, followed by 45 cycles of 95°C for 10 seconds and 60°C for 1 minute.

Genomic copies quantification

ASFV p72 genomic copy number (CN) was calculated using reference standard curve methodology using a reference standard curve composed from ten-fold serial dilutions performed in triplicate of the quantitated ASFV p72 plasmid DNA control. Copy number for samples were mathematically determined using the PCR-determined cycle threshold (Ct) for ASFV p72 (two PCR well replicates) and the slope and intercept of the ASFV p72 DNA standard curve. Data are reported as PCR determined copy number per mL of solution recovered from environmental swab sample processing.

Statistical analysis

Data were analyzed as 4 × 5 factorial arrangement with 4 sampling surfaces, and 5 batches of feed not including the initial negative control samples. Individual sample collected from a surface for a specific batch was considered the experimental unit.

Visualization on data was performed using the ggplot2 package using the RStudio environment (Version 1.2.1335, RStudio, Inc., Boston, MA) using R programming language [Version 3.6.1 (2019-07-05), R Core Team, R Foundation for Statistical Computing, Vienna, Austria]. The proportion of PCR reactions positive for detectable ASFV DNA are reported as # of PCR positive reactions/total # of PCR reactions. The proportion of PCR reactions having detectable ASFV DNA was fit using the glmer function in the lme4 package using a binomial distribution with the fixed effects of sampling zone, batch of feed, and the associated interaction with a random effect of environmental swab to indicate the appropriate level of experimental replication given the duplicate qPCR analysis of environmental swabs.

Genomic CN/mL and Ct were analyzed using a linear mixed model fit using the lme function in the nlme package using similar fixed and random effects as previously mentioned.

Results of Ct and p72 genomic CN/mL data are reported as least squares means ± standard error of the mean. Samples not containing detectable ASFV DNA were assigned a value of 45 because that was the greatest number of cycles the qPCR assay performed before concluding a sample did not have detectable ASFV DNA. Genomic CN/mL values were $\log_{10}$ transformed prior to data analysis to satisfy the assumption of normality. All statistical models were evaluated using visual assessment of studentized residuals and models accounting for heterogeneous residual variance were used when appropriate. A Tukey multiple comparison adjustment was incorporated when appropriate. Results were considered significant at $P \leq 0.05$ and marginally significant between $P > 0.05$ and $P \leq 0.10$.

Results

As expected, environmental swabs collected prior to inoculation had no detectable ASFV DNA (Table 3). Environmental swabs collected after the manufacture of ASFV-contaminated feed showed presence of ASFV-specific DNA in all zones with 38% (95% confidence limit = 6.4-78.3%) to 100% (95% confidence limit = 0-100%) of qPCR reactions resulting in detectable ASFV DNA depending on the contact surface. There was no evidence of a sampling zone × batch of feed interaction for prevalence of qPCR reactions detecting ASFV DNA ($P = 0.912$), log10 genomic copies/mL ($P = 0.625$), or Ct value ($P = 0.608$). Additionally, there was insufficient evidence to conclude that the proportion of qPCR positive reactions was affected by sampling zone ($P = 0.701$) or batch of feed ($P = 1.000$).

Batch of feed influenced the Ct value for environmental samples ($P = 0.037$), with samples collected after manufacture of the ASFV- contaminated batch of feed having a lower Ct value compared to the environmental swabs collected after sequence 3 ($P < 0.05$; Table 4). Environmental swabs collected after other sequences (1, 2, 4) were intermediate in terms of Ct

value. There was marginally significant evidence that batch of feed influenced $\log_{10}$ p72 genomic copy/mL ($P = 0.059$), however no significant pairwise differences were detected when using a Tukey multiple comparison adjustment.

There was a significant difference in both the Ct value and $\log_{10}$ genomic copy/mL values between sampling zones ($P < 0.0001$), with the transient surfaces having lower Ct values ($P < 0.05$) and greater $\log_{10}$ p72 genomic copies/mL ($P < 0.05$) compared to all other sampling zones. This indicates that the soles of worker boots contained a greater quantity of detectable ASFV DNA compared to all other sampling zones, including feed contact and non-feed contact surfaces.

Discussion

African swine fever is a devastating disease not only for substantial morbidity and mortality, but also serious economic consequences associated with global trade [5]. The virus is a double stranded DNA virus of the family Asfarviridae and has an external lipid envelope [17]. Multiple routes of transmission to domestic swine have been characterized including domestic Swine to domestic swine, wild suids to domestic swine, and soft tick of the Ornithodoros genus [6]. Within these, multiple mechanisms of transmission can occur whereby domesticated swine become infected with ASFV including direct animal contact, contact with contaminated fomites, or exposure to contaminated feedstuffs or water [7]. Extended stability of ASFV within pork products has been widely documented [8], detection of ASFV DNA within pork products crossing international borders has been documented [9, 10], and strong evidence of ASFV transmission via contaminated food products has been documented [11, 12]. Thus, understanding the risk for ingestion of ASFV contaminated feedstuffs is very important to prevent infection with ASFV in swine populations. While it has clearly been demonstrated that consumption of

contaminated food products can result in ASFV transmission, the risk of feedstuffs serving as a potential vector for pathogen transmission in modern swine production with limited access to potentially contaminated pork products is not as well characterized to date.

Traditionally, biosecurity for the swine industry has focused on preventing pathogen entry onto the farm by controlling the safety of incoming animals, personnel, and supplies. However, the entry and spread of PEDV throughout North American in 2013–2014 speculated that feed could serve as a fomite and the feed supply chain could help spread the virus but ultimately, research demonstrated that feed could serve as a potential fomite for viral transmission and contaminate the feed delivery supply chain [1]. The introduction and dissemination of PEDV in North America served to shift the mindset of swine producers and feed manufacturers and a greater degree of attention was directed towards a framework for extending biosecurity practices to feed and feed mills. Previous research using other viruses [18–22] has established that mitigation techniques are largely expensive and impractical, so prevention of pathogen introduction into the feed supply chain is critical. Lessons learned through experiences with PEDV in regards to feed biosecurity served to shift the mindset and practices of the swine feed industry.

Previous work with PEDV has demonstrated that once viral contamination is introduced into feed manufacturing equipment, the contamination can be detected on surfaces after several subsequent batches of feed [2]. Furthermore, contamination on non-feed contact surfaces persists longer than contamination of feed-contact surfaces due to the abrasive and dilution properties of successive batches of feed. This can be observed as a reduction of detection of PEDV RNA as subsequent batches of feed are manufactured [3], while still being able to detect contamination on environmental surfaces. These findings are hugely problematic because they describe that if

PEDV enters a feed mill environment, the risk of transmission is not just with one batch of feed containing a contaminated ingredient, but that risk may persist across multiple batches of feed, including those that do not directly contain the suspect ingredient. Prior to this experiment, it was not known whether ASFV would have similar characteristics to PEDV and if biosecurity measures in place to detect PEDV would also be effective for ASFV detection within a feed manufacturing environment.

In the current experiment, it is evident that distribution of ASFV into the feed manufacturing environment is widespread and persists even after manufacturing additional feed batches initially free of ASFV. This is similar to what is observed with PEDV [3]. This indicates that it is extremely important for the US to prevent the entry of ASFV into US feed mills since once ASFV is in a feed mill, it will remain in its environment for an extended period of time. This knowledge is important to consider when designing and implementing surveillance and monitoring programs for ASFV as currently being investigated in ASFV endemic regions [15].

The present study demonstrates that transient surfaces had the highest amount of detectable ASFV DNA across all zones. This indicates that people and personal protective equipment (PPE) have a high potential to spread viruses within the feed mill. This is a consistent finding because it was previously reported that moving objects of a farm, like trucks and feed, contributed to the spread of PEDV, and that PPE and people transmitted PEDV to naïve herds [23, 24]. An understanding of the contamination within the feed mill environment is vital due to how the US manufactures and distributes feed within the swine industry. If a feed truck is contaminated, there is a risk that it could contaminate the production site it is delivering to, but it also could potentially contaminate the feed mill when returning from a production site currently experiencing a disease outbreak. Additionally, recent information from Vietnam has indicated

that feed trucks are an area where contamination with ASFV can be found [15]. This current study along with previous studies highlight the importance of understanding the epidemiological interaction of the US feed delivery system regardless of the virus of concern.

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

In conclusion, this study reveals that contamination with ASFV was rapid and widespread within the swine feed manufacturing facility after introduction through inoculated feed and presence of ASFV-specific DNA minimally changed with each subsequent batch. This study also proved that if there is viral contamination within the feed mill environment, it can be found with environmental swabs. In areas where ASFV is considered endemic, environmental swabs can be incorporated into surveillance programs or feed mill audits to understand the potential contamination within the feed mill and respective delivery system. In the present study, it was also demonstrated that transient surfaces play an important role in the spread of virus through the feed mill. Moving objects like people, PPE, and trucks should be taken in account

when designing feed biosecurity protocols and feed/feed mill surveillance could be pivotal in maintaining appropriate feed biosecurity.

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Table 5.1 Diet composition (as-fed basis)

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

[1] Conventional dehulled, solvent extracted soybean meal.

[2] Each kg of premix contains 73 g Fe, 73 g Zn, 22 g Mn, 11 g Cu, 198 mg I, and 198 mg Se.

[3] Each kg of premix contains 1,650,000 IU vitamin A, 8,800 IU vitamin E, 88 mg biotin, 396 mg pyridoxine, 880 mg folic acid, 220,000 mg choline, 79 mg chromium, 19,800 mg L-carnitine.

[4] Each kg of premix contains 1,650,000 IU vitamin A, 660,000 IU vitamin D3, 17,600 IU vitamin E, 1,320 mg menadione, 3,300 mg riboflavin, 11,000 mg d-pantothenic acid, 19,800 mg niacin, 13 mg vitamin B12.

[5] HiPhos 2700 (DSM Nutritional Products, Parsippany, NJ).

[6] NRC. 2012. Nutrient Requirements of Swine, 11th ed. Natl. Acad. Press, Washington D.C.

Table 5.2 Location of environmental swabs and grouping by zone.

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

Table 5.3 Interactive effect of feed batch and zone on detection of African swine fever virus (ASFV) during manufacture of virus inoculated feed[1,2]

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

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

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

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

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

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

Table 5.4 Main effect of feed batch and zone on detection of African swine fever virus (ASFV) during manufacture of virus inoculated feed[1,2]

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

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

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

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

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

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

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

Chapter 6 - Effect of mixing and feed batch sequencing on the prevalence and distribution of African swine fever virus in swine[5] feed

Abstract

It is critical to have methods that can detect and mitigate the risk of African swine fever virus (ASFV) in potentially contaminated feed or ingredients bound for the United States. The purpose of this work was to evaluate feed batch sequencing as a mitigation technique for ASFV contamination in a feed mill, and to 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, Kansas. 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 final concentration of 5.6×10^4 $TCID_{50}$/g. Then, four subsequent ASFV-free batches of feed were manufactured. After discharging each batch into a collection container, 10 samples were collected in a double 'X' pattern. Samples were analyzed using a qPCR assay for ASFV p72 gene then the cycle threshold (Ct) and Log10 genomic copy number (CN)/g of feed were determined. The qPCR Ct values (P<.0001) and the Log10 genomic CN/g (P<.0001) content of feed samples were impacted based on the batch of feed. Feed samples obtained after manufacturing the ASFV-contaminated diet contained the greatest amounts of ASFV p72 DNA

[5] This work was published in *Transboundary and Emerging Disease.* Elijah CG, Trujillo JD, Jones CK, Kwon T, Stark CR, Cool KR, Paulk CB, Gaudreault NN, Woodworth JC, Morozov I, Gallardo C, Gebhardt JT, Ricjt JA. (2021). Effect of mixing and feed batch sequencing on the prevalence and distribution of African swine fever virus in swine feed. *Transboundary and Emerging Disease,* 1-6. doi:10.1111/tbed,14177

across all criteria ($P < .05$). Quantity of ASFV p72 DNA decreased sequentially as additional batches of feed were manufactured, but was still detectable after batch sequence 4. This subsampling method was able to identify ASFV genetic material in feed samples using p72 qPCR. In summary, sequencing batches of feed decreases concentration of ASFV contamination in feed, but does not eliminate it. Bulk ingredients can be accurately evaluated for ASFV contamination by collecting 10 subsamples using the sampling method described herein. Future research is needed to evaluate if different mitigation techniques can reduce ASFV feed contamination.

Keywords: African swine fever virus, bulk sampling, feed batch sequencing, feed safety

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 (USDA- APHIS, 2015). This hypothesis was never unequivocally proven, but afterwards the concept of applying biosecurity practices to the United States (US) swine industry feed manufacturing and delivery systems became heavily emphasized. 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; Dee at al., 2018, Stoian et al., 2020). Improved biosecurity practices in the feed industry became particularly important in 2018 when a number of historically ASFV-free countries in Southeast Asia began to report ASFV cases (Gaudreault et al., 2020). 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 United States through the feed supply chain or other avenues. There is no active surveillance for ASFV in feed

or ingredients imported from ASFV-endemic regions, nor is there a validated protocol to sample or analyze for ASFV in a feed or ingredient matrix (USDA-APHIS-VS, 2019). It has been hypothesized that the same methods which demonstrated appropriate sensitivity and specificity for PEDV detection in feed may be applicable to ASFV, but this has not yet been tested. Furthermore, it has been suggested that mitigation measures common in PEDV, such as feed batch sequencing to reduce viral concentration, may be equally effective with ASFV. However, this has also never been evaluated. 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 (KSU) in Manhattan, KS, with approval by KSU'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 ethylendiaminetetraacetic acid (EDTA) from ASFV-infected pigs was mixed in RPMI media to prepare 530 mL of virus inoculum at the final concentration of 2.7×10^6 $TCID_{50}$/mL of ASFV genotype II virus (strain Armenia 2007).

Manufacturing and sampling

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

a. Negative Control (Batch 1) – Priming the feed mill: To initiate the trial, a 25 kg batch of ASFV-free feed was mixed in a 50 kg capacity steel mixer with a 0.113 m^3 electric paddle mixer (H.C Davis Sons Manufacturing, model # SS-L1; Bonner Springs, KS). The feed was mixed for five minutes then discharged at a rate of approximately 4.5 kg/min into the conveyor (Universal Industries, Cedar Falls, IA) that carried 74 buckets (each 114 cm^3) of feed. The feed was conveyed and discharged through a downspout into double-lined bags.

b. Positive Control (Batch 2) - ASFV-contaminated feed: Upon completion of priming the system with the initial batch of ASFV-free feed, 530 mL of a genotype II ASFV (strain Armenia 2007) at a concentration of 2.7×10^6 $TCID_{50}$/mL was then mixed with 4.7 kg of feed in a 5 kg stainless steel mixer (Cabela's Inc., Sidney, NE) to make 5.23 kg of ASFV-contaminated feed. This mixture was subsequently added to 20 kg of feed resulting in a final ASFV concentration of 5.6×10^4 $TCID_{50}$/g, and then mixed, conveyed, and discharged using the same equipment and procedures as previously described for the negative control.

c. Sequences 1-4 (Batch 3, 4, 5, and 6) – Manufacture of subsequent batches of feed: Following discharge of the ASFV-contaminated batch of feed, the same process of mixing, conveying, and discharging 25 kg batches of feed was repeated four additional times using ASFV-free feed. After a batch of feed was discharged, ten feed samples were collected similar as previously described by Jones et al. (2020). Briefly, the ten samples 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 imaginary diagonal lines were drawn from corner to corner to make an 'X'. Samples were taken from the corners each half along with a sample from the middle where the two imaginary diagonal lines crossed. The ten samples were not mixed together but analyzed in separate PCR reactions. This sampling technique resulted in a grand total of 60 feed samples for the entirety of the experiment.

Laboratory Analysis

Feed samples were tested at a BSL-3+ laboratory in the BRI. Briefly, 10 g of each feed sample was put in a tube, suspended with 35 mL of PBS, and the tube was capped and inverted, and incubated overnight at 4ºC. Approximately 10 mL of supernatant was recovered, aliquoted into 5 ml cryovials, and stored at -80°C until processed for qPCR. In preparation for magnetic bead-based DNA extraction, 500µL of PBS eluent was combined with 500µL of Buffer AL (Qiagen, Germantown, MD, USA), briefly vortexed, and incubated at 70°C for 10 minutes in an oscillating heat block. DNA extraction was carried out using the GeneReach DNA/RNA extraction kit on a Taco™ mini automatic nucleic acid extraction system (GeneReach, Boston, MA, USA). The extraction was performed according to the manufacturer's instructions with modifications. Briefly, 200µL of AL/sample lysate was transferred to column A of the taco™ deep-well extraction plate which contained 500µL of the GeneReach lysis buffer and 50µL of

magnetic beads, followed by addition of 200µL of molecular grade isopropanol (ThermoFisher Scientific, Waltham, MA, USA). The extraction consisted of two washes with 750µL of wash buffer A, one wash with 750µL wash buffer B, and a final wash with 750µL of 200 proof molecular grade ethanol (ThermoFisher Scientific). After a five-minute drying time, DNA was eluted with 100 µL elution buffer and subsequently transferred into 1.5mL DNA/RNA- free centrifuge tubes (VWR) for storage. Positive and negative extraction controls were included in sample processing and consisted of a positive extraction control which was a partial sequence of the ASFV p72 gene cloned into plasmid Bluescript II and a negative extraction control, which was PCR-grade water.

Real-time quantitative PCR (qPCR) was carried out using primers and probes designed to detect the gene encoding for ASFV p72 and PerfeCTa FastMix II (Quanta Biosciences, Gaithersburg, MD, USA) on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The qPCR reactions were performed in duplicate with each well containing 5µL of template DNA, 0.2µL (200nM) of each primer (Integrated DNA Technology, Coralville, IA, USA), and 0.4µL (200nM) of FAM probe (Thermo Fisher Scientific) in a total reaction volume of 20 µL. Thermocycling conditions were 95°C for 5 minutes, followed by 45 cycles of 95°C for 10 seconds and 60°C for 1 minute.

ASFV p72 genomic copy numbers (CN) were calculated using reference standard curve methodology using a reference standard curve composed from ten-fold serial dilutions performed in triplicate of the quantitated ASFV p72 plasmid DNA control. The CN for samples was mathematically determined using the PCR-determined cycle threshold (Ct) for ASFV p72 (two PCR well replicates) and the slope and intercept of the ASFV p72 DNA standard curve. Genomic CN/g for each sample was based upon the genomic CN/mL and determined as follows:

the value of genomic CN/mL was multiplied by the value 35 due to feed samples being suspended in 35 mL of PBS then divided by 10 because there was 10 g of feed per suspension in order to get the respective genomic CN/g.

Statistical Analysis

Statistical analysis for this study was performed using R programming language [Version 3.6.1 (2019-07-05), R Core Team, R Foundation for Statistical Computing, Vienna, Austria]. 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.

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

Results were considered significant at $P \leq 0.05$ and marginally significant between $P > 0.05$ and $P \leq 0.10$.

Results and discussion

Outbreaks of PEDV in North America were the first events with a potential link between contaminated feed and transmission of disease to pigs (USDA-APHIS, 2015). Since then, veterinarians, producers, and feed manufacturers have focused their efforts to prevent biological hazard transmission through the feed supply chain using both prevention and biosecurity strategies (USDA-APHIS, 2019; Stewart et al., 2020). Potential solutions to ensure feed safety have routinely included different types of mitigation strategies either utilizing these strategies alone or in combination with each other to reduce potential PEDV contamination within the feed (Cochrane et al., 2015, Cochrane et al., 2017, Gebhardt et al., 2016, and Schumacher et al., 2019). It has become commonplace for swine producers to exclude feed or ingredients from countries that are endemic for viruses currently not present in the US. However, this is sometimes difficult to implement because the U.S. must rely on agricultural trade with countries that are endemic for ASFV. For example, the majority of vitamins used in domestic swine diets are manufactured in ASFV-endemic countries (Shurson et al., 2019). While their manufacture is typically in biosecure laboratories and the ingredients themselves may pose low risk for foreign animal disease transmission, containers carrying these ingredients may become contaminated and thus a potential source of ASFV entry into the U.S. In theory, ingredients could be sampled for ASFV and screened for safety prior to entry into the country, but surveillance of this magnitude has not been implemented, partially due to the lack of validated bulk sampling or extraction methodologies (USDA-APHIS-VS, 2019). The Association of American Feed Control Officials (2014) and the Federal Drug Association (2021) recommend a similar method for

sampling bulk containers to account for the unequal distribution of swine viruses but this method still remain untested for many viruses. Jones et al. (2020) was successful at identifying PEDV in bulk containers utilizing this method but this sample strategy remains unproven for other viruses like ASFV or FMDV. Because of this, one of the intents of this study was to determine if a common bulk sampling strategy that could be used by U.S. regulators to detect feed contaminant levels to consistently detect ASFV contamination. This study also wanted to evaluate a feed mitigation technique of feed batch sequencing, a technique that has been tested with PEDV, to see if this would be a potential useful practice for ASFV-contaminated feed.

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 impacted the Ct value ($P < 0.0001$) and the Log10 genomic CN/g ($P < 0.0001$; Table 2) of samples. Samples taken from the feed manufactured with direct contamination with ASFV contained the greatest amount of ASFV p72 genetic material across all response criteria ($P < 0.05$). Sequence 1 had slightly 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 level 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. This suggests that flushing a feed mill with ASFV free feed after an ASFV contaminated feed will reduce the amount of ASFV in the feed but won't eliminate the virus entirely. Schumacher et al.

(2019) found similar results in their study evaluating sequencing to reduce PEDV contamination. The current study's findings also suggest that the X pattern sampling technique used was able to identify ASFV contamination within feed samples and supports the study by Jones et al. (2020) who had similar success with this sampling method for detecting various levels of PEDV contamination within feed containers. The probability of infection for the feed samples collected in this study could be estimated using the data on ASFV infectious dose and probability of infection recently published by Niederwerder et al. (2019). Based on their ASFV exposure model, the amount of genomic CN/g found in this study's feed samples from sequence 1-4 has an infection probability ranging from 0.25 – 1.00. However, Niederwerder et al. (2019) used genotype II ASFV but a different isolate (Georgia 2007/1) for their study, so infectivity based off their model is an extrapolation.

A limitation of this experiment is the lack of infectivity data associated with the feed samples containing ASFV p72-specific DNA. This research utilized ASFV, a BSL-3 pathogen and a US select agent, meaning to get approval to use this virus is rigorous, requires special laboratories and intensive training. Validating these feed samples for ASFV infectivity is important and will be an area of our future research efforts; however, the focus of this study was to determine if feed sequencing was an effective mitigant strategy for ASFV-contaminated feed and if feed sampling techniques could accurately identify ASFV genetic material. The data presented here provides significant value to the global feed and swine industry by establishing the presence of ASFV DNA in feed after first contaminating and then flushing a feed production system with subsequent batches of "clean" feed, along with the ability to detect ASFV genetic material in the feed which can provide information for urgently needed surveillance programs.

In conclusion, sequencing with four batches of feed after contamination of a feed mill with ASFV can decrease overall ASFV contamination within feed samples but not eliminate it entirely. In addition, collecting 10 evenly distributed samples using a X pattern collection system allows for the detection of ASFV genetic material under the conditions of the current investigation. The findings of this study highlight the importance of excluding ingredients from ASFV endemic countries but also highlights that proper sampling can be an effective tool to detect ASFV contamination. Additional research is necessary to evaluate the combination of mitigation techniques like chemically treating flush diets (similar to what is done with PEDV) on ASFV contaminated ingredients.

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Table 6.1 Detection of African swine fever virus (ASFV) p72 DNA in feed samples

	Batch of feed[†]					
	Negative	Positive	Sequence 1	Sequence 2	Sequence 3	Sequence 4
Positive	0/10	10/10	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

[†]Swine gestation feed was inoculated with African swine fever virus (ASFV) at 5.6×10^4 $TCID_{50}$/gram inoculated feed (positive) following an initial priming of the feed manufacturing equipment with ASFV free feed (negative). Four subsequent batches of feed were manufactured (sequence 1 to 4) and were initially free of ASFV. Ten feed samples were collected from each subsequent 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.

Table 6.2 Concentration of detectable African swine fever virus (ASFV) p72 DNA in feed samples.

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

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

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

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

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

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

Chapter 7 - Detection of African swine fever in feed and feed mill environment following extended storage

Abstract

One way to mitigate risk of feed-based pathogens for swine diets is to quarantine feed ingredients before inclusion in complete diets. Data has been generated evaluating the stability of swine viruses in ingredients, but the stability of African swine fever virus (ASFV) in feed or in a feed manufacturing environment has not been well characterized. Therefore, this study aimed to determine the stability of ASFV DNA in swine feed and on mill surfaces over time. A pilot-scale feed mill was used to manufacture six sequential batches of feed consisting of a batch of ASFV-free feed, followed by a batch inoculated with ASFV (final concentration = 5.6×10^4 $TCID_{50}$/gram), and then 4 subsequent ASFV-free batches. After each batch, 10 feed samples were aseptically collected in a double 'X' pattern. During feed manufacturing, 24 steel coupons were placed on the floor of the manufacturing area and allowed to collect dust during feed manufacturing. Once feed manufacturing was completed, feed samples and steel coupons were stored at room temperature. Three of each were randomly selected from storage on 3, 7, 14, 28, 60, 90, and 180 days after feed manufacturing and analyzed for ASFV DNA. For feed samples, there was evidence of a batch × day interaction ($P = 0.023$) for the quantification of genomic copies/g of feed, indicating that the amount of ASFV DNA present was impacted by both the batch of feed and days held at room temperature. There were no differences of genomic copies/g in early batches, but quantity of detectable ASFV decreased with increasing storage time. In batches 4-6, the greatest quantity of ASFV DNA was detected on the day of feed manufacturing. The lowest quantity was detected at day 7 for batch 4, day 60 for batch 5, and at 28 and 180 days for batch 6. There was no evidence of ASFV degradation on environmental discs across holding

times ($P = 0.433$). In conclusion, the quarantining of feed may help reduce but not eliminate the presence of ASFV DNA in feed over time. Importantly, ASFV DNA was detectable on feed manufacturing surfaces for at least 180 days with no overt evidence of reduction, highlighting the importance of bioexclusion of ASFV within feed manufacturing facilities and the need for thorough/effective decontamination and other mitigation processes in affected areas.
Keywords: African swine fever virus, environmental contamination over time, feed safety, virus stability, feed quarantine

Introduction

African swine fever virus (ASFV) can have devastating agricultural and economic consequences when introduced to a region which puts the United States' swine industry at risk as it maintains trade with ASFV endemic countries for feed ingredients (Gaudreault et al., 2020). Recent data suggests certain feed ingredients have the ability to support virus survival during simulated conditions of transatlantic shipping (Dee et al., 2018; Stoian et al., 2020). To prevent the spread of disease, feed manufacturing facilities hold, or quarantine, these ingredients before including them in complete swine diets (Dee et al., 2018). Periods of quarantine allow for viruses to naturally decay, thereby reducing infectivity (viral loads) within the feed over time (Stoian et al., 2019; Gebhardt et al., 2020). This risk mitigation practice also reduces the risk for a potential introduction by virus contaminated feed dust generated during manufacturing (Gebhardt et al., 2018a). However, by holding feed ingredients for long periods of time, it can reduce product quality because of ingredient instability over time (Saensukjaroenphon et al., 2020a; Saensukjaroenphon et al., 2020b). When considering research that has evaluated ASFV persistence, there is unanimous agreement that ASFV has the ability to persist for long periods of time in cured meats (Petrini et al., 2019), fat sources (McKercher et al., 1987), soil and wild boar

carcasses (Zani et al., 2020), and feces, urine, and oral fluids (Davies et al., 2017). However, when trying to extrapolate these findings to feed ingredients and feed manufacturing facilities, it can be challenging to define the limit to which ASFV is affected since most of this research has focused on porcine epidemic diarrhea virus (PEDV), an enveloped RNA virus. How these studies apply to ASFV, a DNA virus with proven environmental stability, remains uncertain. Therefore, the objectives of this study were (i) to evaluate the impact of storage over a period of six months at room temperature of ASFV-contaminated complete swine feed on ASFV stability and (ii) to evaluate the length of time that ASFV could be detected in a feed mill environment after its introduction through the milling of experimentally ASFV-inoculated swine feed.

Materials and Methods

General

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

Inoculation and Sampling

Eight and a half mL of pooled blood treated with ethylendiaminetetraacetic acid (EDTA) from ASFV infected pigs was mixed in RPMI media to prepare 530 mL of the virus inoculum at the final concentration of 2.7×10^6 $TCID_{50}$/mL of ASFV genotype II virus (Armenia 2007). Feed was manufactured as described by Elijah et al. (2021a, b). Briefly, the feed manufacturing system was first primed with an ASFV-free batch of feed which was subsequently followed by a

second batch of feed that was contaminated with ASFV. Four additional batches of ASFV-free feed were then mixed and discharged through the same equipment without any cleaning or disinfection occurring between batches. For this study, a corn and soybean-meal based diet with a composition normally fed to gestating sows was manufactured at the Kansas State University O.H. Kruse Food Technology Innovation Center (Manhattan, KS) and transported to the BSL-3 facility. Feed manufacturing was structured as follows:

a. Negative Control (Batch 1) – Priming the feed mill: To initiate the trial, a 25 kg batch of ASFV-free feed was mixed in a 50 kg capacity steel mixer with a 0.113 m^3 electric paddle mixer (H.C Davis Sons Manufacturing, model # SS-L1; Bonner Springs, KS). The feed was mixed for five minutes then discharged at a rate of approximately 4.5 kg/min into the conveyor (Universal Industries, Cedar Falls, IA) that carried 74 buckets (each 114 cm^3) of feed. The feed was conveyed and discharged through a downspout into double-lined bags.

b. Positive Control (Batch 2) - ASFV-contaminated feed: Upon completion of priming the system with the initial batch of ASFV-free feed, 530 mL of a genotype II ASFV (strain Armenia 2007) at a concentration of 2.7×10^6 $TCID_{50}$/mL was then mixed with 4.7 kg of feed in a 5 kg stainless steel mixer (Cabela's Inc., Sidney, NE) to make 5.23 kg of ASFV-contaminated feed. This mixture was subsequently added to 20 kg of feed resulting in a final ASFV concentration of 5.6×10^4 $TCID_{50}$/g, and then mixed, conveyed, and discharged using the same equipment and procedures as previously described for the negative control.

c. Sequences 1-4 (Batch 3, 4, 5, and 6) – Manufacture of subsequent batches of feed: Following discharge of the ASFV-contaminated batch of feed, the same process of mixing, conveying, and discharging 25 kg batches of feed was repeated four additional times using ASFV-free feed.

After a batch of feed was discharged, ten feed samples were collected similar as previously described by Jones et al. (2020). Briefly, the ten samples 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 imaginary diagonal lines were drawn from corner to corner to make an 'X'. Samples were taken from the corners each half along with a sample from the middle where the two imaginary diagonal lines crossed. The ten samples were not mixed together but analyzed in separate PCR reactions. This sampling technique resulted in a grand total of 60 feed samples for the entirety of the experiment.

Nine stainless steel coupons (Built So-Well Manufacturing, Manhattan, KS), 10 cm × 10 cm in size, referred to as environmental discs, were placed at floor level in 3 different locations within the BSL-3 Ag large animal room (1 location near the feed manufacturing equipment and 2 locations in different corners of the room, outside the working area). Environmental discs (n=27) were allowed to collect dust during the feed manufacturing process and rested overnight. The following day all environmental discs were placed into a storage container and stored at RT in a locked cabinet. At day 0, and 3, 7, 14, 28, 60, 90, and 180 days after feed manufacturing, 1 environmental disc from each of the 3 locations were randomly selected and sampled (n=3) using a 10 cm × 10 cm cotton gauze as previously described (Elijah et al., 2021a). Remaining environmental discs not used for this analysis were discarded at the conclusion of the study following BSL-3 laboratory protocols for disposal.

Laboratory Analysis

Feed samples and environmental swabs from environmental discs were processed and tested in a BSL-3 laboratory in the BRI. For the feed samples, each 10 g sample was put in a 50 ml conical tube, suspended in 35 mL of PBS, the tube capped and inverted, then incubated overnight at 4ºC.

Approximately 10 mL of supernatant was recovered, aliquoted into 5 ml cryovials, and stored at -80°C until processed for qPCR. For environmental swabs from environmental discs, 20 mL of phosphate buffered solution was added to each swab within a 50 ml conical tube, tube capped, inverted, and incubated overnight in 4ºC. Tubes were vortexed for about 30 seconds and held upright for 5 minutes. Approximately 10 mL of supernatant was recovered, aliquoted into 5 ml cryovials, and stored at -80°C until processed for qPCR.

Feed samples and environmental disc samples were processed in a similar manner for the recovery of DNA for PCR testing. In preparation for magnetic bead-based DNA extraction, 500µL of PBS eluent was combined with 500µL of Buffer AL (Qiagen, Germantown, MD, USA), briefly vortexed, and incubated at 70°C for 10 minutes in an oscillating heat block. DNA extraction was carried out using the GeneReach DNA/RNA extraction kit on a Taco™ mini automatic nucleic acid extraction system (GeneReach, Boston, MA, USA). The extraction was performed according to the manufacturer's instructions with modifications. Briefly, 200µL of AL sample lysate was transferred to column A of the taco deep-well extraction plate which contained 500µL of the GeneReach lysis buffer and 50µL of magnetic beads and mixed by pipetting. Two hundred µL of molecular grade isopropanol (ThermoFisher Scientific, Waltham, MA, USA) was added to this well prior to extraction. The extraction consisted of two washes with 750µL of wash buffer A, one wash with 750µL wash buffer B, and a final wash with 750µL of 200 proof molecular grade ethanol (ThermoFisher Scientific). After a five-minute drying time, DNA was eluted with 100 µL elution buffer and subsequently transferred into 1.5mL DNA/RNA- free centrifuge tubes (VWR) for storage. Positive and negative extraction controls were included in sample processing and consist of the positive extraction control, a plasmid containing partial sequence of the ASFV p72 and PCR-grade water (negative).

Real-time quantitative PCR (qPCR) was carried out using primers and probes designed to detect the gene encoding for ASFV p72 and PerfeCTa® FastMix II® (Quanta Biosciences, Gaithersburg, MD, USA) on the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The qPCR reactions were performed in duplicate with each well containing 5µL of DNA, 0.2µL (200nM) of each primer (Integrated DNA Technology, Coralville, IA, USA), and 0.4µL (200nM) of FAM probe (Thermo Fisher Scientific) in a total reaction volume of 20 µL. Thermocycling conditions were 95°C for 5 minutes, followed by 45 cycles of 95°C for 10 seconds and 60°C for 1 minute.

ASFV p72 genomic copy number (CN) was calculated using reference standard curve methodology using a 10-point reference standard curve composed from ten-fold serial dilutions performed in triplicate of a quantitated ASFV p72 plasmid DNA control. CN for samples were mathematically determined using the PCR-determined mean cycle threshold (Ct) for ASFV p72 (two PCR well replicates) and the slope and intercept of the ASFV p72 DNA standard curve. Data are reported as PCR determined copy number per mL. Genomic CN/g for each sample was based upon the genomic CN/mL of solution recovered during sample processing, multiplied by the volume of phosphate buffered solution added during sample processing (35 mL), then divided by the amount of feed per suspension (10 g).

Statistical Analysis

Statistical analysis was performed using R programming language [Version 3.6.1 (2019-07-05), R Core Team, R Foundation for Statistical Computing, Vienna, Austria]. Experimental units were the feed and environmental samples. Each feed and environmental sample had one extraction for PCR assay and each extraction was run in duplicate for PCR analysis. However,

for feed sample results from batch 2 on day 1, each feed sample had two extractions for PCR assay and both extractions were run in duplicate for PCR analysis.

For feed samples, response values for the ASFV P72 gene were analyzed using a linear model fit with the lme function in the nlme packing and a normal distribution with the fixed effect as batch, day, and the associated interaction with a random effect of sample to indicate the appropriate level of experimental replication given the duplicate qPCR analysis of feed samples. For environmental discs, response values for the ASFV P72 gene were analyzed using a linear model fit with the lme function in the nlme packing and a normal distribution with the fixed effect as day with a random effect of sample.

Results of Ct and quantification of genomic copies are reported as least square means ± standard error of the mean. Samples not containing detectable ASFV DNA were assigned a value of 45 because that was the greatest number of cycles the qPCR assay was performed before concluding a sample did not have detectable ASFV DNA. Genomic copy data were transformed with $\log_{10}$ function and analysis included PCR negative reactions using a value of 0 for the quantified genomic CN/mL or CN/g. All statistical models were evaluated using visual assessment of studentized residuals and models accounting for heterogeneous residual variance were used when appropriate. A Tukey multiple comparison adjustment was incorporated into all statistical models. Results were considered significant at $P \leq 0.05$ and marginally significant between $P > 0.05$ and $P \leq 0.10$.

Results and Discussion

African swine fever virus is the only member of the viral family *Asfaviridae* with *Ornithodoros* ticks serving as biological vectors and reservoir hosts besides wild boars, wart hogs, and other wild suid native African pig species (Gaudreault et al., 2020). Transmission of ASFV has been

documented via direct contact with infected pigs or indirect contact through contaminated fomites. Additionally, ASFV can be detected in various feed matrices subjected to transatlantic shipping conditions and has been shown to be highly stable within the environment in a feed production system, specifically in areas of high foot traffic and on worker's clothing (Dee et al., 2018; Stoian et al., 2019; Blome et al., 2020; Stoian et al., 2020; Niederwerder et al., 2021 Gebhardt et al., 2022). An understanding of ASFV properties in swine feed and a feed mill environment is pivotal since the US relies on trade with ASFV endemic countries for feed ingredients for swine feed (Shurson et al., 2019). While these feed ingredients are manufactured in settings that are regulated and controlled by third party auditors, there is still the potential that these feed ingredients could become contaminated with ASFV during shipping and transportation. Due to the necessity of this trade relationship, research has focused on understanding the implications of ASFV contamination into a feed mill, how feed batch sequencing helps reduce contamination in the subsequent batches and how this impacts the feed mill environment (Elijah et al., 2021a; Elijah et al., 2021b). This work is critical to prevent further spread of ASFV, which has expanded to regions of Asia and Europe and confirmed to be present in the western hemisphere (Hispaniola island) for the first time in forty years (Gaudreault et al., 2020; Paulino-Ramirez and Jimenez, 2021). However, there are gaps in understanding the impacts of holding times on ASFV contaminated feed and how long ASFV can persist in a feed mill environment. When considering previous research, it has been documented that feed mills could harbor PEDV in the feed mill environment for long periods of time and decontamination of the feed mill would be largely impractical if contaminated with PEDV (Huss et al., 2017; Schumacher et al., 2018). When comparing the two viruses, it could be assumed that ASFV could persist within a feed mill environment and feed for longer periods of time due to innate

qualities of this virus but there is limited research on this topic at this time. Therefore, this study sought to understand how sequencing batches of feed impacted quantity of ASFV in swine feed over various holding times and how long ASFV can be detected in environmental samples collected from feed manufacturing surfaces.

Batch 1 feed samples were PCR negative, as expected, since this was the priming batch and manufactured before ASFV was introduced into the feed manufacturing system. For batch 2 feed samples, ASFV DNA was detected in feed samples across all holding times (day 0 – 180). After each successive batch of feed, the quantity of ASFV DNA generally decreased as holding times increased (Table 1). For ASFV P72 genetic material, there was a marginally significant batch × day interaction for Ct value ($P = 0.072$) and a significant batch × day interaction for $\log_{10}$ genomic CN/g ($P = 0.023$) in feed samples, indicating the batch of feed and days held at room temperature impacted the quantity of ASFV DNA. Quantity of $\log_{10}$ genomic CN/g in feed samples from batch 2 and 3 did not differ across holding dates ($P > 0.05$). For batch 4, the quantity of ASFV detected was lower ($P < 0.05$) on day 7 compared to day 1 with the other days of analysis being intermediate. In batch 5, the quantity of ASFV detected was lower ($P < 0.05$) on day 60 compared to day 1 with the other days of analysis being intermediate. While in batch 6, the quantity of ASFV detected was lower ($P < 0.05$) on days 28 and 180 compared to day 1 with the other days of analysis being intermediate. The variability of ASFV DNA in the feed samples for each batch after different holding times is most likely due to how feed samples were collected for PCR analysis and how each successive batch started with an unknown, diluted, amount of ASFV DNA. In general, these findings are similar to Gebhardt et al. (2018b) where holding time influenced the amount of PEDV detected within feed samples. In Gebhardt et al.

(2018b) the authors only held feed for 42 days but the findings were similar suggesting holding feed is a potential technique to lessen contamination risk for feed mills to employ.

The main effect of batch ($P < 0.0001$) and day ($P = 0.0001$) were statistically significant for Ct values (Table 2). In batch 2, the quantity of ASFV DNA detected was greater than batch 4, 5, and 6 ($P < 0.05$) indicating that the batch that was experimentally inoculated had the greatest amount of ASFV DNA while subsequent non-inoculated batches had lower amounts of ASFV DNA. For holding dates, day 1 feed samples had more ASFV DNA compared to days 7, 60, and 180 ($P < 0.05$) with all other holding dates intermediate ($P < 0.05$) indicating that feed samples analyzed on the day of feed manufacturing had greater amounts of ASFV DNA detected and that the quantity of ASFV DNA decreased as feed was held for periods of time.

For $\log_{10}$ genomic copies/g of feed, main effects of batch ($P < 0.0001$) and day ($P < 0.0001$) were statistically significant. Similar to the Ct values, batch 2 feed samples had greater quantities of ASFV detected compared to batch 4, 5, and 6 ($P < 0.05$) indicating that the batch that was experimentally inoculated had the greatest amount of ASFV DNA while subsequent ASFV-free batches had lower amounts of ASFV DNA. For holding dates, day 1 feed samples had greater amounts of ASFV DNA compared to all other holding dates except for day 7 which was intermediate ($P < 0.05$) indicating that amount of ASFV DNA was greatest on the day of manufacturing and decreased as feed samples were held for extended periods of time suggesting natural decay of the virus occurred.

When introduced into the feed mill environment after experimental inoculation, ASFV DNA was detected on environmental discs even after long periods of RT storage (Table 3). For ASFV P72 genetic material, there was no evidence of a change in Ct value ($P = 0.449$) or $\log_{10}$ genomic CN/mL ($P = 0.433$) of environmental samples from environmental discs over time. Thus, even

after 180 days of RT storage, the amount of ASFV DNA detected on environmental surfaces was equal to the amount detected on the day of feed manufacture. These findings are similar to the work by Nuanualsuwan et al. (2022) who detected experimentally inoculated ASFV DNA on metal surfaces that were held for extended time at different temperatures. However, it should be noted that Nuaanualsuwan et al. (2022) evaluated viral persistence for only seven days and then calculated a model to determine the length of time that ASFV could be detected on various surfaces. To our knowledge, the study reported herein is the first work to document detectable ASFV DNA introduced through contaminated feed in environmental samples after holding the samples at RT for this length of time. These results indicate that environmental samples could be utilized to detect ASFV DNA contamination within feed mill environments for at least 6 months after initial contamination.

A limitation of this experiment is the absence of infectivity data associated with the feed and environmental samples, which is an important part to fully understand risk. However, the focus of this experiment was to evaluate the detection of ASFV DNA which is a rapid and practical method that could be readily employed to screen samples as opposed to virus isolation which requires a BSL-3 facility with special clearances. The data presented provides significant value by establishing the presence of ASFV in feed and the feed mill environment after its introduction through contaminated feed, throughout subsequent batches, and over time at RT storage. This information can be used to help guide epidemiological investigations as the current data shows that ASFV DNA is extremely stable in swine feed and on feed manufacturing surfaces. Thus, if ASFV contamination were present within a feed manufacturing facility, this data has demonstrated the feasibility of detecting also infectious virus in the environment utilizing the simple and convenient sampling methods listed herein.

In conclusion, holding feed for periods of time at RT can decrease ASFV DNA contamination but doesn't necessarily eliminate ASFV DNA entirely. This study also provides evidence that once ASFV is introduced into the feed mill environment, it will remain in the feed mill for long periods of time (at least 180 days under the conditions used in this study). Fortunately, these data also highlight the fact that ASFV markers can be detected over long periods of time in feed and environmental samples by methods described here. Further research is needed to evaluate potential methods to reduce ASFV contamination either in feed or the environment that is applicable for commercial feed mills.

Acknowledgements

Funding for this work was obtained from the NBAF Transition Funds from the state of Kansas and by the National Pork Board (Award #20-018), the Department of Homeland Security Center of Excellence for Emerging and Zoonotic Animal Diseases under grant number HSHQDC 16-A-B0006 and the AMP Core of the NIGMS COBRE Center on Emerging and Zoonotic Infectious Diseases (CEZID) under award number P20GM13044.

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Table 7.1 Proportion of qPCR positive and interactive means of cycle threshold (Ct) value and log_{10} genomic copies/g of feed samples for ASFV DNA survival after experimental inoculation of swine feed and subsequent feed batch sequencing.

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

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

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

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

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

Table 7.2 Proportion of qPCR positive and main effects of batch and day on cycle threshold (Ct) value and log_{10} genomic copies/g for feed held in room temperature storage for ASFV DNA survival after experimental inoculation of swine feed and subsequent feed batch sequencing.

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

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

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

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

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

Table 7.3 Proportion of qPCR positive and main effects of location on cycle threshold (Ct) and log_{10} genomic copies/mL of environmental discs for ASFV DNA survival after experimental inoculation of swine feed and subsequent feed batch sequencing.

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

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

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

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