Mitigation of swine pathogens in feed and feed manufacturing systems

by

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B.S., Kansas State University, 2020
M.S., Kansas State University, 2021

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Animal Sciences and Industry
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2024

Abstract

Contaminated fomites such as feed, vehicles, and worker clothing increase the risk pathogen transmission within and between farms as feed mills. Maintaining prevention-based biosecurity practices and implementing mitigation as needed can reduce the risk of pathogen transmission through feed mills and potentially contaminated feed. Chemical disinfectants and mitigants have frequently been evaluated in laboratory settings but evaluating these products in commercial-like settings have been limited.

A literature review was conducted to understand the link between *Salmonella enterica* 4,[5],12:i:- (STM) contaminated feed and pork products and human *Salmonella* outbreaks. The STM stain has been detected in swine farms, feed mills, swine feed, and in pork products themselves which suggest a link between the samples identified in the pork supply chain and foodborne illness in humans. However, the causal link between STM contamination in swine feed and STM cases in the human population has not been fully elucidated and is in need of continued research. Chapter 2 evaluates the efficacy of boot baths as a preventative biosecurity measure using either the control (no disinfection), liquid disinfectant, or dry powdered disinfectant on boots inoculated with porcine epidemic diarrhea virus (PEDV) and porcine reproductive and respiratory syndrome virus (PRRSV). Overall, the boot bath with dry powder was the most efficacious in reducing the detectable viral RNA on both boots and subsequent surfaces.

In chapter 3, a data analysis of imported non-animal origin feed ingredients was conducted as the United States Department of Agriculture (USDA) categorizes the risk of African swine fever virus (ASFV) entry into the United States through these ingredients as “negligible to moderate, with high uncertainty”. As regulators and industry consider a potential

pathway forward, the objective of this manuscript is to describe a process to determine if a voluntary or regulatory import policy is warranted by the United States. In 2020, soybean co-products and unprocessed grains and oilseeds from ASFV-positive countries represented 3.1% of all ingredients imported into the United States. Industry representatives from Canada and Australia, both countries which have policies in place to prevent ASFV entry, consistently stated their policies would not be feasible in the United States due to the differences in cost and complexity of the swine and feed industries. Overall, unprocessed grains and oilseeds and their co-products from ASFV positive countries represent a low percentage of imported ingredients into the United States; however, cautionary procedures may still be warranted given industry demand.

A series of experiments were conducted in Chapter 4-6 to evaluate mitigation and disinfection strategies to reduce PEDV, PRRSV, and Seneca Valley virus 1 (SVV1) presence if feed, and subsequently the feed mill, were to become contaminated. The objectives of the experiments included 1) the use of flush batches to reduce viral presence, 2) pelleting as a thermal processing method, and 3) physical cleaning and decontamination strategies to disinfect feed manufacturing equipment. Samples were collected during each experiment and were analyzed via PCR for the quantity of detectable RNA and via a swine bioassay to determine viral infectivity. In Experiment 1, the use of formaldehyde as a chemical mitigant and the implementation of a flush batch reduced the quantity of viral RNA for PEDV, PRRSV, and SVV1; however, viral presence was still observed in feed and the dust on non-feed contact surfaces which could pose as a source of contamination if re-introduced into finished feed. Overall, pelleting reduced the quantity of detectable viral RNA and reduced the risk of infectivity in Experiment 2; however, small quantities of viral RNA remaining in the feed and

environment following pelleting may increase the risk of re-contamination. In Experiment 3, complete facility decontamination (removal of organic matter with heated pressure washing, disinfection with 1% Virkon, disinfection with 5% household bleach, environmental heat held at 140°F for 48 hours) was the only decontamination treatment where PEDV, PRRSV, and SVV1 RNA was non-detectable after completion of all steps. Chlorine dioxide and heat treatments reduced the detectable quantity of RNA, but viral particles were still detectable across mill surfaces. During the bioassay, SVV1 and PEDV replication was not observed in pigs inoculated with samples from any experiment; however, PRRSV replication was noticed across multiple mitigation and decontamination treatments. At this time, it is unclear whether the observed PRRSV replication was due to cross-contamination or if infectious PRRSV RNA was capable of surviving undetected by PCR in the collected samples. Overall, feed mitigation using either flushes or thermal processing and feed mill decontamination strategies were able to reduce the overall presence of viral RNA and demonstrated the ability to reduce the risk of viral infection.

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Acknowledgements

There is a long list of people that deserve endless appreciation for the support they have provided throughout my graduate program. Firstly, I want to thank my major professor, Dr. Cassie Jones for her endless support and her ability to always look on the bright side of situations. I would also like to thank my committee members, Drs. Jordan Gebhardt, Chad Paulk, and Jason Woodworth for their expertise, guidance, and help while working in the FSRC.

There are few people who understand the struggles of graduate school more than your peers working alongside you. Thank you to Payton Dahmer, Grace Houston, Skyler Scotten, and Allison Blomme for all their help during my projects in the feed mill. I also want to thank all of the Applied Swine Nutrition and Feed Science graduate students for all the fun memories outside of research.

Most importantly I want to thank my friends and family. Ashton, Jenna, Claudia, and Cole were some of the first friends I made in Manhattan, and I would not have gotten where I am today without them. Thank you to the Jones family who are the reason I started working with pigs in the first place and who kickstarted my career trajectory back when I was in junior high. You all supported me no matter what I chose, and I can never thank you enough for everything you have done for me. There are not enough words to express my gratitude to my aunts and uncles, cousins, siblings, and my parents. No matter the distance, they were always there for me.

There will never be enough space to thank everyone for their role they have played in my graduate program, but I will forever be grateful to everyone that was a part of this journey.

Dedication

To Mom, Andrew, Lauren, and Ashton. They usually had no idea what I was talking about, but they listened anyway.

Preface

This dissertation is original work completed by the author, O.L. Harrison. Chapters 1 (doi: 10.4315/JFP-21-400), Chapter 2 (doi: 10.1093/tas/txac150), and Chapter 3 (doi: 10.1111/tbed.14473) were published in Journal of Food Protection, Translational Animal Science, and Transboundary and Emerging Diseases, respectively. Each of the chapters were formatted according to the required standards of the corresponding journal.

Chapter 1 - *Salmonella enterica* 4,[5],12:i:- an emerging threat for the swine feed and pork production industry[1]

Abstract

Salmonella continues to be a significant cause of foodborne illnesses in human medicine. The Centers for Disease Control and Prevention reported *Salmonella* as the second leading cause of foodborne illness in the United States, and the leading cause of both hospitalizations and deaths. *Salmonella enterica* 4,[5],12:i:- (STM) is a monophasic variant of *S.* Typhimurium and it is an emerging threat to both human and animal health. STM was first identified in the 1980's from poultry products and has become increasingly prevalent in meat products including pork. STM has also been identified in swine farms as well as feed manufacturing environments and feed itself. Similar pulse-field gel electrophoresis profiles have been observed between human clinical cases and the STM samples originating from swine feed. These related profiles suggest a link between swine ingesting contaminated feed and the source of foodborne illness in human. The objective of this article was to better understand the history of STM and the possible pathway between swine feed to table. Continued research is necessary to better understand how STM can enter both the feed supply chain and the pork production chain to avoid contamination of pork products destined for human consumption.

[1] This work has been published in *Journal of Food Protection*: Harrison, O.L, S. Rensing, C.K. Jones, and V. Trinetta. 2022. *Salmonella* enterica 4,[5],12:i:- an emerging threat for the swine feed and pork production industry. J. Food Protect. 85(4):660-663. doi: 10.4315/JFP-21-400.

History of STM

Salmonella infection is a significant cause of food-borne gastroenteritis in humans and a growing global public health problem (*6*). In the United States, the most recent 2017 report from the Centers for Disease Control and Prevention (CDC) listed *Salmonella* as the second most common cause of foodborne disease outbreaks with 113 (29%) outbreaks and 3,007 (34%) illnesses (*6*). Furthermore, among the confirmed, single-source outbreaks, *Salmonella* was the leading cause of hospitalizations (472 hospitalizations, 66%). The CDC estimates that *Salmonella* cause approximately 1.35 million illnesses, 26,500 hospitalizations, and 420 deaths each year in the United States (*7*). Of the 112 *Salmonella* outbreaks in 2017 with a serotype reported, *Salmonella* Typhimurium accounted for 14 (13%) of the outbreaks, which was the second-most common (*6*).

The last thirty years have seen the emergence of *Salmonella* Typhimurium 4,[5],12:i:- (STM), a monophasic serovar of Typhimurium, as a prevalent multi-drug resistant serovar and a growing food safety concern, especially in pork products and swine (*37*). STM is lacking the second-phase flagellin or the 1,2 phase 2H antigens (*38*); nevertheless, STM is capable of adhesion and invasion of intestinal cells similar to its ancestor *S.* Typhimurium (*10*). The research article that first isolated STM in the recent era was a study of *Salmonella* in chicken carcasses in Portugal (*26*). This study tested 300 chicken carcasses in 1986-87 and found *Salmonella* in 57% of samples. Since the identification of STM, it has quickly become one of the most prevalent *Salmonella* serotypes in Europe, the United States, and Asia. In their 2009 review of the emergence and distribution of STM, Switt et al. did note that a few reports of serotype 4,5,12:i:– appeared in peer-reviewed research from the mid-twentieth century, but these reports were not sufficiently detailed (*38*).

By the late 1990s, a growing number of researchers were studying the spread of STM and its antibiotic resistance patterns. Boonmar et al. compared specimens from 1308 humans and 407 frozen chickens in 1993 and 1994 to measure increasing antibiotic resistance of 1715 *Salmonella* strains, including *S*. Typhimurium (*2*). By 1999, Echeita et al. traced the rapidly increasing prevalence of STM in Spain and were the first to detail an ACSuGSTSxT multidrug resistance pattern; which concluded that STM was quickly becoming the most prevalent *Salmonella* serotype of concern in Spain's epidemiological surveillance system, and named contaminated pork as the most probable source (*14*). Multiple STM serotypes have also been identified with either pansusceptible or multidrug resistant antimicrobial profiles (*21, 40*), showing resistance to several antimicrobials (*35*) such as ampicillin, streptomycin, and tetracycline, one of the most widely consumed antibiotics in swine production (*24*).

In the United States, at roughly the same time (January 1998 to December 2000), Public Health Laboratories were noting a dramatic increase in STM cases stemming from a food poisoning outbreak involving over 100 people in Queens, New York (*1*). Agasan et al. reported that this outbreak resulted in 86 people reporting gastrointestinal illness and that 31 of them had symptoms severe enough to require hospitalization (*1*). The Centers for Disease Control and Prevention (CDC) also began reporting an uptick in STM cases in the United States, from 34 in 1998 to 44 in 1999 (*5*). By the early 2000s, an emerging consensus was developing that further solidified the connection between STM and the swine food system industry, with human infections increasingly traced back to pork and pork products (*12*). By 2010, Hauser et al. completed a comprehensive analysis of STM isolates in the food chain compared to clinical cases from outbreaks in humans (*20*). Their analysis conclusively demonstrated transmission of STM from the farm to fork, but since data from isolates from feed were not collected before

2007, Hauser concluded that, “The role feeding stuff may play in dissemination remains to be elucidated” (*20*).

The rise of STM prevalence in pork products and swine occurred within the context of a rapidly globalizing pork industry. Corporate consolidation within the pork industry accelerated throughout the 1990s, especially after the devastating price drops in 1998 which resulted in many small farmers in the United States declaring bankruptcy. Between 1992 and 2009, the number of hog farms in the United States declined by over 70% while pork production stayed stable (*29*). While these industrial structural changes produced significant gains in productivity in the pork industry, vertical integration within the industry also increased reliance on purchasing animal feed on the global market, rather than sourcing feed locally from small farms. As the scale of industrial pork production increased, so too did the overuse of antibiotics among pork producers, which fueled the rise of multi-drug resistant strains of *Salmonella*. By the mid-2010s, Elnekave et al. were warning of an emerging multi-drug resistant clade found in livestock, such as cattle, swine, and poultry, in the midwestern United States (*15*). Subsequent Bayesian analysis by Elnekave et al. confirmed that the outbreaks of STM in the United States shared a European origin and they suggested that one driver of multi-drug resistance “might be the approval for enrofloxacin use in swine in the United States since 2008” (*16*). A 2018 report from the National Resources Defense Council sounded the alarm on the overuse of antibiotics in the American pork industry, specifically noting with concern that U.S. pork producers “use about double the antibiotics per kilogram of pig as are used in the United Kingdom, more than three times as much as in France, and more than seven times the levels used in Denmark or the Netherlands” (*33*). Further research has also linked the increased resistance of STM to heavy metals such as mercury and arsenic to the overuse of such metals in herbicides and pesticides in grains intended

for animal consumption (*4*). The accumulation of metals in grains and oilseeds led to a rise in genetic mutations when feed and feed ingredients were contaminated with *Salmonella* serotypes (*4*).

Feed as a vehicle for *Salmonella* contamination

Salmonella contamination in feed ingredients can be traced back at least 50 years (*18*). Animal derived products such as meat and bone meal or rendered fat can be frequent sources of contamination (*22, 27*). The thermal and chemical processing used to manufacture animal-derived proteins is extreme enough to kill most bacteria present (*42*). However, re-contamination of the product can occur via contaminated dust or equipment during post-processing (*13*). Similarly, non-animal origin products are at risk for *Salmonella* contamination. Oilseeds, such as soybeans, are commonly used as vegetable protein sources in swine diets (*32)*. Soybean meal and oil are the typical feed ingredients added into swine diets instead of whole oilseeds. Like animal-derived proteins, these vegetable protein sources go through either chemical or thermal processing before being added to the diet. Grains and oilseeds, themselves, are seldom contaminated with *Salmonella*, but can become contaminated during the processing of by-products as animal-derived proteins (*17*).

The presence of both contaminated animal-derived proteins and plant-based ingredients post-processing can lead to *Salmonella* positive complete feed being fed to pigs. Li et al. found 8.1% of livestock complete feed was *Salmonella* positive during a 7-year period (*25*). A much lower incidence of contamination was observed from six feed mills in Ireland, where only 0.95% (3/317) of complete feed samples were *Salmonella* positive (*3*). Other research has found intermediate ranges between 0.9-1.6% *Salmonella* positive complete feed (*19, 23*). It is important

to note however that the isolation and enumeration of feed samples might present some challenges. The current procedures set by the U.S. Food and Drug Administration (FDA) incorporate all animal feeds such as pet, cattle, horse, as well as swine (*41*). The diversity in diet composition may affect the sensitivity and accuracy when using a standardized procedure as animal-derived protein sources are likely to have a higher prevalence of *Salmonella* than plant-derived (*17*). A lower prevalence of *Salmonella* may therefore increase the need for enrichment procedures when attempting to accurately detect *Salmonella* prevalence within feed which is not differentiated in current FDA protocols (*41*).

Similar to processing facilities for animal-derived proteins, *Salmonella* contamination may occur after thermal processing. Contamination within mills was observed in the cooler of pellet mill, which circulates cool air after complete feed has undergone high conditioning temperatures (*11*). Other sources of contamination post-pelleting can include dust present in the environment and contamination within the delivery trucks themselves (*44*). Magossi et al. found environmental *Salmonella* contamination on the exterior of the pellet mill, finished product boot bin, and load out auger (*28*). Transient surfaces such as worker shoes and brooms have the potential to contaminate the finished product, but also transfer *Salmonella* to other areas of the mill, such as receiving and storing of unprocessed feed ingredients (*28*).

STM in feed and feed ingredients

Multiple *Salmonella* serotypes have been identified in feed and feed ingredients over the years. Trinetta et al. and Li et al. have identified multiple serotypes of *Salmonella* within the feed and the environment at swine feed mills throughout the United States (*24, 39*). Furthermore, increased prevalence of STM has been observed also in feed and feed ingredients at mills (*3, 24,*

39, 43). Burns et al. isolated STM from both feed ingredients and complete feed; however, STM was observed in only 0.6% and 0.95% of the samples, respectively (*3*). Mourão et al. observed similar pulsed-field gel electrophoresis (PFGE) profiles between human clinical samples and animal feed from a swine farm (*30*). Environmental samples were STM positive at feed mills in Kansas and Iowa, but no STM contaminated feed was found during a yearlong surveillance program across 5 states (*39*).

Research conducted on STM inoculated pigs has focused on identifying pathogenicity of the serotype and clinical signs in infected animals. As a serovar of *S.* Typhimurium, STM inoculated pigs display similar clinical signs such as fever, increased incidence of diarrhea, fecal shedding of STM, and STM colonization within the lymph nodes and digestive tract (*8, 9*, *31, 36*). STM shedding in feces was observed intermittently up to 84 days post inoculation (dpi) (*9*). Currently, no studies have observed STM colonization in skeletal muscles, where reports of STM contaminated products have been reported (*8, 9, 36*). As of yet, no studies have focused on fomites such as feed or the environment as sources of STM exposure in swine. While the presence of STM in swine diets has been observed on both farms and feed mills, it is unclear whether these are the source of contamination in human products. Nevertheless, similar profiles between human clinical cases and those in swine diets do allude to swine as possible reservoirs for STM (*20, 30*).

Conclusions

According to the Food and Agriculture Organization of the United Nations (FAO) forecast, global pork production is expected to increase by 13.1% by 2030 and account for 34% of the global meat products consumed (*34*). The United States Department of Agriculture

(USDA) predicts an increase in both pork production and global trade of feed ingredients such as soybeans and corn (*40*). As pork production increases, there is an increased need to reduce pathogen entry into the supply chain.

Continued research is needed to better understand the passage of STM contaminated feed through swine and into the pork supply chain, as well as in other animal species products. Recently, Food Safety and Inspection Service (FSIS) announced a stronger effort to reduce *Salmonella* illnesses associated with poultry products. The agency will focus on *Salmonella* serotypes and virulence factors that pose the greatest public health risk. This initiative involves collaboration and ongoing dialogue with stakeholders and one of the key components is pre-harvest control. As highlighted in the present review, *Salmonella* contamination of feed ingredients and complete feed poses a risk to both human and animal health.

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Chapter 2 - Evaluating a dry vs. wet disinfection in boot baths on detection of porcine epidemic diarrhea virus and porcine reproductive and respiratory virus RNA[2]

Abstract

Maintaining biosecurity between swine barns is challenging, and boot baths are an easily implementable option some utilize to limit pathogen spread. However, there are concerns regarding their efficacy, especially when comparing wet or dry disinfectants. The objective of this study was to evaluate the efficacy of boot baths in reducing the quantity of detectable porcine epidemic diarrhea virus (PEDV) and porcine reproductive and respiratory syndrome virus (PRRSV) genetic material using wet or dry disinfectants. Treatments included 1) control, 2) dry chlorine powder (Traffic C.O.P., PSP, LLC, Rainsville, AL), and 3) wet quaternary ammonium/glutaraldehyde liquid (1:256 Synergize, Neogen, Lexington, KY). Prior to disinfection, rubber boots were inoculated with 1 mL of a co-inoculants of PRRSV (1×10^5 $TCID_{50}$/mL) and PEDV (1×10^5 $TCID_{50}$/mL) and dried for 15 minutes. After the drying period, a researcher placed the boot on the right foot and stepped directly on a stainless steel coupon (control). Alternatively, the researcher stepped first into a boot bath containing either the wet or dry sanitizer, stood for 3 s, and then stepped onto a steel coupon. After one minute, an environmental swab was then collected and processed from each boot and steel coupon. The procedure was replicated 12 times per disinfectant treatment. Samples were analyzed using a

[2] This work has been published in Translational Animal Science: Harrison, O.L., G. E. Houston, A.K. Blomme, H.K. Otott, J. Bai, E.G. Poulsen Porter, J.C. Woodworth, C.B. Paulk, J.T. Gebhardt, and C.K. Jones. 2022. Evaluating dry vs. wet disinfection in boot baths on detection of porcine epidemic diarrhea virus and porcine reproductive and respiratory virus RNA. Transl. Anim. Sci. 6:1-4. doi: 10.1093/tas/txac150

duplex qPCR at the Kansas State Veterinary Diagnostic Laboratory. Cycle threshold values were analyzed using SAS GLIMMIX v 9.4 (SAS, Inc., Cary, NC). There was no evidence of a disinfectant × surface × virus interaction ($P > 0.10$). An interaction between disinfectant × surface impacted ($P < 0.05$) the quantity of detectable viral RNA. As expected, the quantity of the viruses on the coupon were greatest in the control, indicating that a contaminated boot has the ability to transfer viruses from a contaminated surface to a clean surface. Comparatively, the dry disinfectant treatment resulted in no detectable viral RNA on either the boot or subsequent coupon. The wet disinfectant treatment had statistically similar ($P > 0.05$) viral contamination to the control on the boot, but less viral contamination compared to the control on the metal coupon. In this experiment, a boot bath with dry powder was the most efficacious in reducing the detectable viral RNA on both boots and subsequent surfaces.

Introduction

Disease spread between populations of animals is a major concern for many swine producers. Protocols like changing clothes and wearing plastic boot covers help reduce farm-to-farm disease spread between production sites (Otake et al., 2002; Dee et al., 2004). However, room-to-room disease spread within a single production site is typically limited due to challenges in infrastructure and practicality of implementation. One easily implemented option is to place a boot bath between rooms with the intent to sanitize the boot bottoms of personnel as they move from one room to another.

The efficacy of the boot bath in a production system is dependent on the disinfectant utilized, the pathogen of concern, and the maintenance of the system itself. Muckey et al. (2021) saw that surface type largely affected the ability of different disinfectants to reduce the presence

of porcine epidemic diarrhea virus (PEDV). As expected, smooth surfaces are easier to clean than textured ones, such as rubber boots, because the texture requires additional steps to remove organic matter for effective sanitation (Amass et al., 2000; Huss et al., 2017).

Boot baths have been demonstrated to be effective at preventing fomite transmission of porcine reproductive and respiratory disease syndrome (PRRSV), highly pathogenic and low pathogenic avian influenza, and aerobic bacteria (Dee et al., 2004; Hauck et al., 2017; Nasr et al., 2018). However, their usefulness is often questioned due to the maintenance needed to maintain efficacy (Amass et al., 2000; Amass et al., 2001; Bashandy et al., 2017). Historically, most boot baths have contained wet sanitizer, which can pose a slip hazard and quickly accumulates organic matter, potentially reducing its efficacy over time. Alternate dry powder disinfectants have recently become available, but there is little data to compare the efficacy of the dry powder compared to the wet disinfectant. Therefore, the objective of this study was to evaluate the efficacy of boot baths, using either wet or dry disinfectants, on the detectability of PEDV and PRRSV genetic material.

Materials and Methods

Experimental Design

All experimental procedures were approved by the Institutional Biosafety Committee at Kansas State University (IBC #1511) and were conducted in the Cargill Feed Safety Research Center (FSRC) at the Kansas State University O.H. Kruse Feed Technology Innovation Center in Manhattan, KS.

Preparation of Inoculum

Prior to the experiment, 4 mL of 1.33×10^6 $TCID_{50}$/mL PEDV (USA/Co/2013) and 4 mL of 1.33×10^6 $TCID_{50}$/mL PRRSV (wild type 1-7-4) were individually diluted with 36 mL phosphate buffer solution (PBS) in separate 50 mL conical tubes for an approximate final concentration of 1×10^5 $TCID_{50}$/mL. Viruses were further divided into 10 mL aliquots and stored at -80°C until the start of the experiment. The inoculants were tested via PCR at the time of sample analysis to validate the presence of viral RNA prior to disinfectant exposure.

Preparation of Surfaces

At the start of the experiment, aliquots of each virus were thawed at room temperature in a biosafety cabinet within the BSL-2 facility. Next, 9 mL of each virus was combined in a single container and gently agitated to create a single container with 18 mL of a PEDV/PRRSV co-inocula. From this container, 1-mL aliquots were drawn into individual 2 mL Monject syringes and stored in the biosafety cabinet for a maximum of 15 minutes.

Meanwhile, boots, boot baths, and surfaces were prepared within the BSL-2 facility but outside the biosafety cabinet. Thirty-six boots (size 12, right foot only; Tingley, Tingley Rubber Company, Piscataway, NJ) were placed upside down on a boot drying rack and dusted with approximately 2 g of autoclaved ground corn (600 microns) to disrupt the rubber surface tension of the boot prior to viral inoculation. Twenty-four plastic containers (35.5 cm × 25.4 cm × 10.2 cm; Van Ness Small Liter Pan, Van Ness Pets, Clifton, New Jersey) were filled with approximately 2.5 cm of either dry disinfectant (Traffic C.O.P., PSP, LLC, Rainsville, AL) or wet disinfectant (Synergize, Neogen, Lexington, KY). The dry disinfectant was a dry powder containing chlorine, silicates, and acid-impregnated zeolites used directly as received from the manufacturer. The wet disinfectant was a quaternary ammonium/glutaraldehyde liquid that

required 1:256 dilution with water per the manufacturer's directions. Thirty-six stainless steel coupons (10 cm × 10 cm; Built So-Well, LLC, Manhattan, KS) were autoclaved and placed at least 10 cm apart from one another.

Surface Inoculation

One mL of the co-inoculant was distributed in the same location across the sole of each boot in two even lines. Boots were then allowed to air-dry for 15 minutes at room temperature (approx. 22-25°C). After the drying period, a single designated researcher placed the boot on the right foot and stepped directly on a stainless-steel coupon (control). Alternatively, the researcher stepped first into a dry or wet boot bath and stood for 3 s before stepping onto the steel coupon. Boots were then placed back on the drying rack and surfaces were allowed to air-dry for 1 minute at ambient temperature and humidity. Next, an environmental swab was collected and processed from each boot and steel coupon using procedures described by Elijah et al. (2021b). Briefly, a 10 cm × 10 cm surgical cotton gauze swab was pre-moistened with PBS and stored in a 50 mL conical tube prior to the experiment. During sampling, the gauze was aseptically removed and swabbed across the sampling surface in an approximately 10 cm × 10 cm area before the swab was returned to the conical tube. Swabs were returned to the biosafety cabinet, where 20 mL of PBS was added to the conical tube. Conical tubes were then inverted for 10-15 sec and incubated at room temperature for 1 hour. After incubation 1.75 mL of supernatant were aliquoted into cryovials. Upon exiting the facility, samples were placed in a -80°C freezer until analysis. Other than temperature to slow viral degradation, no additional stop agents were used to end the disinfectant process. These procedures were repeated 12 times. Altogether, 72 environmental swabs were collected, representing 12 replicates of 3 boot bath treatments

(control, dry disinfectant, or wet disinfectant) and two surfaces (rubber boot and stainless-steel coupon).

Quantitative Viral Analysis

Environmental swabs were analyzed for quantitative real-time polymerase chain reaction (qRT-PCR) for PEDV and PRRSV at the Kansas State University Veterinary Diagnostic Laboratory using procedures similar to those described by Elijah et al. (2021a). First, 50 µL of supernatant was placed in a deep well plate and RNA extracted using a Kingfisher Flex magnetic particle processor (Fisher Scientific, Pittsburgh, PA) and a MagMAX-96 Viral Isolation Kit (Life Technologies, Grand Island, NY). The final elution volume was reduced to 60 µL, and extracted RNA was stored at -80°C until analyzed for PEDV or PRRSV using a qRT-PCR duplex assay with a maximum cycle threshold of 45. Results were reported as the number of samples considered PCR positive and the cycle threshold (Ct) at which either PEDV or PRRSV RNA was detected.

Statistical Analysis

Results were analyzed as a split plot design with boot bath pan as the main experimental unit and surface (either boot or coupon) as the sub-plot using the GLIMMIX procedure of SAS version 9.4 (SAS Institute Inc., Cary, NC). Fixed effects included disinfectant (control, dry, or wet), surface type (boot or steel), virus (PEDV or PRRSV), and their associated interactions. Random effect included boot bath pan. There were two response criteria considered, the proportion of PCR positive samples and the quantity of detectable viral RNA. To estimate the proportion of PCR positive samples, the number of samples with detectable PEDV or PRRSV RNA was placed in ratio to the number of total samples. Data were analyzed by fitting to a binary distribution, logit link, Laplace approximation, and ridge-stabilized Newton-Raphson

algorithm. As a binary distribution model data were fit by each individual interaction, starting with the disinfectant × surface type × virus interaction, and their subsequent main effects. To estimate the quantity of detectable viral RNA, the Ct of each sample was used. If no PEDV or PRRSV RNA was detected, samples were assigned a value of 45.0. A Kenward-Roger denominator degree of freedom adjustment was used, as well as a Tukey-Kramer multiple comparison adjustment. Results were considered significant at $P \leq 0.05$.

Results and Discussion

There was no evidence of a disinfectant × surface × virus interaction ($P > 0.05$) for either the proportion of positive samples or their quantity of detectable viral RNA (Table 1). However, there was a disinfectant × surface interaction ($P < 0.05$) for both response criteria. There was no evidence ($P > 0.05$) that the proportion of PCR positive samples differed between samples collected from boots or steel coupons for the control treatment or the boots for the wet disinfectant treatment. However, these all had a greater ($P < 0.05$) proportion of PCR positive samples than the steel surface after the boot bath with wet disinfectant. There were no PCR positive samples for either the boot or steel surface after the boot bath with dry disinfectant. The quantity of viral RNA was greater ($P < 0.05$) for the boots and steel coupons from the control treatment and the boots from the wet disinfectant treatment as compared to the steel coupons from the wet disinfectant treatment and either surface from the dry disinfectant treatment (Table 1).

These results are similar to those reported by Hauck et al. (2017), which determined a dry chlorine-based disinfectant had greater efficacy at sanitizing high or low pathogenic avian influenza virus compared to either a wet quaternary ammonium/glutaraldehyde disinfectant or a

control. Rabbe et al. (2012) also observed a reduced proportion of PRRSV-positive sample when dry disinfectant was used compared to wet disinfectant in the presence of fecal matter. However, in the absence of fecal matter, our results are contrary to Rabbe et al. (2012) as dry disinfectant had a greater proportion of PRRSV-positive samples as compared to the wet disinfectant. One possible explanation for this discrepancy is that the active ingredients in the dry disinfectant used by Rabbe et al. (2012) were reported as phosphates, silicates, copper, and iron, whereas the current study utilized a product with sodium sulfate, sodium chloride, calcium hypochlorite, calcium chlorate, calcium chloride, calcium hydroxide, and calcium carbonate.

The observed differences across surface types are consistent with those previously reported (Huss et al., 2017; Muckey et al., 2021). It is important to reiterate that the rubber boot, but not the stainless-steel coupon, was directly inoculated with PEDV and PRRSV. Therefore, any viral RNA present on the steel surface was transferred from the boot. Dee et al. (2004) reported similar results, where surfaces that were affected by cross-contamination had less detectable viral RNA than those that were experimentally inoculated. Huss et al. (2017) identified smooth rubber as an ideal candidate for equipment that can easily be decontaminated, but the crevices of boots in this study may prevent fully effective disinfection. Muckey et al. (2021) also observed differences in detectable PEDV RNA between surface types when quaternary ammonium/glutaraldehyde was used as a disinfectant. In both Huss et al. (2017) and Muckey et al. (2021), however, the authors compared disinfectants on varying surface types that had been directly inoculated. Whereas this study only directly inoculated the rubber boot and relied on cross-contamination to spread the virus to the steel surface.

In addition to the disinfectant × surface interaction reported above, the quantity of detected viral RNA in this study was also affected by a disinfectant × virus interaction ($P < 0.05$;

Table 2). Specifically, there were greater ($P < 0.05$) quantities of PEDV detected in the control samples than of PRRSV in the control or PEDV in samples from the boot bath with wet disinfectant. Again, no PEDV or PRSSV was detected in samples from the boot bath with dry disinfectant.

Our results indicate using a wet disinfectant was able to reduce both PEDV and PRRSV viral RNA, but not to the extent observed with the dry disinfectant. Few studies have compared sanitizer efficacy in PEDV and PRRSV simultaneously. Elijah et al. (2021a) evaluated sanitizer efficacy on vehicle interior surfaces and reported that surface type and sanitizer both impacted the proportion of positive PEDV- or PRRSV-positive samples. However, all the surfaces in the Elijah et al. (2021a) experiment were inoculated directly, so it is probable that the initial Ct values of each virus is driving this interaction.

Limitations

One of the major challenges of using boot baths is the buildup of organic matter during use, which may impact sanitizer efficacy. This project initially intended to include an organic matter (an equal combination of soil and fecal matter) component; however, the organic matter results had to be omitted because the particles in the organic matter interfered with the qRT-PCR assay. Furthermore, these results only report the quantity of detected viral RNA as determined by Ct, not the infectivity of these samples. It is hypothesized that chemical sanitizers are effective for PEDV or PRRSV because the sanitizer disrupts the viral envelope and prevents fomite-based transmission of infectious particles. This would likely result in one of two options: the fragments of viral RNA can be detected by qRT-PCR but are no longer capable of causing infection or the viruses can cause infection, but the viral particles present were below infectious dosages.

Response criteria that evaluates infectivity, such as a bioassay or cell culture virus isolation assay, are necessary to draw full conclusions. However, others have reported that the reduction of viral RNA is indicative of infectivity, and so while these results are limited, they are nevertheless important in better understanding practical biosecurity measures. Continued research is necessary to improve both the practicality and efficacy of on-farm biosecurity measures, as well as the laboratory procedures used to test, apply, and interpret supported results.

Conclusions

Boot baths are an easily-implemented biosecurity measure to reduce room-to-room viral transfer on swine farms and other facilities. A boot bath containing a dry chlorine powder in this experiment surpassed the performance of a boot bath containing a wet quaternary ammonium/glutaraldehyde liquid disinfectant. While the wet disinfectant reduced the quantity of viral RNA compared to the control, it did not reduce viral RNA of either virus beyond detectable limits. Future research should focus on the utilization of dry disinfectant in production settings and in the presence of organic matter, as well as to evaluate viral infectivity.

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Table 2.1 Detection of viral RNA on boots or subsequent steel surfaces after stepping in a boot bath containing a wet or dry disinfectant[1]

		Boot Bath Disinfectant Type		
Item		Control	Dry	Wet
PCR positive[2]				
	Boot	19/24[c]	0/24[a]	21/24[c]
	Steel	22/24[c]	0/24[a]	9/24[b]
Ct[3]				
	Boot	37.0[c]	45.0[a]	38.1[c]
	Steel	34.0[d]	45.0[a]	42.2[b]

[1]Boots were inoculated with 1 mL of a PEDV/PRRSV co-inoculant and were randomly subjected to one of three boot bath disinfectants. Boots were stepped onto a stainless-steel coupon (10 × 10 cm) after submersion in the boot bath. The dry disinfectant was a powder containing chlorine, silicates, and acid-impregnated zeolites (Traffic C.O.P., PSP LLC, Rainsville, AL). The wet disinfectant was liquid quaternary ammonia and glutaraldehyde blend (Synergize, Neogen, Lexington, KY). Samples with no detectable RNA were assigned a Ct value of 45.0. Disinfectant × surface × virus, $P > 0.05$.
[2]PCR positive: Disinfectant × Surface, $P = 0.0154$
[3]Ct is the average cycle threshold value for both PEDV and PRRSV. Disinfectant × Surface, $P = 0.0001$; SEM = 0.60674.
[abcd] Means with differing superscripts differ significantly.

Table 2.2 Detection of PEDV and PRRSV RNA after stepping in a boot bath containing a wet or dry disinfectant[1]

		Boot Bath Disinfectant Type		
Item		Control	Dry	Wet
PCR positive[2]				
	PEDV	20/24	0/24	19/24
	PRRSV	21/24	0/24	11/24
Ct[3]				
	PEDV	34.0[d]	45.0[a]	38.0[c]
	PRRSV	37.0[c]	45.0[a]	42.3[b]

[1]Boots were inoculated with 1 mL of a PEDV/PRRSV co-inoculant and were randomly subjected to one of three boot bath disinfectants. Boots were stepped onto a stainless-steel coupon (10 × 10 cm) after submersion in the boot bath. The dry disinfectant was a powder containing chlorine, silicates, and acid-impregnated zeolites (Traffic C.O.P., PSP LLC, Rainsville, AL). The wet disinfectant was liquid quaternary ammonia and glutaraldehyde blend (Synergize, Neogen, Lexington, KY). Samples with no detectable RNA were assigned a Ct value of 45.0. Disinfectant × surface × virus, $P > 0.05$.

[2]PCR positive: Disinfectant × virus, $P > 0.05$.

[3]Ct is the average cycle threshold value for both boot and stainless-steel surfaces. Disinfectant × virus, $P = 0.0019$; SEM = 0.6574.

[abcd] Means with differing superscripts differ significantly.

Chapter 3 - Developing a gateway program for importing non-animal origin ingredients from regions with African swine fever virus[3]

Abstract

The United States Department of Agriculture (USDA) categorizes the risk of African swine fever virus (ASFV) entry into the United States through non-animal origin feed ingredients as "negligible to moderate, with high uncertainty". Both Canada and Australia have implemented policies that are suggested to reduce the risk of ASFV entry through feed ingredients, but the United States has not because of scientific limitations that have been addressed by recent publications. As regulators and industry consider a potential pathway forward, the objective of this manuscript is to describe a process to determine if a voluntary or regulatory import policy is warranted by the United States. Initially, the volume and types of non-animal origin feed ingredients imported from countries with ASFV were quantified and assigned a level of risk (high risk: unprocessed grains and oilseeds, moderate risk: soybean co-products (meals, oil, and oilcake), and low risk: amino acids, vitamins, and other synthetically-produced products from countries that have ASFV). In 2020, moderate and high-risk ingredients from ASFV-positive countries represented 3.1% of all ingredients imported into the United States. Policies from Canada and Australia were evaluated for practicality of implementation by U.S. Government officials. Industry representatives from both countries consistently stated their policies would not

[3] This work has been published in Transboundary and Emerging Diseases: Harrison, O.L., J.T. Gebhardt, C.B. Paulk, J.C. Woodworth, and C.K. Jones. 2022. Developing a gateway program for importing non-animal origin ingredients from regions with African swine fever virus. Trans. Emerg. Dis. 69:e1407-e1416. doi: 10.1111/tbed.14473

be feasible in the United States due to the differences in cost and complexity of the swine and feed industries. Overall, unprocessed, or high-risk, ingredients from ASFV positive countries represent a low percentage of imported ingredients into the United States; however, cautionary procedures may still be warranted given industry demand.

Introduction

Currently, African Swine Fever virus (ASFV) has been confirmed in pigs in more than 50 countries. As the number of affected countries grows, so does the risk for virus entry into the United States. The most likely method of ASFV transmission is direct contact between pigs. In countries free from disease, prevention of entry is focused on preventing fomite-based transmission. One potential route for fomite-based transmission is the feed supply chain. The USDA cites that used tote bags carrying feed ingredients was the most likely source of porcine epidemic diarrhea virus (PEDV) entry into the United States from China in 2013 (USDA-APHIS, 2015). The virus was then spread throughout North America and remains endemic throughout the continent today. Research has shown that feed ingredients have the capability to sustain not only PEDV, but a host of other viruses, including ASFV (Dee et al., 2018).

In order for a feed ingredient to serve as a fomite in disease transmission, it must first be contaminated by the pathogen, that pathogen must survive transport to the point of consumption by an animal, and the quantity of virus consumed by an animal must be substantial enough to cause disease. These factors can vary widely in different types of ingredients. For example, wild boar or feral pigs affected by ASFV often seek food and shelter in fields. Their resulting carcasses may contaminate grains or seeds during harvest. Due to these differences, unprocessed grains or seeds from ASFV-endemic regions are considered to be at a greater risk to be contaminated with the pathogen than manufactured ingredients, such as synthetic amino acids or

vitamins, derived from the same region (EFSA, 2021). Virus survival also differs based on ingredient type. For example, the half-life of ASFV can range from 9.6 to 12.9 d in conventional vs. organic soybean meal (Stoian et al., 2019). Research has demonstrated that if ingredients are contaminated with a high level of ASFV, the virus can survive theoretical trans-oceanic shipment to the United States (Dee et al., 2018). Finally, feed contaminated with ASFV has been demonstrated to cause infection in pigs when sufficient quantities of virus are ingested (Niederwerder et al., 2019).

These studies have raised concern that the feed supply chain may be a realistic route of ASFV entry into the United States. Both Canada and Australia have regulations to reduce this risk. Scientific limitations have slowed the implementation of similar rules in the United States. For example, there are no approved sampling or extraction protocols for ASFV detection in ingredients. Recent publications have helped to address these issues, and while some challenges still remain, there is greater confidence that a representative sample can be collected and analyzed for ASFV if testing were allowed (Jones et al., 2019, Dee et al., 2021). This scientific progress led to the United States Animal Health Association passing a resolution in 2020 that requested the federal government to, "restrict the import of feed and/or a feed ingredient from countries that are positive for African swine fever and to create enforceable standards for those countries to reduce the contamination threat during harvest and processing of the feed and feed ingredients." However, the government responded that there is too great of cost to both government and industry at the current time to implement such a program. Furthermore, the types of enforceable standards requested were too broad; there are mixed messages from within both the swine and feed industries about the types of standards that should be implemented. As regulators and industry consider a potential pathway forward, the objective of this manuscript is

to describe a process to determine if a voluntary or regulatory import policy is warranted by the United States.

Materials and Methods

To make the determination if an industry-wide policy should be adopted, and what it should address, it was important to first quantify the sources and volumes of non-animal origin ingredients imported into the US from ASFV-positive regions. Next, an assessment of the regulatory framework incorporated by other ASFV-free countries were considered. Finally, a working group representing the swine industry made recommendations for a pilot import program and its eventual incorporation into regulatory policy.

The volume and path of non-animal origin feed and ingredients imported from countries with ASFV

Import data from 2016 through 2020 was collected from the United States International Trade Commission (USITC DataWeb) with the Harmonized Tariff Schedule (HTS) found in Table 1. Disease presence was based off OIE-World Organization for Animal Health (OIE-WAHIS) disease reporting database. Imported ingredients were separated into three categories based off their perceived risk of contamination or re-contamination upon importation into the United States (Table 2). 'Low risk' ingredients were those that were least likely to be contaminated with ASFV, such as ingredients that are synthetically manufactured (amino acids, vitamins, enzymes) or undergo substantial thermal or chemical processing that would likely render pathogen inactive (oils and most oilseed cakes or flours). 'Moderate risk' ingredients were those that undergo thermal or chemical processing, but allow for the risk of post-processing cross-contamination and demonstrate a capacity for significant viral survival over time (soybean oilcake and soybean meal). 'High risk' ingredients were those that have a risk for natural

contamination by affected animals and undergo minimal processing (whole grains or seeds). Notably, an exception was made to also characterize choline as a 'high risk' ingredient. Similar to vitamins, the synthetic production of choline poses little risk for ASFV contamination. While most vitamins are transported to the United States in their highly concentrated form and diluted upon arrival to the United States, choline is typically applied to a corn cob or rice hull carrier shortly after manufacturing because it is highly hygroscopic. This application maximizes its stability during transoceanic shipment. The carrier to which choline applies has a similar probability of contamination as whole grains or seeds, so the imported product category of choline was included in the 'high risk' category.

Current regulations for non-animal origin feed and ingredients imported from ASFV-positive regions

To understand the complex environment of importation requirements across three countries, the guidelines from the United States, Canada, and Australia were initially reviewed. Canada and Australia were selected for comparison because, similar to the United States, they are countries free from ASFV but depend on imports from ASFV-positive countries. They also had implemented significant policies to prevent disease entry through ingredients. Independent interviews were also conducted with representatives in each country representing the federal government, feed industry, and swine industry. Positions interviewed to compile this information from the United States include: USDA APHIS-PPQ National Policy Manager, USDA APHIS-VS Director of Animal Product Imports, American Feed Industry Association Vice President of Public Policy and Education, and National Grain and Feed Association Senior Vice President of Feed Services. Positions interviewed from Canada include Canadian Food Inspection Agency Programs and Policy Branch National Manager, Canadian Food Inspection Agency Animal

Health Risk Assessment and Intelligence Section Risk Analyst, Canadian Food Inspection Agency Foreign Animal Disease Section National Manager, Canadian Pork Council, Animal Nutrition Association of Canada Technical Services Director, and Animal Nutrition Association of Canada Executive Director. Positions interviewed from Australia include: Australian Department of Agriculture, Water, and the Environment Data Analyst, Australia Department of Agriculture Program Lead for Biosecurity and Social Science, SunPork Australia Veterinarian and African Swine Fever Liaison to Australian Pork Limited, SunPork Australia Nutritionist, Australian Pork Limited Manager of Production Stewardship, and Stock Feed Manufacturers' Council of Australia Executive Officer.

Results and Discussion

The volume and path of non-animal origin feed and ingredients imported from countries with ASFV

From 2016 to 2020, the quantity of feed ingredients imported into the United States ranged from 11.6 to 12.8 million metric tons per year (Figure 1). This quantity does not differentiate between those ingredients intended for human consumption, swine diets or other livestock and pet food diets. During the same time frame, the total quantity imported from ASFV-positive countries ranged from 0.8 to 1.1 million metric tons per year. The greatest quantity imported from ASFV-positive countries was in 2020, with the increase from recent years due to the growing number of United States' trade partners that were impacted by the disease. In 2020, the United States imported ingredients that could be used in swine feed from 29 of the 50 countries that were ASFV-positive, some of which are critical to United States trade (Figure 2). The greatest quantity of high risk imported ingredients came from Germany, with all

the incoming product consisting of grains. Relatively significant quantities of soybeans were imported from Russia, Ukraine, and India in 2020, as well as grains from Romania. Choline was largely sourced from Belgium. Notably, fewer than 5,000 metric tons of high risk ingredients were imported from China in 2020. These high-risk ingredients were imported into 23 different US ports (Figure 3). The Port at New Orleans imported the greatest quantity of high-risk ingredients from ASFV-positive countries at more than 114,000 metric tons of product. These shipments contained 9 metric tons of barley imported from Italy and likely destined for human food and 114,775 metric tons of certified organic soybeans from Ethiopia, Russia, and Ukraine (Table 3). The source of these ingredients (i.e. region of a specific country) and transportation methods could not be further determined prior to their Port of Loading. Interestingly, the Ports of Loading for these shipments were from India and Turkey. This demonstrates that even when a country of origin can be determined for an ingredient, its growing conditions, transportation, and other factors relating to the likelihood of ASFV contamination is unlikely to be known. These three shipments arrived at the Port of New Orleans in jumbo bags (45) or bulk (1) consignments after at least 32.4 days at sea. Part or all of each shipment was purchased by a single company, Sunrise Foods International, an entity based in Canada. These soybeans would likely require crushing prior to use in swine diets because whole, unprocessed soybeans have poorly available nutrients to pigs (Liener, 1994). Therefore, it is unlikely that any of the high risk ingredients received into the Port of New Orleans in 2020, which represents 31.4% of all high risk ingredients imported, was consumed by pigs. Moreover, it is improbable that the remaining high risk ingredients imported into other ports in 2020 were consumed by pigs due to the small volume of each consignment. For example, the Port of New York had the greatest number of consignments, or individual shipments of high-risk ingredients. However, these consignments

totaled to fewer than 14,000 metric tons of product, with most of the product arriving in small quantities that would be unlikely to be destined for animal feed.

The moderate risk ingredients were imported from 10 different ASFV-positive countries (Figure 4). Soy oilcake was imported from China and Nigeria, soy flour and meals were imported from Belgium, Bulgaria, China, Estonia, Poland, Rwanda, South Korea, and Ukraine, and soy oil was imported from Italy and South Korea. The combined quantity of moderate risk ingredients from ASFV-positive countries totaled less than 1,500 metric tons. While a risk exists that these non-animal origin ingredients may be contaminated with ASFV, these data support the USDA's assertion that there is a negligible risk for ingredients to serve as a fomite for ASFV entry into naïve pigs in the United States (USDA-APHIS-VS, 2019).

Current regulations for non-animal origin feed and ingredients imported from ASFV-positive regions

United States of America

Non-animal origin feed ingredients imported into the US falls under two government jurisdictions. The FDA is responsible for the importation of non-animal-origin ingredient and complete feeds. Alternatively, the USDA Animal & Plant Health Inspection Service (APHIS) includes foreign animal disease prevention and surveillance and partners with U.S. Customs and Border Protection to enforce importation requirements. Two agencies within USDA-APHIS share this responsibility. Veterinary Services (VS) "regulates the importation of animals and animal-derived materials to ensure that exotic animal and poultry diseases are not introduced into the United States (USDA APHIS-VS, 2021)." Currently, USDA APHIS-VS prohibits most animal-derived ingredients from being imported into the United States. In the rare instances

where animal-derived ingredients are allowed, such as vitamins coated in porcine-derived gelatin to improve stability, special conditions or permits are issued by USDA APHIS-VS to ensure the product has been processed completely to minimize the risk of disease. Meanwhile, another division of USDA APHIS, Plant Protection and Quarantine (PPQ) "regulates the importation of plants and plant products… from the risks associated with the entry, establishment, or spread of animal and plant pests or noxious weeds (USDA APHIS-PPQ, 2021)". The USDA APHIS-PPQ specifies in its *Seeds not for Planting* guide that seeds from certain countries to be accompanied by phytosanitary certificates to eliminate the risk of pests, such as Khapra beetles in soybeans from Nigeria (USDA APHIS, 2020). When necessary, products may need to undergo more strenuous processes. In their *Miscellaneous and Processed Products Import Manual*, APHIS-PPQ specifies that peanuts from Argentina or Brazil must be commercially blanched at 84˚C for 12 minutes to alleviate concerns of peanut stripe virus (USDA APHIS, 2017). These manuals do not currently have requirements that address animal pathogens in plant-based products.

Canada

Canada developed their risk management strategy based on the concept of strengthening the country's protection against ASFV entry in ingredients, but not eliminating the risk altogether. Their import policy was implemented after an extensive literature review and importer surveys to better understand the number of importers affected and their understanding of pathogen transmission in feed. Canada requires an import permit for any raw or unprocessed grains and oilseeds and any plant-based meal of concern imported from an ASFV-positive country. They also recognize regionalization with 12 countries. Regionalization is permitted in countries who participate in the European Union's Commission Implementing Regulation 2021/605 and 2021/1205, which designates restricted zones within a country based on the

disease status of the wild and domestic swine populations. This permit must be accompanied by a completed questionnaire demonstrating certain conditions have been met either in the country of origin and certified by a competent authority (a representative of a governing body guaranteeing requirements have been met) or upon arrival at a Canadian port. The required conditions include: 1) heat treatment with a minimum temperature of 70°C for 30 minutes or 85°C for 5 minutes or holding of the product should occur for a minimum of 20 days at 20°C or 100 days at 10°C; and 2) prevention of cross-contamination via unidirectional traffic at the processing facility, single use containers or bulk containers with plastic liners, and no additions of unprocessed ingredients added to the finished product.

Canada did not introduce a new regulation to implement their import policy. Under their *Health of Animals Act* and *Health of Animals Regulations*, the Minister of Agriculture can declare Secondary Control Zones (SCZ) to prevent disease entry into Canada. By declaring certain zones or countries areas of concern regarding ASFV, the Canadian Food Inspection Agency can create restrictions when importing certain ingredients from ASFV-positive countries. The amendments to *Health of Animals Act* and *Health of Animals Regulations* allowed for rapid implementation of the new policies; however, more than just the swine industry would be affected by the new import requirements. The swine and feed industry met with the National Farm Animal Care Council (NFACC), a unified organization representing all livestock sectors, to discuss their need for import policies from ASFV-positive countries. There was no opposition from any other industries which also eased the way for the Minister of Agriculture to approve the new policies. Canada only has 6 marine ports compared to 50 within the United States. Canada, like the United States also imports a relatively low amount of bulk grains and oilseeds (Figure 5).

Those interviewed could not estimate a cost associated with the import policy. Any costs associated with increased holding time at port or additional processing are incurred by the importer. Increased costs at the port would trickle down to the buyers of the finished product; however, no analysis has been conducted to better understand the economic difference since this program has been implemented. All Canadian representatives stated that they did not believe a policy similar to Canada's would be practical to implement in the United States due to the greater complexity of grain trade and marine ports that exist in the U.S.

Australia

Australia implemented import control measures approximately 20 years ago to reduce the risk of introducing foot and mouth disease virus, bovine spongiform encephalopathy, and plant pests, such as the Khapra beetle. After ASFV was identified in Southeast Asia, the Australian Department of Agriculture reviewed their policies and confirmed their existing rules would effectively prevent ASFV entry in ingredients so no new policies were needed. Current policies include that all imported cereal grains and oilseeds must be either processed at the port of entry or have an import permit from the country of origin signed by a competent authority that the product was effectively processed. All foreign facilities that process ingredients bound for Australia must be audited by the Australian government every two years at the cost to the importing facility (~$10,000 AUD/audit). Accepted processing methods include: 1) Heating the product to a core temperature of 85°C for 10 minutes if it originates from a low-risk country or to 100°C for 30 minutes if it originates from a high-risk country; 2) pH reduction by 3.3; or 3) irradiation of 50 kGy. Australian pork industry officials interviewed described that even with these import restrictions in place, they frequently implemented their own holding time mitigation strategies due to their lack of confidence in effective processing by importing countries. The

Australian government is currently conducting a cost:benefit analysis to determine the value of mitigation strategies to prevent ASFV. However, their analysis does not consider the role of feed or ingredients as a vector of ASFV entry or transmission. All Australian representatives stated that they did not believe a policy similar to Australia's would be practical to implement in the United States due to the size of the U.S. feed and pork industries. In fact, both regulators and industry representatives alike expressed their concern that existing policies reduced the ability for pork producers to have access to low cost ingredients, which limited the industry's domestic growth and international competitiveness.

In comparison to Canada and Australia, the U.S. regulations may appear lacking. However, there is a growing proportion of production companies taking innovative steps to secure their ingredient supply chains. In a preliminary survey, which represented companies owning one-third of the pigs marketed annually, over half of the participants utilized holding times for imported vitamins, minerals, and supplements from ASFV-positive countries. Moreover, half of the participants were working towards sourcing only domestic grains and oilseeds. Other companies have privately been tracing their ingredient supply chain back to the original sources as well as quarantining those ingredients in segregated warehouses. Ingredient tracing programs allow the entire supply chain, including the veterinarians and producers to have more confidence in the quality and disease status of imported ingredients (Patterson et al., 2019). Furthermore, the swine industry has continued to support proactive research to lessen any gaps of knowledge. Blomme et al., (2022) looked specifically at soy ingredients imported into the United States from foreign animal disease positive countries beyond the ASFV positive countries mentioned in this study. While Schambow et al., (2022) developed a model to predict ASFV entry more accurately through non-animal origin feed ingredients. Innovative steps such as these

can take the place of government regulations and can instead demonstrate the unity and forward thinking of the swine industry.

Limitations

As with any study, there are limitations which must be addressed. Due to time restraints, a limited number of individuals were able to be interviewed. As the objectives were primarily focused on regulatory structure in certain countries, the interviews primarily focused on governing regulatory bodies and national organizations. This limited representation from individual swine producers and veterinarians, which may view the challenges facing the swine industry differently than those interviewed.

Conclusion

There continues to be a small, but realistic threat for African swine fever virus entry into the United States through non-animal origin feed ingredients. In 2020, moderate and high-risk ingredients from ASFV-positive countries represented only 3.1% of all ingredients imported into the United States. While the United States has policies to prevent foreign entry of plant pests and diseases, Canada and Australia have been more aggressive in their regulatory approach to prevent animal pathogens in plant-based ingredients. Industry led practices can be developed to disallow use of high and medium risk ingredients from ASFV-positive countries in swine feeds and require the use of mitigation strategies in low risk ingredients if there is a high demand from producers and industry leaders.

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Table 3.1 HTS codes utilized and their product descriptions

HTS code	Product Description
1001.19	DURUM WHEAT, OTHER THAN SEED
1001.99	WHEAT AND MESLIN, NOT DURUM WHEAT, OTHER THAN SEED
1002.9	RYE, OTHER THAN SEED
1003.9	BARLEY, OTHER THAN SEED
1004.9	OATS, OTHER THAN SEED
1005.9	CORN (MAIZE), OTHER THAN SEED CORN
1006.1	RICE IN THE HUSK (PADDY OR ROUGH)
1007.9	GRAIN SORGHUM, OTHER THAN SEED
1008.1	BUCKWHEAT
1008.6	TRITICALE
1103	CEREAL GROATS, MEAL AND PELLETS
1104	CEREAL GRAINS, OTHERWISE WORKED (HULLED, ROLLED ETC.), EXCEPT RICE (HEADING 1006); GERM OF CEREALS, WHOLE, ROLLED, FLAKED OR GROUND
1107	MALT, WHETHER OR NOT ROASTED
1109	WHEAT GLUTEN, WHETHER OR NOT DRIED
1208	FLOURS AND MEALS OF OIL SEEDS OR OLEAGINOUS FRUITS, OTHER THAN THOSE OF MUSTARD
1201.90.0005	SOYBEAN SEEDS OF A KIND USED AS OIL STOCK, WHETHER OR NOT BROKEN
1201.90.0010	SOYBEANS, CERTIFIED ORGANIC, WHETHER OR NOT BROKEN, EXCEPT SEEDS OF A KIND USED FOR SOWING OR USED AS OIL STOCK
1201.90.0090	SOYBEANS, WHETHER OR NOT BROKEN, OTHER THAN CERTIFIED ORGANIC, NESOI

1204.00.0025	CERTIFIED ORGANIC FLAXSEED (LINSEED) FOR USE AS OIL STOCK, WHETHER OR NOT BROKEN
1204.00.0035	FLAXSEED (LINSEED) FOR USE AS OIL STOCK, WHETHER OR NOT BROKEN
1205.10.0020	RAPE OR COLZA SEEDS FOR USE AS OIL STOCK, LOW ERUCIC ACID, WHETHER OR NOT BROKEN
1205.90.0020	RAPE OR COLZA SEEDS FOR USE AS OIL STOCK, NESOI, WHETHER OR NOT BROKEN
1206.00.0020	SUNFLOWER SEEDS, WHETHER OR NOT BROKEN, FOR USE AS OIL STOCK
1207.60.0000	SAFFLOWER (CARTHAMUS TINCTORIUS) SEEDS, WHETHER OR NOT BROKEN
1507.10.0000	SOYBEAN OIL AND ITS FRACTIONS, CRUDE, WHETHER OR NOT DEGUMMED
1507.90.4020	SOYBEAN OIL AND ITS FRACTIONS, ONCE-REFINED (SUBJECT TO ALKALAI OR CAUSTIC WASH BUT NOT BLEACHED OR DEODORIZED), NOT CHEMICALLY MODIFIED
1507.90.4040	SOYBEAN OIL AND ITS FRACTIONS, FULLY REFINED, WASHED, BLEACHED OR DEODORIZED BUT NOT CHEMICALLY MODIFIED, NESOI
2302.50.0000	BRAN, SHARPS (MIDDLINGS) AND OTHER RESIDUES, WHETHER OR NOT IN PELLETS, DERIVED FROM SIFTING, MILLING OR OTHER WORKINGS OF LEGUMINOUS PLANTS
2303.10.0010	CORN GLUTEN FEED, WHETHER OR NOT IN PELLETS
2303.10.0020	CORN GLUTEN MEAL, WHETHER OR NOT IN PELLETS
2304.00.0000	SOYBEAN OILCAKE AND OTHER SOLID RESIDUES RESULTING FROM THE EXTRACTION OF SOY BEAN OIL, WHETHER OR NOT GROUND OR IN THE FORM OF PELLETS
2306.10.0000	COTTON SEED OILCAKE AND OTHER SOLID RESIDUES RESULTING FROM THE EXTRACTION OF COTTON SEED OIL, WHETHER OR NOT GROUND OR IN THE FORM OF PELLETS
2306.20.0000	LINSEED OILCAKE AND OTHER SOLID RESIDUES RESULTING FROM THE EXTRACTION OF LINSEED OIL, WHETHER OR NOT GROUND OR IN THE FORM OF PELLETS
2306.30.0000	SUNFLOWER SEED OILCAKE AND OTHER SOLID RESIDUES RESULTING FROM THE EXTRACTION OF SUNFLOWER SEED OIL, WHETHER OR NOT GROUND OR IN THE FORM OF PELLETS

2306.41.0000	RAPE OR COLZA SEED OILCAKE AND OTHER SOLID RESIDUES, LOW ERUCIC ACID, RESULTING FROM THE EXTRACTION OF RAPE OR COLZA SEED OIL, WHETHER OR NOT GROUND
2306.49.0000	RAPE OR COLZA SEED OILCAKE AND OTHER SOLID RESIDUES, NESOI, RESULTING FROM THE EXTRACTION OF RAPE OR COLZA SEED OIL, WHETHER OR NOT GROUND/IN PELLETS
2306.90.0120	CORN (MAIZE) GERM OILCAKE AND OTHER SOLID RESIDUES RESULTING FROM THE EXTRACTION OF CORN OIL, WHETHER OR NOT GROUND OR IN THE FORM OF PELLETS
2309.90.7000	PREPARATIONS, WITH A BASIS OF VITAMIN B12, FOR SUPPLEMENTING ANIMAL FEED
2922.41.0010	LYSINE AND ITS ESTERS; SALTS THEREOF,MEETING REQUIREMENTS OF FOOD CHEMICAL CODEX, CODEX ALIMENTARIUS OR UNITED STATES PHARMACOPEIA
2922.41.0090	LYSINE AND ITS ESTERS; SALTS THEREOF,NESOI
2922.49.4950	AMINO ACIDS, NON-AROMATIC, NESOI
2923.10.0000	CHOLINE AND ITS SALTS
2930.40.0000	METHIONINE
2933.99.1200	AROMATIC OR MODIFIED AROMATIC HETEROCYCLIC COMPOUNDS WITH NITROGEN HETERO ATOM(S) ONLY, EXCEPT ACRIDINE, INDOLE AND CARBAZOLE
2936.21.0000	VITAMINS A AND THEIR DERIVATIVES UNMIXED
2936.22.0000	VITAMIN B1 (THIAMINE) AND ITS DERIVATIVES
2936.23.0000	VITAMIN B2 (RIBOFLAVIN) AND ITS DERIVATIVES
2936.24.0000	D- OR DL-PANTOTHENIC ACID (VITAMIN B3 OR VITAMIN B5) AND ITS DERIVATIVES
2936.25.0000	VITAMIN B6 (PYRIDOXINE AND RELATED COMPOUNDS WITH VITAMIN B6 ACTIVITY) AND ITS DERIVATIVES
2936.26.0000	VITAMIN B12 (CYANOCOBALAMIN AND RELATED COMPOUNDS WITH VITAMIN B12 ACTIVITY) AND ITS DERIVATIVES
2936.27.0000	VITAMIN C (ASCORBIC ACID) AND ITS DERIVATIVES
2936.28.0000	VITAMIN E (TOCOPHEROLS AND RELATED COMPOUNDS WITH VITAMIN E ACTIVITY) AND ITS DERIVATIVES

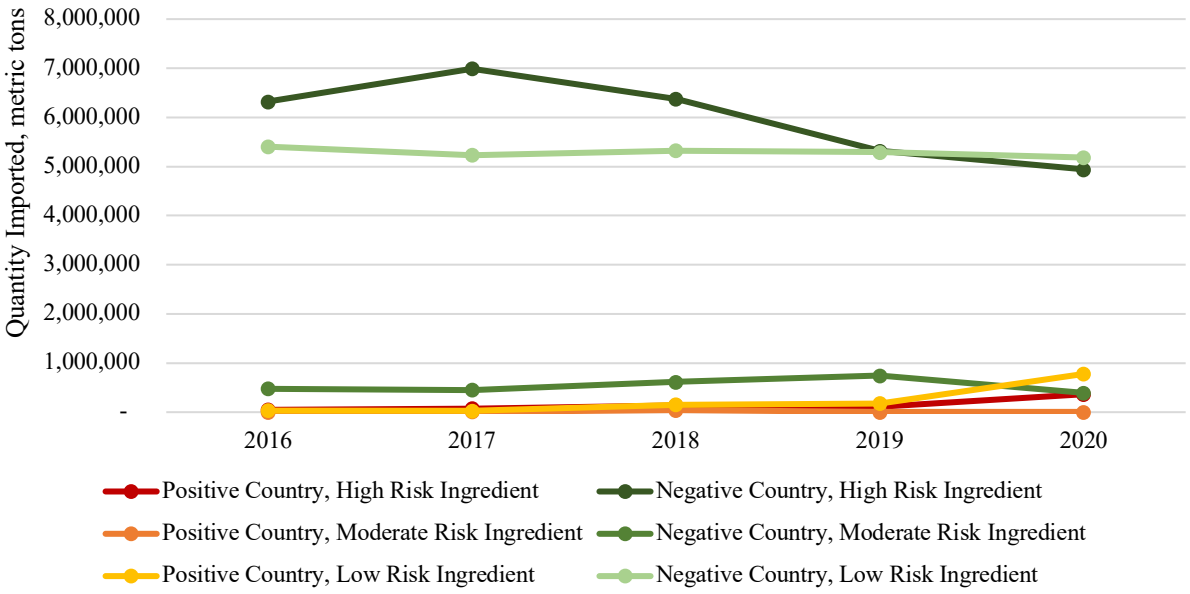
2936.29.1000	FOLIC ACID
2936.29.1510	NIACIN (PHARMACEUTICAL GRADE)
2936.29.1520	NIACIN (EXCLUDING PHARMACEUTICAL GRADE)
2936.29.1530	NIACINAMIDE
2936.29.2000	OTHER AROMATIC OR MODIFIED AROMATIC VITAMINS AND THEIR DERIVATIVES
2936.29.5020	VITAMINS D AND THEIR DERIVATIVES
2936.29.5050	OTHER VITAMINS (EXCLUDING AROMATIC AND MODIFIED AROMATIC) AND THEIR DERIVATIVES
2936.90.0110	PROVITAMINS
2936.90.0150	OTHER INCLUDING NATURAL CONCENTRATES
3004.50.5005	OTHER MEDICAMENTS CONTAINING VITAMINS OR OTHER PRODUCTS OF HEADING 2936 NESOI, FOR VETERINARY USE
3004.50.5010	SINGLE VITAMINS COMBINED WITH MINERALS OR OTHER NUTRIENTS
3004.50.5020	SINGLE VITAMINS, NOT COMBINED WITH MINERALS OR OTHER NUTRIENTS
3004.50.5030	MULTIPLE VITAMINS COMBINED WITH MINERALS OR OTHER NUTRIENTS
3004.50.5040	MULTIPLE VITAMINS NOT COMBINED WITH MINERALS OR OTHER NUTRIENTS

Table 3.2 Perceived risk of ingredients imported into the United States

Low[1]	Moderate[2]	High[3]
Amino acids (aromatic, Lys, Met)	Oilseed meals and flours	Choline
Grain co-products (flaked, germ, gluten feed, malt)	Soy oil	Grain co-products (groats, hulls, middlings)
Oil cakes (corn, cottonseed, rapeseed, sunflower)	Soy oilcake	Oilseed co-products (hulls)
Oils (flaxseed, linseed, safflower, sunflower)		Whole and ground grains
Vitamins (A, B1, B2, B3, B3 and B5, B6, B9, C, D, E, aromatic, other)		Whole oilseeds

[1] Processed ingredients which undergo either thermal or chemical processing during the manufacturing process.
[2] Processed ingredients which undergo thermal or chemical processing but are able to maintain virus for an extended period of time.
[3] Unprocessed ingredients

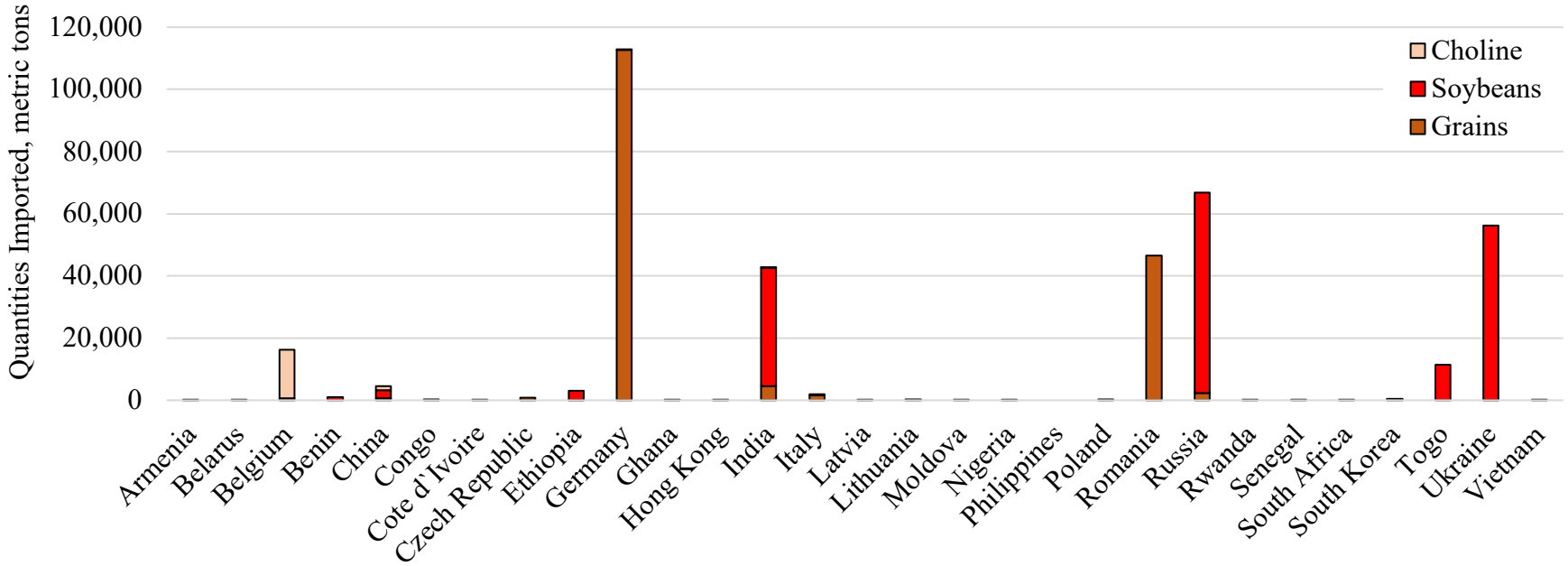
Figure 3.1 Ingredients imported from ASFV-free and positive countries from 2016 to 2020[a,b]



[a] Quantities (metric tons) imported were obtained from the United States International Trade and Tariff Database.

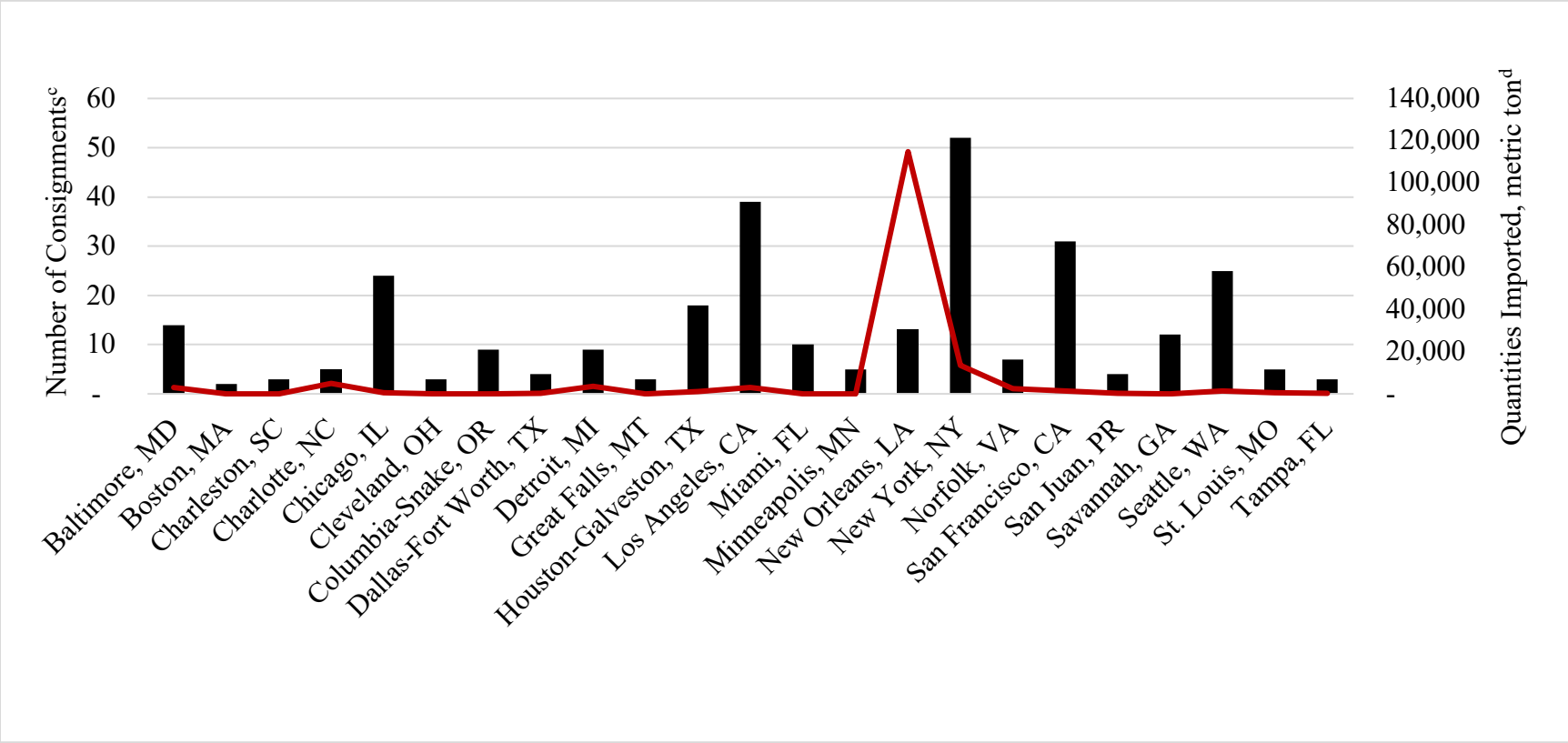
[b] African swine fever status was determined based on presence of cases in a country during each year as reported by the OIE-WAHIS Quantitative Data database.

Figure 3.2 High-risk ingredients imported from ASFV-positive countries in 2020[a,b]



[a] Quantities (metric tons) imported were obtained from the United States International Trade and Tariff Database.
[b] African swine fever status was determined based on presence of cases in a country during each year as reported by the OIE-WAHIS Quantitative Data database.

Figure 3.3 Port of entry high-risk ingredients imported into the United States from ASFV-positive countries in 2020[a,b]



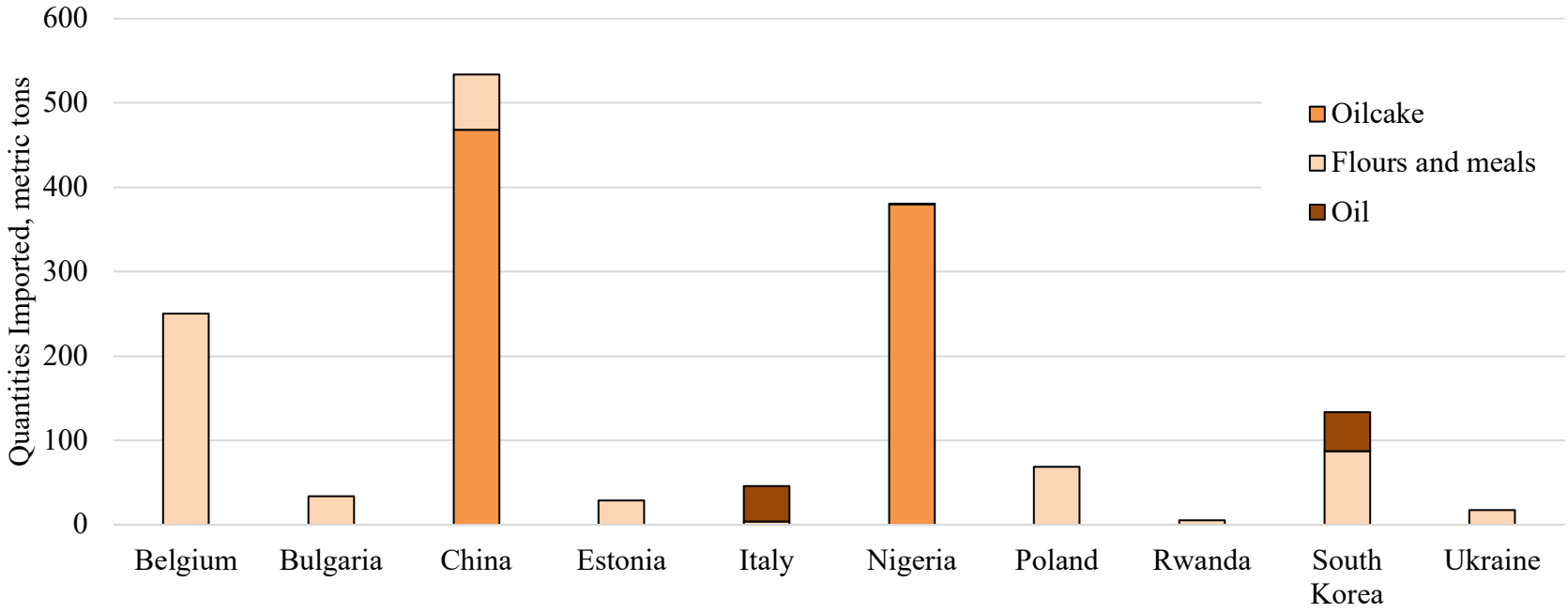
[a] Quantities (metric tons) imported were obtained from the United States International Trade and Tariff Database.
[b] African swine fever status was determined based on presence of cases in a country during each year as reported by the OIE-WAHIS Quantitative Data database.
[c] Number of consignments are represented by the black bars
[d] Quantities imported are represented by the red line

Table 3.3 Unprocessed ingredients from ASFV-positive countries imported into the Port of New Orleans in 2020[a]

Ingredient	Country of Origin	Port(s) of Loading	Days at Sea	Consignments	Form	Volume, metric ton	Importer	Buyer
Barley	Italy	Sines, Portugal	22.2	29	Boxes	9	Italfoods, Inc	Frantoio Oleario Bartolini Emilio
Certified Organic Soybeans	Ethiopia	Jawaharal Nehru, India	45.1	1	Jumbo bags	220	Sunrise Foods International	Sunrise Foods International
	Russia	Jawaharal Nehru, Valencia, and Mundra, India	45.0	29	Jumbo bags	63,462	Leche Gloria Sociedad Anonima Glorai S A, Sunrise Foods International	Leche Gloria Sociedad Anonima Glorai S A and Norman Krieger, Inc., Sunrise Foods International
	Ukraine	Samsun, Turkey	32.4	16	Jumbo bags and bulk	51,093	Sunrise Foods International	Sunrise Foods International

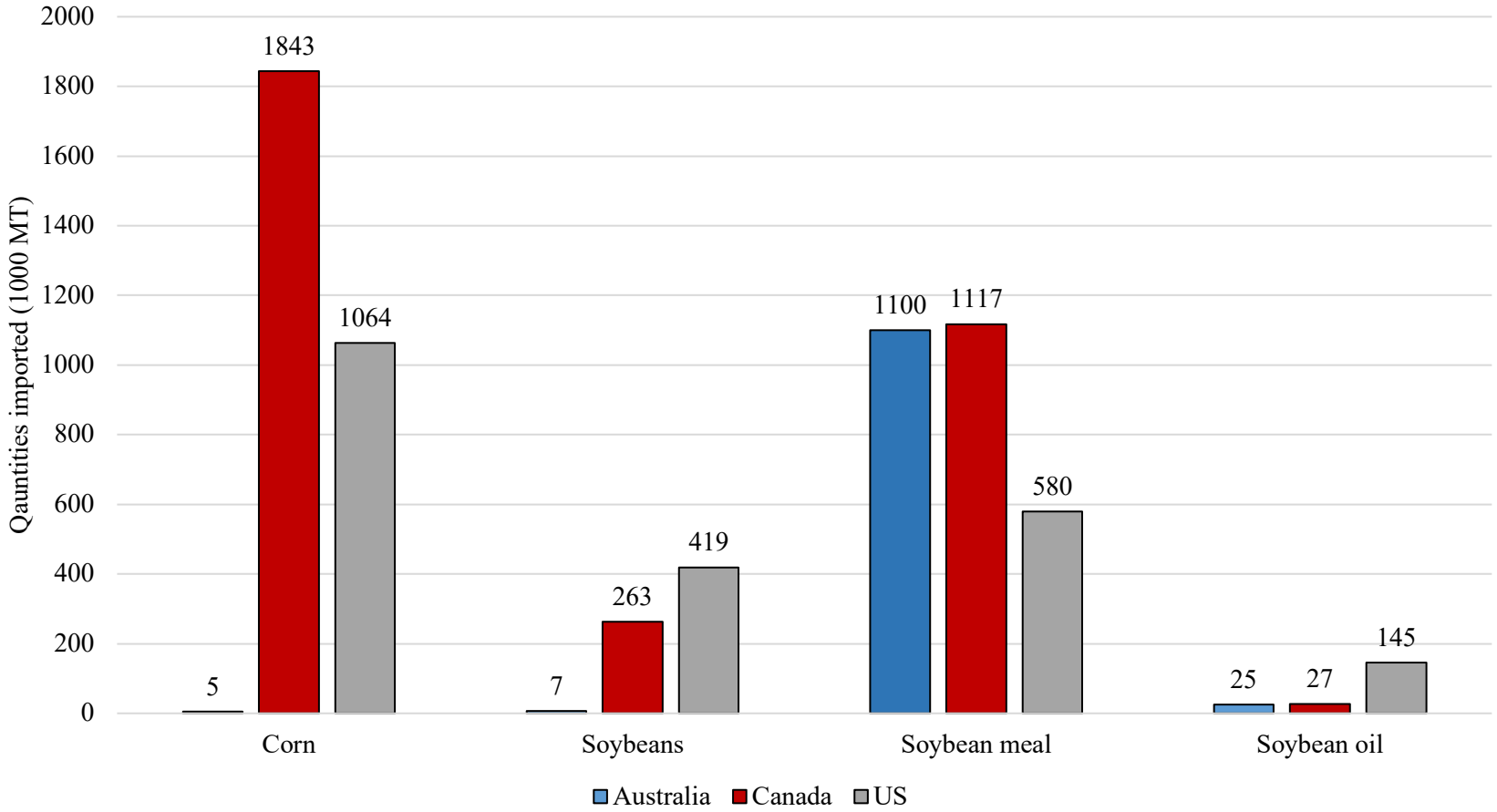
[a] Importer and buyer information was obtained from the United States Import database

Figure 3.4 Moderate risk ingredients imported from ASFV-positive countries in 2020[a,b]



[a] Quantities (metric tons) imported were obtained from the United States International Trade and Tariff Database.
[b] African swine fever status was determined based on presence of cases in a country during each year as reported by the OIE-WAHIS Quantitative Data database.

Figure 3.5 Bulk commodities imported into Australia, Canada, and the United States in 2019/2020[a]



[a] Quantities imported were obtained from the USDA Foreign Agricultural Service – Production, Supply and Distribution database

Chapter 4 - Evaluating chemical mitigation flush strategies in a feed manufacturing facility after manufacturing feed contaminated with porcine epidemic diarrhea virus, porcine reproductive and respiratory syndrome virus, and Seneca Valley virus 1

Abstract

An understanding of pathogen mitigation strategies is needed to reduce the risk of virus transmission between feed mills and swine farms. The objective of this trial was to evaluate different chemical mitigation strategies on the detection of porcine epidemic diarrhea virus (PEDV), porcine reproductive and respiratory syndrome virus (PRRSV), and Seneca Valley virus 1 (SVV1) inoculated feed in the feed and environment of a feed mill. Feed was inoculated with PEDV, PRRSV, and SVV1 and manufactured using a mixer and bucket elevator. Following the inoculated batch, a flush treatment of 1) ground corn, 2) ground corn + liquid formaldehyde (SalCURB LF Liquid, Kemin, Des Moines, IA), 3) ground corn + liquid formaldehyde + abrasive material, 4) ground corn + abrasive material flush followed by ground corn + liquid formaldehyde flush, or 5) dry formaldehyde (SalCURB F2 Dry, Kemin, Des Moines, IA) was utilized, followed by 3 virus-free batches of feed. Feed and environmental samples were collected following each batch and dust samples were collected following the negative, inoculated, and final feed batches. A flush treatment × batch interaction was observed for SVV1 where greater quantities of viral RNA ($P < 0.05$) were present in the inoculated batches and the ground corn flush than those which used chemical mitigants or the post-flush batches. The quantity of viral RNA for all viruses was decreased ($P < 0.05$), but still detectable, in dust from

the last batch of feed compared to the inoculated batch. A batch effect ($P < 0.05$) was observed in all sample matrices for PEDV and PRRSV as RNA decreased after the flush regardless of treatment. The use of chemical mitigants and the implementation of a flush batch reduced the quantity of viral RNA; however, viral presence was still observed in feed and dust which could act as a source of contamination if re-introduced into finished feed.

Introduction

Complete feed has been identified as a potential vector for several swine specific viruses (Dee et al., 2014a; Dee et al., 2018; Dee et al., 2023), such as Seneca Valley virus 1 (SVV1), porcine epidemic diarrhea virus (PEDV), and porcine reproductive and respiratory syndrome virus (PRRSV). In an experimental feed mill setting, PEDV RNA was found in the first batch of feed following the initial contamination (Schumacher et al., 2018), but remained on surfaces within the feed mill up to the fourth and final batch (Schumacher et al., 2017). Similarly, African swine fever virus (ASFV) DNA was present in all four batches of feed following the initial contamination (Elijah et al., 2022) and was found to remain stable in the environment up to 180 days after contamination (Houston et al., 2023b)

To reduce the risk of virus transmission in the feed, multiple mitigation strategies have been evaluated. Flushing, or running feed or an ingredient through the feed manufacturing equipment between batches, can help remove residual virus particles from the equipment and dilute remaining virus concentrations in subsequent batches (Huss et al., 2018; Martinez et al., 2018). A rice hull flush, with or without chemical additives, reduced the presence of PEDV in both the flush batch and subsequent batches of feed (Gebhardt et al., 2018). These chemical mitigants

have a residual, longer lasting effect than other mitigation strategies and can often be combined with flushing or thermal processing to increase the effectiveness of the viral mitigation. Formaldehyde has been identified as one of the most effective chemical mitigants for ASFV (Tran et al., 2020; Niederwerder et al., 2021; Le et al., 2022; Ly et al., 2022), SVV1 and PRRSV (Dee et al., 2021), and PEDV (Dee et al., 2014b; Cochrane et al., 2015; Dee et al., 2015; Dee et al., 2021). However, the efficacy of different flushes with or without the addition of formaldehyde has not been evaluated for viruses currently endemic in the United States. Therefore, the objective of this study was to evaluate different physical and chemical flushes' ability to reduce the viral presence and infectivity of SVV1, PEDV, and PRRSV contaminated feed when introduced into a feed manufacturing facility.

Materials and methods

Feed inoculation and sample collection was conducted at the Kansas State University Cargill Feed Safety Research Center (FSRC), a 3-story biosafety level 2 biocontainment laboratory. Biocontainment was entered 10 separate times, representing 10 inoculation cycles that were needed to complete two replicates of each flush treatment. Following each inoculation cycle, decontamination was conducted following procedures by Huss et al. (2017). Biocontainment was disabled after decontamination procedures were completed and environmental swabs of the facility had been confirmed negative for all viruses via qRT-PCR at the Kansas State University Veterinary Diagnostic Laboratory. All protocols were approved by the Kansas State University Institutional Biosafety Committee (IBC-1636).

Inoculum preparation

An equal volume of SVV-1 (GenBank: KX7780101.1), PEDV CO-isolate (GenBank KF272920), and PRRSV 1-7-4 (GenBank: PP239061) were obtained from the Animal Disease Research and Diagnostic Laboratory at South Dakota State University. The original stock contained 1 × 108 50% tissue culture infectious dose/mL (TCID50/mL) SVV-1, 1 × 107 TCID50/mL PEDV, and 1 × 108 TCID50/mL PRRSV. Viruses were individually packaged into 25 mL aliquots, shipped to KSU on dry ice, and stored at -80°C until used in the FSRC. One aliquot of each virus was removed from storage on the day of inoculation, transported the FSRC, and allowed to thaw at room temperature.

Swine Diet

A corn-soybean meal (79.0 and 17.3%, respectively) mash gestation diet with no additional fat or feed additives was manufactured at Hubbard Feeds (Beloit, Kansas) with similar diet composition as Elijah et al. (2022). Feed samples were collected from multiple bags after feed delivery and submitted for PCR analysis to confirm SVV1, PEDV, and PRRSV negative status prior to entering the FSRC.

Feed Inoculation

The inoculated batch was inoculated similar to methods by Cochrane et al. (2017) and Schumacher et al. (2018). Viruses were combined (23 mL each) in 613 mL of phosphate buffer solution (PBS) to achieve an adequate volume for efficient mixing into the feed. Aliquots of each virus prior to dilution and the combined inoculum in PBS were retained for analysis. The 682 mL inoculum was added to 2.2 kg of feed in a 5 kg benchtop stainless steel paddle mixer

(Cabela's, Inc., Sidney NE) and mixed for 5 minutes creating 2.8 kg of feed. The contents of the benchtop mixer were added to 19.9 kg of feed and mixed for 5 minutes. In summary, 23 mL of each virus at an initial concentration of 1 × 108 TCID50/mL (PEDV at 1 × 107 TCID50/mL) evenly distributed in 22.1 kg of feed represents an approximate final concentration of 1 × 105 TCID50/g of feed for SVV1 and PRRSV and 1 × 104 TCID50/g of feed for PEDV.

Feed manufacturing

Feed batch sequencing was conducted similarly to Schumacher et al. (2018) and (Elijah et al., 2022), wherein the feed manufacturing equipment was primed with a virus negative batch of feed (primer batch), followed by the previously described batch of feed inoculated with equal volume of SVV-1, PEDV, and PRRSV (inoculated batch), followed by a flush treatment, and three subsequent batches of virus negative feed (Sequence 1, 2, and 3). During each batch, feed was mixed for 5 minutes in a 22.7 kg mixer (Hayes & Stolz Industrial Manufacturing Co. LLC, Burleson, TX) and discharged at a rate of 4.5 kg/min into a feed bin. Feed was then poured into the hopper of a bucket elevator (Universal Industries, Cedar Falls, IA) with 74 buckets (each 114 cm3) at an equal discharge rate (4.5 kg/min) and conveyed through a downspout into a fresh feed bin.

Flush treatments

Five flush treatments were included in the study, with two replicates of each treatment. Flush treatments were manufactured following the same procedures as mixing of the primer and inoculated batches of feed. The application and composition of each flush treatment were as follows:

1. Ground corn: 22.7 kg of ground corn (500 μm).
2. Liquid formaldehyde: A liquid applicator pump and spray nozzle (Lenze, Aerzen, Germany) was connected to the 22.7 kg mixer. A pre-measured 369 g aliquot of 10% liquid formaldehyde (Sal CURB LF Liquid, Kemin Industries, Inc., Des Moines, IA) was dispensed onto 22.7 kg of ground corn. Once dispensed, the ground corn + liquid formaldehyde solution was allowed to mix for 5 minutes.
3. Liquid formaldehyde + physical abrasive: A 25% abrasive mixture of 5.6 kg calcium limestone (Shell and Bone Builder, Fehrway Feeds & Livestock Equipment, Winkler, MB, Canada) and 16.8 kg ground corn was added to the mixer. A pre-measured 369 g aliquot of 10% liquid formaldehyde (Sal CURB LF Liquid, Kemin Industries, Inc., Des Moines, IA) was dispensed onto the abrasive mixture. Once dispensed, the abrasive mixture + liquid formaldehyde solution was allowed to mix for 5 minutes.
4. Double flush – physical abrasive followed by liquid formaldehyde: The first flush, a 25% abrasive mixture of 5.6 kg calcium limestone (Shell and Bone Builder, Fehrway Feeds & Livestock Equipment, Winkler, MB, Canada) and 16.8 kg ground corn, was allowed to mix for 5 minutes before discharged through the bucket elevator. Following the physical abrasive flush, a pre-measured 369 g aliquot of 10% liquid formaldehyde (Sal CURB LF Liquid, Kemin Industries, Inc., Des Moines, IA) was dispensed onto 22.7 kg of ground corn. Once dispensed, the ground corn + liquid formaldehyde solution was allowed to mix for 5 minutes.
5. Dry formaldehyde: 22.7 kg of a 4% dry formaldehyde product (Sal CURB F2 Dry, Kemin Industries, Inc., Des Moines, IA) was added to the mixer and allowed to mix for 5 minutes.

Safety precautions

The current approved application of liquid formaldehyde is 3 kg/MT in the diet. The inclusion used in the liquid formaldehyde treatments was 5 times the recommended levels at 15 kg/MT inclusion in the diet. For the researchers' safety, full face respirators (6800 Series, 3M, St. Paul, MN) with disposable cartridges (Multi Gas/Vapor Cartridge/Filter 60926, P100, 3M, St. Paul, MN) were used once either liquid or dry formaldehyde products were implemented. Environmental formaldehyde levels were monitored throughout feed manufacturing of subsequent batches with a formaldehyde meter (ENMET, Ann Arbor, MI).

Feed Sample Collection

Feed samples were collected from the mixer and the bucket elevator during every batch for a total of 12 feed samples per inoculation cycle (14 feed samples for the double flush treatment). Samples from the mixer and bucket elevator were collected using the cut stream method. During discharge a 2 oz plastic cup (Great Value Cups, 2 oz, Walmart, Bentonville, AR) was filled; this process was repeated twice more every 90 seconds. The three individual samples from each piece of equipment were combined into one composite sample weighing 50 g in a Whirl-pak bag (Nasco, Fort Atkinson, WI).

Environmental Sample Collection

Environmental samples were collected from the ribbon of the mixer, the boot of the bucket elevator, and a bucket of the bucket elevator, for a total of 18 feed samples per inoculation cycle (21 environmental samples for the double flush treatment). Environmental samples were taken following procedures similar to Huss et al. (2017). Briefly, a 10 cm × 10 cm cotton surgical

gauze pre-moistened with 5 mL of PBS was prepared and stored in a 50 mL conical tube prior to sampling. A new glove was applied prior to aseptically removing the gauze and swabbing the designated area. The gauze was returned to the conical tube and the glove was discarded.

Dust sample collection

Dust samples were collected from non-feed contact surfaces, such as the top grate of the mixer, the top lip of the discharge bin following the bucket elevator down spout, and the floor surrounding the bucket elevator discharge bin. Samples were collected following the primer batch, the inoculated batch, the flush batch, and Sequence 3 for a total of 12 dust samples per inoculation cycle (15 dust samples for the double flush treatment). Similar to Gebhardt et al. (2018), accumulated dust was collected onto a fresh index card; approximately 2 mL of dust was placed in a 15 mL conical tube for PCR analysis while the remaining dust (between 2-12 mL) was retained in a separate conical tube for a bioassay.

Sample processing

All samples were double bagged, sprayed with 1% peroxygen (Virkon S, Lanxess, Germany) before entering the changing room, submerged in a 1% peroxygen solution while researchers showered out, placed on ice in a biohazard cooler, and transported to a biosafety level-2 laboratory in the KSU Veterinary Diagnostic Laboratory for further processing. Each 50 g feed sample was transferred into 500 mL high density polyurethane bottles with 200 mL of PBS to create a 20% suspension. Similarly, varying quantities of PBS was added to the dust samples directly in the conical tubes to create a 20% suspension. Feed and dust samples were shaken for approximately 10 sec and stored at 4°C overnight. The next day, 25 mL of the supernatant from

the feed samples were transferred to a fresh 50 mL conical tubes. The feed samples were centrifuged at 4,000 × g for 10 minutes at 8°C. Two 300 µL aliquots of the supernatant were retained for PCR analysis and 20 mL of supernatant was transferred to a fresh 50 mL conical tube for a bioassay (Dee et al., 2022; Houston et al., 2023a). Dust samples were similarly centrifuged with 2-300 µL aliquots of the supernatant retained for PCR; undiluted dust was already retained for the bioassay (Gebhardt et al., 2018). For environmental samples, 20 mL of PBS was added and each tube vortexed for 10 sec. Environmental samples were allowed to incubate at room temperature for one hour. After one hour, a 20 mL aliquot was transferred to a fresh 50 mL conical tube. Centrifugation and sample allocation followed the feed sample procedures (Khanal et al., 2022; Houston et al., 2023a).

Quantitative polymerase chain reaction analysis

Samples were analyzed for detection of SVV1, PEDV, and PRRSV using quantitative reverse transcription real-time polymerase chain reaction (qRT-PCR) at the Kansas State University Veterinary Diagnostic Laboratory. First, 50 µL of supernatant was placed in a deep well plate and RNA extracted using a Kingfisher Flex magnetic particle processor (Fisher Scientific, Pittsburgh, PA) and a MagMAX-96 Viral Isolation Kit (Life Technologies, Grand Island, NY). The final elution volume was reduced to 60 µL, and extracted RNA was stored at -80°C until analyzed for SVV1, PEDV, or PRRSV using a qRT-PCR tri-plex assay with a maximum cycle threshold of 45. Results were reported as the number of samples considered positive and the cycle threshold (Ct) below 45 at which either SVV1, PEDV, or PRRSV RNA was detected.

Bioassay

A pilot bioassay was conducted at Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL) to verify the inoculation methods planned for the experimental bioassay. The pilot bioassay included four treatment rooms with 3 mixed sex 10-day old piglets in each room. The treatments included 1) pure virus positive control with an equal volume SVV1, PEDV, and PRRSV diluted to the approximate concentration of the inoculated feed batch, 2) feed samples from the inoculated feed batch, 3) dust samples from the inoculated feed batch, and 4) environmental samples from the inoculated feed batch. All samples were collected during the final inoculum cycle and were collected in addition to the samples normally collected each inoculation cycle. Each pig was inoculated with 2 mL intramuscularly, 2 mL intranasally (1 mL/nostril) and either 10 mL oral gavage (feed and environmental samples) or a 10 mL dust slurry fed to the piglet to allow natural swallowing reflexes. Prior to piglets arriving at ISU-VDL, serum from the sows the piglets were nursing was tested for PRRSV with an enzyme-linked immunosorbent assay and SVV1 and PEDV with an indirect fluorescent antibody assay. Additionally, sow serum and rectal swabs were tested for PRRSV, SVV1, and enteric coronaviruses via PCR. Prior to inoculation, individual piglet serum and feces were also tested for PRRSV, SVV1, and enteric coronaviruses via PCR. All sows and piglets tested negative for SVV1, PEDV, and PRRSV prior to inoculation. During the bioassay, rectal swabs were collected day 1-7 post-inoculation, blood samples were collected 4 and 7 dpi. Tonsils, lung tissue, jejunal and cecal tissue and cecal contents were collected at necropsy. Day 0 inoculum, rectal (SVV1 and PEDV), and serum (PRRSV) samples were analyzed via PCR at the ISU-VDL.

The experimental bioassay included 12 treatment rooms with 3 mixed sex 10-day old piglets in each room. The inoculation treatments included 1) negative control, 2) pure virus positive control with an equal volume SVV1, PEDV, and PRRSV diluted to the approximate concentration of the inoculated batch of feed, 3) feed samples from the inoculated batch, 4) dust samples from the inoculated batch, 5) feed from the ground corn sequence 1 batch, 6) feed from the ground corn sequence 3 batch, 7) feed from the SalCURB sequence 1 batch, 8) feed from the dry formaldehyde sequence 1 batch, 9) feed from the dry formaldehyde sequence 3 batch, 10) dust from the ground corn sequence 3 batch, 11) dust from the SalCURB sequence 3 batch, and 12) dust from the dry formaldehyde sequence 3 batch. The inoculation and sampling methods followed the same protocol and timeline as the pilot bioassay.

Statistical analysis

Results were analyzed as a split plot design with the inoculation cycle as the experimental unit for the flush treatment, and individual sample as the experimental unit for sampling location and batch. Fixed effects included flush treatment, location, batch, and their associated interactions. Inoculation cycle was included in the model as a random effect. Data were separated and individually analyzed based on the sample type (feed, environmental, or dust) and virus (SVV1, PEDV, and PRRSV). Two response criteria were considered, the number of PCR positive samples and the quantity of detectable viral RNA. Data were analyzed by fitting to a binary distribution, logit link, Laplace approximation, and ridge-stabilized Newton-Raphson algorithm. As a binary distribution model, data were fit by each individual interaction, starting with the treatment × location × batch interaction, and their subsequent main effects. To estimate the quantity of detectable viral RNA, the Ct of each sample was used. If no viral RNA was detected,

samples were assigned a value of 45. A Kenward-Roger denominator degree of freedom adjustment was used, as well as a Tukey-Kramer multiple comparison adjustment. All data were analyzed using GLIMMIX procedure of SAS version 9.4 (SAS Institute Inc., Cary, NC) and results were considered significant at $P \leq 0.05$.

Results and discussion

PCR Results

As expected, SVV1, PEDV, and PRRSV RNA was not detected in any primer batch of feed nor the environment during the ten inoculation cycles. There was no evidence of a treatment × batch × location interaction ($P > 0.05$) for any of the sampling matrices (feed, environmental, or dust) regardless of the virus analyzed, nor were 2-way interactions observed for either PEDV or PRRSV. A treatment × batch interaction was observed for SVV1 feed samples (Table 1), where the inoculated batch had the greatest quantity of viral RNA ($P < 0.05$) and feed samples from the ground corn flush had more detectable SVV1 RNA than either the liquid or dry formaldehyde flushes. Similarly, detectable SVV1 RNA was greatest in the inoculated batch dust samples, with the ground corn flush having the greatest quantity of viral RNA ($P < 0.05$) compared to all other flush treatments. For both feed and dust samples, there was no treatment difference ($P > 0.05$) in detectable SVV1 RNA when measured in the subsequent sequence batches.

Formaldehyde has often been found to be an effective chemical mitigant for multiple swine viruses including ASFV (Niederwerder et al., 2021), SVV1, PEDV, and PRRSV (Dee et al., 2021). Specifically, formaldehyde can interact and bind to proteins, DNA, or RNA within the virus, causing mutations, and thereby limiting the ability of the virus to replicate (McDonnell and

Russell, 1999). Gebhardt et al. (2018) observed an initial decrease in PEDV positive samples following a rice hull flush which was further reduced in subsequent negative batches of feed. The quantity of PCR negative samples was increased when either formaldehyde or medium chain fatty acids were included in the flush (Gebhardt et al., 2018), similar to the quantity of detectable SVV1 RNA following a formaldehyde flush in either its liquid or dry form. The dry formaldehyde product used in the experiment is not currently approved for use in the United States but may have a similar efficacy to liquid formaldehyde (Tran et al., 2020); however the high inclusion level of either product poses a health hazard. Immediately following product application in the mixer, environmental formaldehyde levels reached a high of 24.7 and 185 mg/kg or the liquid and dry formaldehyde treatments, respectively. Following the final batch of the day, environmental formaldehyde was 0.4 ppm following the liquid treatment which is below the 0.5 mg/kg permissible exposure limit for an 8-hour day (OSHA, 2011). During the dry formaldehyde treatment environmental formaldehyde concentrations were above 3 ppm following the final batch of feed and remained above 2 ppm 24 hours after the initial product application.

In the environment, there was significantly more ($P < 0.05$) SVV1 positive samples in the two ground corn inoculation cycles (20/36) than the dry formaldehyde cycles (6/36) with all other flush treatments being intermediate (Table 2). While this is the first study to evaluate SVV1 RNA presence and concentration in the environment following a flush, it is not surprising that less RNA was detected following the dry formaldehyde flush than the ground corn. Physically, the fine particle size of the dry formaldehyde product coated the feed contact and non-feed contact surfaces of the equipment more so than the ground corn and liquid formaldehyde

treatments. The efficacy of the dry formaldehyde is therefore due to both its chemical mitigation efficacy and its physical capabilities to spread throughout the feed mill prolonging the mitigation contact time.

Differences in both the quantity of detectable RNA and the proportion of samples PCR positive based off location were noticed as the mixer, a metal surface, had less detectable SVV1 RNA ($P < 0.05$) and fewer PCR positive environmental samples ($P < 0.05$) than the plastic bucket within the bucket elevator, with the metal boot of the bucket elevator being intermediate (Table 3). Schumacher et al. (2017) also noticed a greater proportion of PCR samples and subsequently more PEDV RNA on the surfaces within the bucket elevator as compared to the mixer. While there is some evidence virus concentrations differ between surfaces (Muckey et al., 2021), the increased presence of viral RNA is most likely due to the design of the bucket elevator itself. The bucket elevator has a constant level of feed remaining in the boot unless the feed is manually removed and cleaned. This residual feed comes in contact with subsequent batches and can re-contaminate the surfaces of the elevator as the contaminated feed is disturbed and moved. Similar instances of re-contamination have been noticed in the bucket elevator when drug-carryover has been evaluated (Martinez et al., 2018; Akoto and Maier, 2023).

The drug-carryover model by Akoto and Maier (2023) can also describe the batch effect demonstrated by all viruses in each sampling matrices (Table 4), with the exception of PRRSV environmental sample as no viral RNA was detected in any environmental sample. The inoculated batch consistently had greater quantities of the respective viral RNA ($P < 0.05$) which decreased with subsequent batches. However, viral RNA was still present after the final batch for

SVV1 feed, environmental, and dust samples and PEDV feed samples. As expected, the greatest quantity of PEDV RNA (Schumacher et al., 2018) and ASFV DNA (Elijah et al., 2022) was in the experimentally contaminated batch of feed which decreased in subsequent negative batches of feed. Similar to PEDV and PRRSV in this study, Schumacher et al. (2018) did not detect PEDV RNA after the second non-inoculated batch of feed. However, ASFV DNA was detected in all subsequent non-inoculated batches of feed (Elijah et al., 2022) comparable to the consistent SVV1 presence observed in feed from this study. Environmental contamination of PEDV and ASFV was more persistent throughout the non-inoculated batches of feed (Schumacher et al., 2017; Elijah et al., 2021b), but neither study utilized chemical flushes which would tangentially decrease viral concentrations as subsequent batches of feed were manufactured. Even with chemical flushes, SVV1 RNA was still found in dust and environmental samples which could re-contaminate the feed or be tracked to other areas of the mill on workers shoes, brooms, or other transient equipment.

Bioassay Results

The results of the pilot bioassay are found in Table 5. As expected, pigs inoculated with the pure virus demonstrated viral replication of SVV1, PEDV, and PRRSV throughout the 7 d bioassay. In the other treatment rooms, only signs of SVV1 viral replication and shedding were observed. Although feed, dust, and environmental samples were PCR positive for both PEDV and PRRSV, no signs of infection or viral replication were observed. It is difficult to determine the minimum Ct needed for successful infection as assays may vary and PCR is only able to detect genetic material, not determine whether the material remains infectious. Nevertheless, Schumacher et al. (2016) estimated a Ct of at least 37.0 was required for successful PEDV infection. The Ct of both

the PEDV and PRRSV inoculums were near this upper threshold which suggests that the quantity of PEDV and PRRSV RNA was not sufficient to cause infection following inoculation in this experiment; however, caution should be used when interpreting infectivity based solely on Ct values.

Results of the swine bioassay are found in Table 6. Pigs inoculated with the pure virus positive control showed no signs of shedding PEDV. Later testing confirmed the PEDV stock virus used as the pure virus positive control was still infectious in a virus isolation assay, so it is unknown why infection did not occur during the bioassay. Pure virus, feed, and dust positive controls were able to cause infection and shedding for both SVV1 and PRRSV. No other treatments displayed signs of SVV1 shedding. PRRSV viremia was noticed in all feed and dust treatment rooms except the dry formaldehyde sequence 3 feed room and ground corn sequence 3 dust room. Interestingly, only two ground corn sequence 3 dust inoculums were PCR positive at either the KSU- or ISU-VDL using PCR with all other feed or dust treatment inoculums being PCR negative. Formaldehyde at lower concentrations has been found to be an effective mitigant against PRRSV (Dee et al., 2021; Elijah et al., 2021a), so why PRRSV RNA remained undetected in PCR but was still infectious in the bioassay at the greater formaldehyde concentrations used in the flushes remains unclear. Cross-contamination between rooms could have also occurred, but retracing employee and researcher movements did not highlight any obvious cross-contamination scenarios. These results highlight the sensitivity and necessity for swine bioassays to better understand infectivity compared to only laboratory assays such as PCR.

Conclusion

Flush batches between the purposefully inoculated batch and subsequent virus-free batches of feed were able to reduce viral RNA regardless of treatment type. Chemical mitigants reduced viral RNA, specifically SVV1 RNA, more quickly than the non-chemical ground corn treatment; however, viral RNA was still present in feed and within the environment after the final batch. The flush treatments were able to reduce SVV1 infectivity of the subsequent feed and dust samples when given to pigs in a bioassay, but non-detectable PRRSV RNA was still infectious when consumed by pigs. Flushing feed manufacturing systems following a pathogen introduction can reduce the initial contamination but did not completely eliminate the risk of recontamination in both the feed and the environment.

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Table 4.1 Effect of treatment and batch on the relative quantification of Seneca Valley virus 1 (SVV1) in feed and dust.[1]

	Batch					
	Primer[2]	Inoculated	Flush	Sequence 1	Sequence 2	Sequence 3
Feed[3]						
Ground corn	45.0	27.8^{c}	34.1^{bc}	38.9^{ab}	45.0^{a}	41.3^{a}
Liquid formaldehyde[4]	45.0	27.1^{c}	43.6^{a}	45.0^{a}	45.0^{a}	45.0^{a}
Liquid formaldehyde + abrasive[5]	45.0	27.7^{c}	40.5^{ab}	43.4^{a}	43.3^{a}	45.0^{a}
Double flush[6]	45.0	27.3^{c}	39.8^{ab}	45.0^{a}	45.0^{a}	43.3^{a}
Dry formaldehyde[7]	45.0	27.2^{c}	45.0^{a}	45.0^{a}	45.0^{a}	43.5^{a}
Dust[8,9]						
Ground corn	45.0	30.3^{e}	31.9^{de}	—	—	37.6^{bcd}
Liquid formaldehyde[4]	45.0	31.6^{de}	42.2^{abc}	—	—	43.5^{ab}
Liquid formaldehyde + abrasive[5]	45.0	29.8^{e}	38.1^{abcd}	—	—	43.4^{ab}
Double flush[6]	45.0	28.0^{e}	37.3^{cd}	—	—	43.8^{ab}
Dry formaldehyde[7]	45.0	29.1^{e}	45.0^{a}	—	—	43.8^{ab}

[1] A 22.7 batch of feed was inoculated with Seneca Valley virus 1, porcine epidemic diarrhea virus, and porcine reproductive and respiratory syndrome virus, followed by a flush treatment and three virus-negative batches of feed. Feed, environmental, and dust samples were taken following each batch of feed. A cycle threshold (Ct) value of 45.0 is considered negative with no detectable viral RNA.
[2] Samples from the primer batch were excluded from the statistical analysis as all samples were considered negative.
[3] Feed Ct value: Treatment × batch, $P = 0.011$, SEM = 1.90
[4] Liquid formaldehyde flush: 15 kg/MT liquid formaldehyde (Sal CURB LF Liquid, Kemin, Industries, Inc. Des Moines, IA) in 22.7 kg of ground corn.
[5] Liquid formaldehyde + abrasive flush: 15 kg/MT liquid formaldehyde (Sal CURB LF Liquid, Kemin, Industries, Inc. Des Moines, IA) in 17.0 kg of ground corn and 5.6 kg calcium limestone.
[6] Double flush: Flush 1) 17.0 kg of ground corn and 5.6 kg calcium limestone followed by Flush 2) 15 kg/MT liquid formaldehyde (Sal CURB LF Liquid, Kemin, Industries, Inc. Des Moines, IA) in 22.7 kg of ground corn.
[7] Dry formaldehyde: 22.7 kg of dry formaldehyde product (Sal CURB F2 Dry, Kemin Industries, Inc., Des Moines, IA)
[8] Dust Ct value: Treatment × batch, $P = 0.0004$, SEM = 2.00
[9] Dust samples were not collected during Sequence 1 or 2 feed batches.
^{abcde} means with differing superscripts within the "Feed" or "Dust" matrix differ significantly, $P < 0.05$

Table 4.2 Effect of treatment on the proportion PCR positive of Seneca Valley virus 1 (SVV1) in environmental samples from a feed manufacturing facility after inoculation.[1,2]

	Ground corn	Liquid formaldehyde[3]	Liquid formaldehyde + abrasive[4]	Double flush[5,6]	Dry formaldehyde[7]
Proportion positive	20/30[a]	13/30[ab]	9/30[ab]	16/36[ab]	6/30[b]

[1] A 22.7 kg batch of feed was inoculated with Seneca Valley virus 1, porcine epidemic diarrhea virus, and porcine reproductive and respiratory syndrome virus, followed by a flush treatment and three virus-negative batches of feed. Environmental samples were taken from three locations following each batch of feed. A cycle threshold (Ct) value of 45.0 is considered negative with no detectable viral RNA. Proportion positive of SVV1: Batch, $P = 0.022$

[2] Samples from the primer batch (n=6 for each treatment) were excluded from the statistical analysis as all samples were considered negative.

[3] Liquid formaldehyde flush: 15 kg/MT liquid formaldehyde (Sal CURB LF Liquid, Kemin, Industries, Inc. Des Moines, IA) in 22.7 kg of ground corn.

[4] Liquid formaldehyde + abrasive flush: 15 kg/MT liquid formaldehyde (Sal CURB LF Liquid, Kemin, Industries, Inc. Des Moines, IA) in 17.0 kg of ground corn and 5.6 kg calcium limestone.

[5] Double flush: Flush 1) 17.0 kg of ground corn and 5.6 kg calcium limestone followed by Flush 2) 15 kg/MT liquid formaldehyde (Sal CURB LF Liquid, Kemin, Industries, Inc. Des Moines, IA) in 22.7 kg of ground corn.

[6] This treatment has an additional 6 samples due to the double flush.

[7] Dry formaldehyde: 22.7 kg of dry formaldehyde product (Sal CURB F2 Dry, Kemin Industries, Inc., Des Moines, IA)

[ab] means with differing superscripts within row differ significantly $P < 0.05$

Table 4.3 Effect of location on the proportion of PCR positive samples and relative quantification of Seneca Valley virus 1 (SVV1) in environmental samples from a feed manufacturing facility after inoculation.[1,2]

	Mixer	Boot – bucket elevator	Bucket – bucket elevator
SVV1 Ct[3]	42.2[a]	41.4[ab]	39.6[b]
Proportion positive[4]	14/52[b]	20/52[ab]	30/52[a]

[1] A 22.7 kg batch of feed was inoculated with Seneca Valley virus 1, porcine epidemic diarrhea virus, and porcine reproductive and respiratory syndrome virus, followed by a flush treatment and three virus-negative batches of feed. Environmental samples were taken from three locations following each batch of feed. A cycle threshold (Ct) value of 45.0 is considered negative with no detectable viral RNA.

[2] Samples from the primer batch (n=10 for each location) were excluded from the statistical analysis as all samples were considered negative.

[3] SVV1 Ct: Batch, $P = 0.0297$, SEM = 0.8065

[4] Proportion positive: Batch, $P = 0.005$

[ab] means with differing superscripts within row differ significantly $P < 0.05$.

Table 4.4 Effect of batch on the relative quantification of Seneca Valley virus 1 (SVV1), porcine epidemic diarrhea virus (PEDV), and porcine reproductive and respiratory syndrome virus (PRRSV) RNA in feed, environmental samples, and dust samples.[1]

	Batch							
	Primer[2]	Inoculated	Flush	Sequence 1	Sequence 2	Sequence 3	SEM	*P* =
SVV1								
Feed samples	45.0	27.5^{c}	40.6^{b}	43.5^{a}	44.7^{a}	43.6^{a}	0.60	< 0.0001
Environmental samples	45.0	33.6^{c}	41.1^{b}	42.6^{ab}	43.4^{a}	44.0^{a}	0.84	< 0.0001
Dust samples[3]	45.0	29.8^{c}	38.7^{b}	—	—	42.4^{a}	0.97	< 0.0001
PEDV								
Feed samples	45.0	36.5^{b}	44.8^{a}	45.0^{a}	45.0^{a}	44.9^{a}	0.47	< 0.001
Environmental samples	45.0	42.8^{b}	44.8^{a}	45.0^{a}	45.0^{a}	45.0^{a}	0.35	< 0.0001
Dust samples[3]	45.0	38.7^{b}	44.7^{a}	—	—	45.0^{a}	0.62	< 0.0001
PRRSV								
Feed samples	45.0	41.3^{b}	44.8^{a}	45.0^{a}	45.0^{a}	45.0^{a}	0.38	< 0.001
Environmental samples[4]	45.0	45.0	45.0	45.0	45.0	45.0	—	—
Dust samples[3]	45.0	43.2^{b}	44.8^{a}	—	—	45.0^{a}	0.36	0.0002

[1] A 22.7 kg batch of feed was inoculated with SVV1, PEDV, and PRRSV, followed by a flush treatment and three virus-negative batches of feed. Feed, environmental, and dust samples were taken following each batch of feed. A cycle threshold (Ct) value of 45.0 is considered negative with no detectable viral RNA.

[2] Samples from the primer batch were excluded from the statistical analysis as all samples were considered negative.

[3] Dust samples were not collected during Sequence 1 or 2 feed batches.

[4] No statistical analysis was conducted for the PRRSV environmental samples since there was no variation between the samples. All Ct values were equal to 45.0 as all samples were negative.

^{abc} means with differing superscripts within row differ significantly $P < 0.05$

Table 4.5 Effects of Seneca Valley virus 1 (SVV1), porcine epidemic diarrhea virus (PEDV), and porcine reproductive and respiratory syndrome virus (PRRSV) infection when inoculated through multiple exposure routes as evaluated in a swine bioassay.[1]

	SVV1			PEDV			PRRSV		
	Inoculum Ct (PCR positive)[2]	4 dpi	7 dpi	Inoculum Ct (PCR positive)[2]	4 dpi	7 dpi	Inoculum Ct (PCR positive)[2]	4 dpi	7 dpi
Pure virus	20.7 (3/3)	+ + +	+ + +	23.2 (3/3)	+ + +	+ + +	24.3 (3/3)	+ + +	+ + +
Feed	25.5 (3/3)	+ + +	+ + +	33.8 (3/3)	– – –	– – –	33.5 (3/3)	– – –	– – –
Dust	29.4 (3/3)	+ + +	+ + –	31.6 (3/3)	– – –	– – –	32.3 (3/3)	– – –	– – –
Environmental	27.8 (3/3)	+ + +	+ – +	34.6 (1/3)	– – –	– – –	35.6 (1/3)	– – –	– – –

[1] Three pigs per each treatment were inoculated on day 0 via intramuscular, intranasal, and either oral gavage (feed and environmental samples) or a slurry (dust samples) and evaluated for 7 days. Rectal samples were taken daily to evaluate SVV1 and PEDV presence and blood was collected on 4 and 7 dpi to assess PRRSV presence.

[2] The cycle threshold (Ct) value is the average Ct of all the PCR positive inoculums within each room as analyzed at Iowa State University Veterinary Diagnostic Laboratory following the d 0 inoculation. If no detectable RNA was present in the inoculum, then it was not included in the average Ct. The numerator represents the number of PCR positive inoculum samples with the denominator representing the total number of inoculum samples.

+/– corresponds to the viral status of each pig in the treatment room where + pigs signify viral RNA was present for the respective virus and – pigs had no detectable viral RNA.

Table 4.6 Effects of physical or chemical flushes as Seneca Valley virus 1 (SVV1), porcine epidemic diarrhea virus (PEDV), and porcine reproductive and respiratory syndrome virus (PRRSV) mitigation as evaluated by a swine bioassay.[1]

	SVV1			PEDV			PRRSV		
	Inoculum Ct (PCR positive)[2]	4 dpi	7 dpi	Inoculum Ct (PCR positive)[2]	4 dpi	7 dpi	Inoculum Ct (PCR positive)[2]	4 dpi	7 dpi
Control treatments									
Negative	(0/3)	– – –	– – –	(0/3)	– – –	– – –	(0/3)	– – –	– – –
Pure virus	23.5 (3/3)	+ + +	+ – –	25.9 (3/3)	– – –	– – –	28.4 (3/3)	+ + +	+ + +
Feed	25.4 (3/3)	– – –	+ + +	35.4 (2/3)	– – –	– – –	35.2 (3/3)	+ + –	– – –
Dust	28.2 (3/3)	+ – –	+ + +	33.9 (3/3)	– – –	– – –	33.9 (3/3)	+ – –	+ + –
Feed treatments									
Ground corn – Sequence 1	38.4 (2/3)	– – –	– – –	39.2 (1/3)	– – –	– – –	(0/3)	+ – –	– – –
Ground corn – Sequence 3	38.4 (2/3)	– – –	– – –	(0/3)	– – –	– – –	(0/3)	+ + +	+ – –
Liquid formaldehyde – Sequence 1	39.3 (1/3)	– – –	– – –	(0/3)	– – –	– – –	(0/3)	– – –	+ – –
Dry formaldehyde – Sequence 1	(0/3)	– – –	– – –	(0/3)	– – –	– – –	(0/3)	+ + –	– – –
Dry formaldehyde – Sequence 3	(0/3)	– – –	– – –	(0/3)	– – –	– – –	(0/3)	– – –	– – –
Dust treatments									
Ground corn – Sequence 3	35.5 (3/3)	– – –	– – –	37.2 (1/3)	– – –	– – –	39.1 (1/3)	– – –	– – –
Liquid formaldehyde – Sequence 3	34.9 (1/3)	– – –	– – –	(0/3)	– – –	– – –	(0/3)	+ – –	– – –
Dry formaldehyde – Sequence 3	(0/3)	– – –	– – –	(0/3)	– – –	– – –	(0/3)	+ – –	– – –

[1] Three pigs per each treatment were inoculated on day 0 via intramuscular, intranasal, and either oral gavage (feed samples) or a slurry (dust samples) and evaluated for 7 days. Rectal samples were taken daily to evaluate SVV1 and PEDV presence and blood was collected on 4 and 7 dpi to assess PRRSV presence.

[2] The cycle threshold (Ct) value is the average Ct of all the PCR positive inoculums within each room as analyzed at Iowa State University Veterinary Diagnostic Laboratory following the d 0 inoculation. If no detectable RNA was present in the inoculum, then it was not included in the average Ct. The numerator represents the number of PCR positive inoculum samples with the denominator representing the total number of inoculum samples.

+/– corresponds to the viral status of each pig in the treatment room where + pigs signify viral RNA was present for the respective virus and – pigs had no detectable viral RNA.

Chapter 5 - Evaluating the reduction of porcine epidemic diarrhea virus, porcine reproductive and respiratory syndrome virus, and Seneca Valley virus 1 RNA in inoculated feed and the environment following thermal processing

Abstract

Pelleting feed has demonstrated to be an effective mitigation strategy for porcine epidemic diarrhea virus (PEDV) contaminated feed but has not been evaluated for other viruses like porcine reproductive and respiratory syndrome virus (PRRSV) or Seneca Valley virus 1 (SVV1). The objective of this experiment was to evaluate the efficacy of pelleting to inactivate PEDV, PRRSV, and SVV1 inoculated feed. Ten replicates were conducted using a pilot scale mixer, bucket elevator, pellet mill (including conditioner and die), and cooler. First, a virus negative batch of gestation feed was run through all equipment, followed by a positive batch of feed inoculated with all three viruses. Feed was conditioned to a minimum of 82°C with a 30 sec retention time; all feed was cooled for 10 min. Feed and environmental samples were taken from each piece of equipment following both batches. Samples were analyzed via PCR at the Kansas State University Veterinary Diagnostic Laboratory. Feed from the mixer and bucket elevator had greater quantities of SVV1, PEDV, and PRRSV RNA ($P < 0.05$) than the other sampling locations. Similarly, environmental samples from the mixer and bucket elevator had greater SVV1 detection ($P < 0.05$) than those collected from pelleting locations. A four-room bioassay was conducted to evaluate the infectivity of the feed samples. Although SVV1 and PEDV RNA were still detectable following pelleting, no pigs inoculated with the pelleted feed showed signs of SVV1 or PEDV clinical infection. Interestingly, PRRSV RNA was not detectable in pelleted

feed samples. However, one pig showed signs of replicating PRRSV virus on d7 of the bioassay. Pelleting reduced the quantity of detectable viral RNA and reduced the risk of infectivity for PEDV and SVV1; yet undetected quantities of PRRSV RNA following pelleting could remain potential contaminants in the finished product.

Introduction

Following the porcine epidemic diarrhea virus (PEDV) outbreak in 2013, feed was identified as a possible vector for swine viruses and research focused on mitigation of this risk in feed and feed ingredients. Pelleting is a form of thermal processing and is considered a point-in-time mitigation as it is capable of inactivating viruses during processing but does not protect the feed from cross-contamination. The effect of pelleting on PEDV infectivity has been variable. At low conditioning temperatures (< 55°C) pelleted feed was still able to cause infection in a swine bioassay while feed conditioned at greater than 55°C failed to cause signs of infection even though viral RNA was detected in the initial inoculum (Cochrane et al., 2017). However, dry heating complete feed at temperatures between 120-145°C took up to 25 min for viral inactivation (Trudeau et al., 2016) compared to the relatively short conditioning time of 30 sec used when pelleting.

Seneca Valley virus 1 (SVV1) has been commonly used as a surrogate for foot and mouth disease and has proven to remain stable in a variety of feed ingredients (Dee et al., 2018). Over extended durations (> 21 d) detectable SVV1 RNA has remained stable in both feed and soybean meal (Dee et al., 2022b) at fluctuating temperatures (range: 1.0-17.5°C). However, SVV1 contaminated soybean meal failed to cause infectivity when held at a constant 23.9°C for 30 days

(Dee et al., 2022a) or 30°C for 21 days (Caserta et al., 2022). Porcine reproductive and respiratory syndrome virus (PRRSV) has similarly failed to cause infection when soybean meal is held at 23.9°C for 30 days (Dee et al., 2022a). While ingredient quarantine has proven to be a viable mitigation strategy for these endemic viruses, pelleting experiments have rarely been conducted due to the specialized equipment and space needed for these virus inoculation studies. Therefore, the objective of this experiment was to evaluate the efficacy of pelleting as a thermal mitigant for PEDV, PRRSV, and SVV1 in inoculated feed and the feed manufacturing environment.

Materials and methods

Feed inoculation, pelleting, and sample collection was conducted at the Kansas State University (KSU) Cargill Feed Safety Research Center (FSRC) which is a 3-story biosafety level 2 biocontainment laboratory. Biocontainment was entered 10 separate times, representing 10 inoculation cycles. After each inoculation cycle, decontamination protocols were followed (Huss et al., 2017). Briefly, the facility was pressure washed with heated water to remove all organic matter, then disinfected with 1% peroxygen (Virkon S, Lanxess, Germany), followed by a rinse and a secondary disinfection with 5% bleach solution (7.5% sodium hypochlorite; Clorox, Oakland, CA), and then the facility was heated to a minimum of 60°C and held at that temperature for 48 hours. To exit containment, environmental swabs of the facility were collected to confirm surfaces were negative for PEDV, PRRSV, and SVV1 via quantitative reverse transcription real-time polymerase chain reaction (qRT-PCR) at the KSU Veterinary Diagnostic Laboratory (KSU-VDL). All protocols were approved by the KSU Institutional Biosafety Committee (IBC-1636).

Inoculum Information

Equal quantities of SVV1 (GenBank: KX7780101.1), PEDV CO-isolate (GenBank KF272920), and PRRSV 1-7-4 (GenBank: PP239061) were used for feed inoculation. The original stock contained 1 × 108 50% tissue culture infectious dose/mL (TCID50/mL) SVV1, 1 × 107 TCID50/mL PEDV, and 1 × 108 TCID50/mL PRRSV. Viruses were individually packaged into 25 mL aliquots, shipped from South Dakota State University to KSU on dry ice, and stored at -80°C until used in the FSRC. One aliquot of each virus was removed from storage on the day of inoculation, transported to the FSRC, and allowed to thaw at room temperature.

Swine Diet

The diet was formulated similar to Elijah et al. (2022), using a 79.0% corn and 17.3% soybean meal mash gestation diet manufactured at Hubbard Feeds (Beloit, Kansas). Feed samples were collected from multiple bags after feed delivery and submitted for qRT-PCR analysis at the KSU-VDL to confirm SVV1, PEDV, and PRRSV negative status prior to entering the FSRC.

Feed Inoculation

All viruses were combined (23 mL each) in 613 mL of phosphate buffer solution (PBS) to create an adequate volume for mixing in 22.7 kg of feed. Aliquots of each virus prior to dilution and the combined viruses with PBS inoculum were retained for analysis. The 682 mL total inoculum was added to 2.2 kg of gestation diet and mixed for 5 minutes in a 5 kg benchtop stainless steel paddle mixer (Cabela's Inc., Sidney, NE) creating 2.8 kg of feed. The inoculated feed was added to an additional 19.9 kg of feed and mixed for another 5 minutes in a 22.7 kg mixer (Hayes &

Stolz Industrial Manufacturing Co. LLC, Burleson, TX). In total, 23 mL of each virus with an initial concentration of 1 × 108 TCID50/mL (PEDV at 1 × 107 TCID50/mL) was evenly distributed in 22.7 of feed which provides an approximate concentration of 1 × 105 TCID50/g feed for SVV1 and PRRSV and 1 × 104 TCID50/g feed for PEDV.

Feed Manufacturing

Feed manufacturing was similar to Cochrane et al. (2017) and Elijah et al. (2022) where equipment was primed with a 22.7 kg virus negative batch of feed (primer batch), followed by 22.7 kg of feed inoculated with equal quantities of SVV1, PEDV, and PRRSV (inoculated batch). During each batch, feed was mixed for 5 min in a 22.7 kg mixer (Hayes & Stolz Industrial Manufacturing Co. LLC, Burleson, TX) and discharged at a rate of 4.5 kg/min into a feed bin. Feed was then poured into the hopper of a bucket elevator (Universal Industries, Cedar Falls, IA) with 74 buckets (each 114 cm3) at an equal discharge rate (4.5 kg/min) and conveyed through a downspout into a fresh feed bin. All feed was then pelleted in a CL-5 Laboratory Pellet Mill (CPM, Waterloo, IA) reaching a conditioning temperature of 82°C with a 30 sec retention time. Feed was dispensed into 6 trays each holding approximately 4 kg of pelleted feed per tray. The trays are rectangular with metal mesh at the bottom to allow for air circulation (Image 1). At one of the short ends of the tray, metal triangles are placed inside both corners, creating a short end with less surface area, hereby referred to as the bottom of the tray. The top of the tray refers to the short end without the metal triangles. As trays were filled, they were then placed in a double door experimental pellet cooler which draws the unfiltered, ambient air into the pellet cooler. The circulating air inside the pellet cooler is then vented back into the ambient air unfiltered. The trays were placed inside the pellet cooler for 10 min before being sampled and discarded into double lined autoclave totes. Trays 1-3 contained pellets which had not been

conditioned to a minimum of 82°C, while trays 4-6 contained pellets which had reached the conditioning temperature of 82°C. Tray 4 was considered the low-temperature (LT) tray, as it shared circulating air in the pellet cooler with trays that had not reached the goal conditioning temperature. Tray 6 was considered the high-temperature (HT) tray as it was placed in the pellet cooler only with trays that had met or exceeded the minimum conditioning temperature of 82°C.

Feed Sample Collection

Feed samples were collected from the mixer, the bucket elevator, the pellet mill conditioner, the pellet die, and two from the pellet cooler (LT tray and HT tray) during the primer and positive batches of each replication for a total of 120 feed samples. Samples from the mixer, bucket elevator, conditioner, and die were collected using the cut stream method. During discharge, a 2 oz plastic cup (Great Value Cups, 2 oz, Walmart, Bentonville, AR) was filled; this process was repeated twice more at 90 second intervals. Three individual feed samples were taken from the LT tray in a "V" pattern, two 2 oz cups were filled with pelleted top of the tray and one from the bottom of the tray (Image 1). This was repeated for the HT tray. The three individual samples from each piece of equipment were combined into one composite sample weighing 50 g in a Whirl-pak bag (Nasco, Fort Atkinson, WI).

Environmental Sample Collection

Environmental samples were collected from the ribbon of the mixer, the boot of the bucket elevator, a bucket of the bucket elevator, the interior of the conditioner in the pellet mill, the pellet die, and the interior non-feed contact surface of the pellet cooler during the primer and positive batches of each replication for a total of 120 environmental samples. Environmental

samples were taken using a cotton surgical gauze pre-moistened with 5 mL of PBS that was prepared and stored in a 50 mL conical tube prior to sampling. The designated area (approximately 100 cm2) was swabbed, and the gauze was returned to the conical tube.

Sample processing

Upon leaving the FSRC, samples were double bagged and underwent disinfection protocols before being placed on ice and transported to a biosafety level-2 laboratory in the KSU-VDL for further processing. Feed samples were transferred to 500 mL high density polyurethane bottles with 200 mL of PBS to create a 20% suspension. Feed samples were shaken for approximately 10 sec and stored at 4°C overnight. The next day, 25 mL of the supernatant from the feed samples was transferred to a fresh 50 mL conical tubes. The supernatant was centrifuged at 4,000 × g for 10 minutes at 8°C. Two 300 µL aliquots were retained for PCR analysis and 20 mL of the supernatant was transferred to a fresh 50 mL conical tube retained for the bioassay. For the environmental samples, 25 mL of PBS was added to each tube and vortexed for 10 sec. Environmental samples were allowed to incubate at room temperature for one hour. After one hour, a 20 mL aliquot was transferred to a fresh 50 mL conical tube. Centrifugation and sample allocation was performed using the same procedures used for feed samples previously described.

Quantitative polymerase chain reaction analysis

Samples were analyzed for detection of SVV1, PEDV, and PRRSV using qRT-PCR at the KSU-VDL. First, 50 µL of supernatant was placed in a deep well plate and RNA extracted using a Kingfisher Flex magnetic particle processor (Fisher Scientific, Pittsburgh, PA) and a MagMAX-96 Viral Isolation Kit (Life Technologies, Grand Island, NY). The final elution volume was 60

μL, and extracted RNA was stored at -80°C until analyzed for SVV1, PEDV, or PRRSV using a qRT-PCR tri-plex assay with a maximum cycle threshold of 45. The tri-plex was designed for experimental use designed to detect SVV1, PEDV, and PRRSV RNA utilizing TxRed, FAM, and HEX probes, respectively. Results were reported as the number of samples considered positive and the cycle threshold (Ct) below 45 at which either SVV1, PEDV, or PRRSV RNA was detected.

Bioassay

A bioassay was conducted at Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL) utilizing 4 treatment rooms with 3 mixed sex 10-day old piglets in each room. Before piglets arrived at ISU-VDL, serum from the sows at the farm the piglets originated from was tested for PRRSV with an enzyme-linked immunosorbent assay and SVV1 and PEDV with an indirect fluorescent antibody assay. Additionally, sow serum and rectal swabs were tested for PRRSV, SVV1, and enteric coronaviruses via PCR. Upon arrival, individual piglet serum and feces were also tested for PRRSV, SVV1, and enteric coronaviruses via PCR. All sows and piglets tested negative for SVV1, PEDV, and PRRSV prior to inoculation. The day 0 inoculation treatments included 1) negative control, 2) pure virus positive control with 15 μL each of SVV1, PEDV, and PRRSV diluted in 15 mL of PBS which was approximately the concentration of the positive batch of feed, 3) feed samples from the positive batch pre-thermal processing (mixer or bucket elevator), and 4) feed samples from the positive batch post-thermal processing (conditioner, pellet die, or LT/HT cooler). Each pig was inoculated with 2 mL intramuscularly, 2 mL intranasally (1 mL/nostril) and a 10 mL oral gavage. Rectal swabs were collected day 1-7 post-inoculation, blood samples were collected 4 and 7 dpi. Tonsils, lung tissue, jejunal and cecal

tissue and cecal contents were collected at necropsy. Day 0 inoculum, rectal (SVV1 and PEDV), and serum (PRRSV) samples were analyzed via PCR at the ISU-VDL.

Statistical analysis

The effects of location and thermal processing (pre- or post-) were analyzed using the GLIMMIX procedure of SAS version 9.4 (SAS Institute Inc., Cary, NC). Samples from the mixer and bucket elevator would be classified as pre-thermal processing while the conditioner, pellet die, and cooler were considered post-thermal processing locations. Fixed effects included location and thermal processing. Inoculation cycle was included in the model as a random effect. Data were separated and analysis performed within sample type (feed or environmental) and virus (SVV1, PEDV, and PRRSV). Two response criteria were considered, the proportion of PCR positive samples and the quantity of detectable viral RNA (Ct value). The proportion of PCR positive samples were analyzed by fitting to a binary distribution, logit link, Laplace approximation, and ridge-stabilized Newton-Raphson algorithm. To estimate the quantity of detectable viral RNA, the Ct of each sample was used and analyzed using a linear mixed model assuming a normal distribution. If no viral RNA was detected, samples were assigned a value of 45.0. A Kenward-Roger denominator degree of freedom adjustment was used, as well as a Tukey-Kramer multiple comparison adjustment. Results were considered significant at $P \leq 0.05$.

Results

There were significant differences ($P < 0.001$) in the quantity of RNA found between the different feed sampling locations regardless of virus (Table 1). Feed from the mixer and bucket elevator had more SVV1, PEDV, or PRRSV RNA ($P < 0.05$) than the other sampling locations.

As these were the feed manufacturing locations prior to thermal processing, the presence of more viral RNA is not surprising. Interestingly, SVV1 RNA was found in feed following thermal processing from the conditioner, pellet die, and from the cooler. Likewise, PEDV RNA was identified in feed from the cooler. Viral RNA was found on all pieces of equipment during environmental sampling, with a noticeable decrease in SVV1 RNA ($P < 0.05$) on equipment used during the pelleting and cooling process (Table 2). Thermal processing (Table 3) was able to decrease detectable RNA ($P < 0.05$) for all viruses regardless of sampling matrix. There was also a greater proportion of pre-thermal processing PEDV positive feed samples ($P < 0.05$) and SVV1 positive environmental samples ($P < 0.05$) than their respective post-thermal processing samples (Table 4).

The results of the bioassay are shown in Table 5. As expected, the negative control pigs, who were given feed samples from the primer batch before virus was introduced, did not display clinical signs of SVV1, PEDV, or PRRSV infection during the study. Pigs given the pure virus (diluted to the approximate concentration of the feed) were SVV1 and PRRSV positive by d4. Interestingly, signs of PEDV infection were not noticed in pigs inoculated with the pure virus even though the pure inoculum itself was found to be infectious via virus isolation. Pigs in the pre-thermal feed treatment room were positive for SVV1 on d 4 and PRRSV on d 7; however, it is unclear why persistent infection of both viruses were not observed for the duration of the bioassay. Although there was detectable SVV1 and PEDV RNA in post-thermal processing feed samples, the swine bioassay showed no signs of clinical infection for those pigs inoculated with these samples. Unlike SVV1 and PEDV, PRRSV RNA was not detectable in the post-thermal feed inoculum, but one pig exhibited clinical signs of PRRSV infection on d 7.

Discussion

To the author's knowledge, Cochrane et al. (2017) is the only other study to evaluate the specific effect of pelleting on virus survival. In their first experiment, signs of infection were not observed in pigs inoculated with PEDV contaminated feed conditioned to at either 68.3, 79.4, or 90.6°C with retention times ranging between 45-180 sec; however, PEDV RNA was detected in the majority of the pelleted feed samples. Utilizing lower temperatures in the second experiment, Cochrane et al. (2015) again detected PEDV RNA in the pelleted feed but found feed conditioned at or greater than 54.4°C was unable to cause infection when consumed by pigs. Likewise, when conditioned at 82°C in this study, viral RNA was still detected in the finished pelleted product but was considered non-infectious for SVV1 and PEDV as no signs of infection were observed in the bioassay.

As Cochrane et al. (2017) and this study both point out, the identification of viral RNA in samples does not equate a bioassay positive response. For infection to occur, an intact virus must come into contact with receptors on a host cell and then enter the host cell and initiate replication. For this process to be carried out, the virus must express surface receptors and have an intact structure. In the case of PRRSV and PEDV, this includes a lipid envelope that is susceptible to damage through processes such as heat or chemical alterations. Specifically, the use of dry heat is more likely to affect viral proteins more so than the viral genome (Wigginton et al., 2012). The addition of steam, or moist heat, works similarly to dry heat by denaturing proteins and enzymes through coagulation limiting the virus' ability to enter the host (Tipnis and Burgess, 2018). Thus, when pelleting is used to inactivate viruses, the viral genetic material may

remain intact and when measured using PCR, fragments of the viral RNA may be detected while the virus particle is no longer whole and therefore unable to cause infection. While PCR results can give us an indication of viral RNA presence, additional tests, like bioassays, are needed to understand the viability of the detected viral RNA.

While little research has focused on pelleting as a means of viral inactivation in feed, the swine industry has evaluated how temperature alone can affect virus survival on a variety of surfaces and matrices. At 30°C, SVV1 showed little viral degradation when feed ingredients were incubated over a period of three weeks (Caserta et al., 2022); however, SVV1 infection was not observed when soybean meal was held at 23.9°C for 30 day (Dee et al., 2022a). Quist-Rybachuk et al. (2015) reported a minimum of two hours were needed for PEDV to be considered non-infectious when incubated in minimum essential medium at 48°C. While these lower temperatures are helpful if the space is available for ingredient storage, the use of higher temperatures is likely to decrease the time needed for viral inactivation. At temperatures closer to the conditioning temperature used in this study, PEDV contaminated feces held at 75°C needed at least 15 minutes before infectious viral particles were no longer present (van Kessel et al., 2021); while slightly less time and a lower temperature (10 minutes at 70°C) were needed as the quantity of PEDV-contaminated feces decreased on stainless steel surfaces (Thomas et al., 2015). Trudeau et al. (2016) reported much higher temperatures (130°C for 15 minutes) were needed to inactivate PEDV in complete feed, but the addition of moisture may reduces the time needed for viral inactivation as moisture increases the rate at which heat penetrates matrices (Tipnis and Burgess, 2018). In fact, only 10 seconds were needed to reduce PEDV infectivity when the virus was introduced to water heated to 76°C. As pelleting uses both heat and moisture

through steam, the time needed to reduce viral infectivity is likely less than the studies using dry heat alone as demonstrated by the reduced PEDV and SVV1 infectivity observed following pelleting in this study.

As mentioned previously, pigs showed no signs of PEDV infection in the bioassay, including the pure virus positive control. Given the pure PEDV was unable to cause infection, it is not surprising that no pigs showed clinical signs of infection in either the pre- or post-thermal feed treatment room. However, the lack of PEDV response does pose questions about whether the bioassay was limited due to the virus itself or the efficacy of feed-based models. Similar difficulties were also observed by Dee et al. (2021) where purposefully inoculated feed showed sporadic infectivity in pens of pigs with little to no PEDV detected on the feed contact areas in the pens. Future research may be necessary to understand how differing PEDV strains and the inoculation method may affect virus viability in bioassays.

The identification of a PRRSV positive pig following consumption of pelleted feed potentially demonstrates the sensitivity of a bioassay over the more cost-effective methods such as PCR, and the challenges described by Davies (2015) when working with samples that have a low individual risk but the collective group risk (risk of a farm becoming positive) is high within the small sample size of a bioassay. At similar temperatures (80°C), van Kessel et al. (2021) saw inactivation of PRRSV in under one minute in tissue culture, but the presence of feed may increase the temperature needed for virus inactivation. As SVV1 is considered more heat tolerant and stable than PRRSV (Dee et al., 2018; Dee et al., 2022a) it is unknown why conditioning at

82°C was able to reduce the infectivity of SVV1 in the bioassay but not completely reduce PRRSV infectivity.

However, the possibility of cross-contamination should also be considered, as this bioassay was a part of a larger study with a total of twenty rooms. During the course of the bioassay, multiple rooms displayed PRRSV infection although PRRSV RNA was not detected in the initial inoculums. Due to the widespread detection of PRRSV, the PRRSV detected in the serum was sequenced and compared to the pure virus used for inoculation. The PRRSV strains matched which reduces the possibility of another PRRSV strain entering the farm. Care was taken to reduce cross-contamination between rooms as limited personnel were allowed access, disposable coveralls and boots were changed between rooms, and the rooms considered the lowest risk of infection (starting with the negative control) were entered first, but these biosecurity measures do not completely eliminate the risk of cross-contamination.

As expected, quantities of viral RNA where greater in environmental samples pre-thermal processing as compared to the post-thermal processing locations. The decreased presence of viral RNA is likely due to the decreased quantity of viral RNA present in the feed post-thermal processing, but additional inactivation may occur once these feed particles settle on the environmental surface itself. On environmental surfaces, the surface temperature of the metal equipment would increase during pelleting but would not reach the conditioning temperature. At room temperature (~25°C), PEDV contaminated metal surfaces were still considered infectious after 10 days (Trudeau et al., 2017). While the time needed for virus inactivation is likely to decrease as temperature increases (van Kessel et al., 2021), viral inactivation due to long-term

exposure to heated metal surfaces has not been evaluated. Therefore, the presence of viral RNA on these surfaces could increase the risk of product re-contamination and efforts should be made to reduce cross-contact between the pre- and post-thermally processed feed.

Conclusion

The use of pelleting reduced the quantity of detectable RNA in the feed and environmental surfaces within the feed mill. The remaining SVV1 RNA was considered non-infectious when pigs were experimentally inoculated; however, PRRSV infection was still observed in the swine bioassay when pigs were inoculated with pelleted feed conditioned to a minimum of 82°C. Furthermore, the accumulation of viral RNA throughout the feed manufacturing facility may further increase the risk of recontamination. Overall, pelleting is a suitable technique for reducing the risk of contamination provided care is taken to avoid contact between the pre-thermal and post-thermally processed feed and sufficient temperatures are utilized.

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Figure 5.1 The trays used in the pellet cooler. The short end without metal triangles is referenced as the top while the end with the triangles is considered the bottom. Samples were collected in a “V” pattern at each point in the imposed black shape.

Table 5.1 Effect of location on the relative quantification of Seneca Valley virus 1 (SVV1), porcine epidemic diarrhea virus (PEDV), and porcine reproductive and respiratory syndrome virus (PRRSV) in feed samples during the pelleting process.[1]

	Mixer	Bucket elevator	Conditioner	Pellet die	Cooler – Low temperature	Cooler – High temperature	SEM	*P* =
SVV1	27.7^{c}	27.3^{c}	43.5^{ab}	42.2^{ab}	45.0^{a}	41.4^{b}	1.10	< 0.0001
PEDV	36.8^{b}	36.1^{b}	45.0^{a}	45.0^{a}	45.0^{a}	44.4^{a}	1.05	< 0.0001
PRRSV	41.6^{b}	40.9^{b}	45.0^{a}	45.0^{a}	45.0^{a}	45.0^{a}	0.94	< 0.0001

[1] A batch of feed inoculated with SVV1, PEDV, and PRRSV was ran through a feed manufacturing system and pelleted at a conditioning temperature of 82°C with a 30 sec retention time. Feed samples from the Cooler – Low Temperature shared circulating air with feed that had not reached the conditioning temperature of 82°C. Cooler- High Temperature samples only shared circulating air with feed that had met or exceeded a conditioning temperature of 82°C. A cycle threshold (Ct) value of 45.0 is considered negative with no detectable viral RNA.

[abc] means with differing superscripts within row differ significantly, $P < 0.05$

Table 5.2 Effect of location on the relative quantification of Seneca Valley virus 1 (SVV1), porcine epidemic diarrhea virus (PEDV), and porcine reproductive and respiratory syndrome virus (PRRSV) in environmental samples during the pelleting process.[1]

	Mixer	Bucket elevator – Bucket	Bucket elevator – Boot	Pellet conditioner	Pellet die	Cooler	SEM	*P* =
SVV1	33.1[b]	34.1[b]	33.5[b]	42.6[a]	41.6[a]	44.4[a]	1.44	< 0.0001
PEDV	42.3	43.3	42.6	43.8	44.5	44.2	1.03	0.231
PRRSV[2]	45.0	45.0	45.0	45.0	45.0	45.0	–	–

[1] A batch of feed inoculated with SVV1, PEDV, and PRRSV was ran through a feed manufacturing system and pelleted at a conditioning temperature of 82°C with a 30 sec retention time. A cycle threshold (Ct) value of 45.0 is considered negative with no detectable viral RNA.

[2] Statistical analysis was not conducted on PRRSV environmental samples as all were considered PCR negative.

[abc] means with differing superscripts within row differ significantly, $P < 0.05$

Table 5.3 Effect of thermal processing on the relative quantification of Seneca Valley virus 1 (SVV1), porcine epidemic diarrhea virus (PEDV), and porcine reproductive and respiratory syndrome virus (PRRSV) in feed and environmental samples during the pelleting process.[1]

	Pre-thermal processing	Post-thermal processing	SEM	*P* =
Feed samples				
SVV1	27.5[b]	43.0[a]	0.73	< 0.0001
PEDV	36.5[b]	44.9[a]	0.62	< 0.0001
PRRSV	41.3[b]	45.0[a]	0.56	< 0.0001
Environmental samples				
SVV1	33.6[b]	42.9[a]	0.83	< 0.0001
PEDV	42.8[b]	44.2[a]	0.58	0.018
PRRSV[2]	45.0	45.0	—	—

[1] A batch of feed inoculated with SVV1, PEDV, and PRRSV was ran through a feed manufacturing system and pelleted at a conditioning temperature of 82°C with a 30 second retention time. Pre-thermal processing samples were taken before heat was introduced into the feed manufacturing system (mixer and bucket elevator) while post-thermal processing samples were taken after the introduction of heat (conditioner, pellet die, and cooler). A cycle threshold (Ct) value of 45.0 is considered negative with no detectable viral RNA.

[2] Statistical analysis was not conducted on PRRSV environmental samples as all were considered PCR negative.

[ab] means with differing superscripts within row differ significantly, $P < 0.05$

Table 5.4 Effect of thermal processing on the proportion of PCR positive samples of Seneca Valley virus 1 (SVV1), porcine epidemic diarrhea virus (PEDV), and porcine reproductive and respiratory syndrome virus (PRRSV) in feed and environmental samples during the pelleting process.[1]

	Pre-thermal processing	Post-thermal processing	*P* =
Feed samples[2]			
SVV1	20/20	11/40	0.843
PEDV	17/20[a]	1/40[b]	0.013
PRRSV	11/20	0/40	0.980
Environmental samples[3]			
SVV1	28/30[a]	9/30[b]	0.0009
PEDV	8/30	4/30	0.075
PRRSV[4]	0/30	0/30	—

[1] A batch of feed inoculated with SVV1, PEDV, and PRRSV was ran through a feed manufacturing system and pelleted at a conditioning temperature of 82°C with a 30 second retention time.

[2] Pre-thermal feed samples included the mixer and bucket elevator (n=20), post-thermal samples included the conditioner, pellet die, and two samples from the cooler (n=40).

[3] Pre-thermal environmental samples included the mixer and two samples from the bucket elevator (n=30), post-thermal samples included the conditioner, pellet die, and the cooler (n=30).

[4] Statistical analysis was not conducted on PRRSV environmental samples as all were considered PCR negative.

[ab] means with differing superscripts within row differ significantly, $P < 0.05$

Table 5.5 Effect of thermal processing as Seneca Valley virus 1 (SVV1), porcine epidemic diarrhea virus (PEDV), and porcine reproductive and respiratory syndrome virus (PRRSV) mitigation as evaluated by a swine bioassay.[1]

	SVV1			PEDV			PRRSV		
	Inoculum Ct (PCR positive)[2]	4 dpi	7 dpi	Inoculum Ct (PCR positive)[2]	4 dpi	7 dpi	Inoculum Ct (PCR positive)[2]	4 dpi	7 dpi
Negative control	(0/3)	– – –	– – –	(0/3)	– – –	– – –	(0/3)	– – –	– – –
Pure virus positive control	23.5 (3/3)	+ + +	+ – –	25.9 (3/3)	– – –	– – –	28.4 (3/3)	+ + +	+ + +
Pre-thermal feed	25.4 (3/3)	– – –	+ + +	35.4 (2/3)	– – –	– – –	35.2 (3/3)	+ + –	– – –
Post-thermal feed	38.3 (3/3)	– – –	– – –	39.1 (1/3)	– – –	– – –	(0/3)	– – –	+ – –

[1] Three pigs per each treatment were inoculated on day 0 via intramuscular, intranasal, and either oral gavage (feed samples) or a slurry (dust samples) and evaluated for 7 days. Rectal samples were taken daily to evaluate SVV1 and PEDV presence and blood was collected on 4 and 7 dpi to assess PRRSV presence.

[2] The cycle threshold (Ct) value is the average Ct of all the PCR positive inoculums within each room as analyzed at Iowa State University Veterinary Diagnostic Laboratory following the d 0 inoculation. If no detectable RNA was present in the inoculum, then it was not included in the average Ct. The numerator represents the number of PCR positive inoculums with the denominator representing the total number of inoculums.

+/– corresponds to the viral status of each pig in the treatment room where + pigs signify viral RNA was present for the respective virus and – pigs had no detectable viral RNA.

Chapter 6 - Decontamination of a feed manufacturing environment following inoculation with porcine epidemic diarrhea virus, porcine reproductive and respiratory syndrome virus, and Seneca Valley virus 1

Abstract

Feed mill decontamination is difficult because equipment is not designed to be cleaned with water. Alternate strategies may improve a mill's ability to decontaminate in the event of viral contamination. The objective of this experiment was to evaluate different decontamination strategies within a mill following the inoculation of swine feed with porcine epidemic diarrhea virus (PEDV), porcine reproductive and respiratory syndrome virus (PRRSV), and Seneca Valley virus 1 (SVV1) ran through feed manufacturing equipment consisting of a mixer, bucket elevator, corn cleaner, drag conveyor, and distributor. Afterwards, decontamination strategies were implemented with environmental samples collected after each step. Strategy treatments included: 1) complete facility decontamination and heating for 48 hours at 60°C; 2) chlorine dioxide application (ProOxine AH, Bio-Cide International, Inc., Norman, OK); 3) organic matter removal using vacuums (Ridge Tool Company, Elyria, OH) and chlorine dioxide application; 4) heat up with portable electric heaters for exactly 48 hours; and 5) organic matter removal and heat up with portable heaters for exactly 48 h. A swine bioassay was completed to determine the infectivity of each treatment after decontamination. A treatment × decontamination step × location interaction was observed ($P < 0.05$) for SVV1, where less RNA was detected post-treatment compared to post-inoculation following the complete facility decontamination treatment on surfaces including the mixer, corn cleaner, drag conveyor, and flooring ($P < 0.05$)

as compared to all other decontamination treatments. Across all treatments, the act of decontamination reduced detectable PEDV ($P < 0.05$) and PRRSV ($P < 0.05$) RNA when compared to samples immediately following inoculation, but complete facility decontamination and heating was the only treatment RNA was non-detectable in all locations. Pigs inoculated with samples collected post-treatment showed no evidence of SVV1 or PEDV infection; PRRSV infection was observed in pigs given the chlorine dioxide with and without organic matter removal treatments and the organic matter removal plus heat treatment. Overall, all treatments reduced detectable RNA for all viruses between the inoculation step and the final decontamination step; however, PRRSV particles remained infectious following decontamination.

Introduction

Feed mill decontamination is a difficult challenge due to the specialized equipment within the mill which is not intended for cleaning, especially with water or liquid disinfectants. Applying water to the equipment increases the risk of rust and mold production, affecting the longevity of the equipment and safety of the manufactured feed. Huss et al. (2017) is currently the only study that evaluated mill decontamination after virus introduction. In order to eliminate porcine epidemic diarrhea virus (PEDV), the feed mill and equipment was power washed, cleaned, disinfected, and held at 60°C for 48 hours (Huss et al., 2017). As this method is not practical for commercial mills, alternative methods must be evaluated.

Chlorine dioxide application has largely been used to decontaminate water and wastewater (Totaro et al., 2021); however, there has been increased usage of gaseous chlorine dioxide as a

means to decontaminate facilities (Lowe et al., 2013). Due to the gaseous application, chlorine dioxide has the ability to reach more areas within a facility than if decontamination were to occur by physical decontamination methods alone, which can be beneficial in large feed mills with complex spouting and machinery. Chlorine dioxide (ClO2) gaseous fumigation has been utilized in the meat industry to reduce bacterial concentrations on food processing equipment (Trinetta et al., 2012) and various surfaces including plastic, stainless steel, and rubber commonly seen in feed mills (Park and Kang, 2017). A variety of enteric, respiratory, and bloodborne viruses have also been inactivated following exposure to chlorine dioxide (Totaro et al., 2021). However, Houston et al. (2024) saw little to no reduction in viral presence via PCR when gaseous ClO2 was used on PEDV and porcine reproductive and respiratory syndrome virus (PRRSV) contaminated surfaces within a truck cab.

When PEDV was exposed to water heated to at least 72°C for a minimum of 10 seconds, PEDV failed to cause infectivity via virus isolation (Zentkovich et al., 2016), but this method would still expose the feed manufacturing equipment to water and would require constant monitoring of the water temperature. Exposure to high temperatures (120-145°C) for 30 minutes has decreased PEDV concentrations in feed (Trudeau et al., 2016), but such high temperatures would be difficult to replicate in commercial settings. Decreased PEDV and PRRSV infectivity (Trudeau et al., 2017) and reduced concentrations of PEDV, PRRSV, and Seneca Valley virus 1 (SVV1), a heat stable virus, (Jayawardena et al., 2022) genetic material (Dee et al., 2022) were observed when virus-contaminated matrices were held for four weeks at room temperature; however, virus inactivation due to extended heat exposure has not yet been evaluated. Therefore, the objective of this study was to evaluate gaseous ClO2 or heat exposure as alternate decontamination

strategies within a mill following the experimental introduction of PEDV, PRRSV, and SVV1 contaminated feed using PCR-based techniques and using a swine bioassay.

Materials and methods

Feed inoculation, decontamination, and sample collection was conducted at the Kansas State University (KSU) Cargill Feed Safety Research Center (FSRC): a 3-story biosafety level 2 biocontainment laboratory. Biocontainment was entered 10 separate times, representing 10 inoculation cycles. After completing each inoculation cycle, facility decontamination was implemented following the protocols outlined by Huss et al. (2017). Prior to disabling the biocontainment settings, environmental swabs were collected aseptically from the feed manufacturing equipment and facility surfaces following disinfection to ensure surfaces were negative for all viruses. Samples were submitted to the KSU Veterinary Diagnostic Laboratory (KSU-VDL) and analyzed via quantitative reverse transcription real-time polymerase chain reaction (qRT-PCR). All protocols were approved by the KSU Institutional Biosafety Committee (IBC-1636).

Inoculum Information

Equal quantities of SVV1 (GenBank: KX7780101.1), PEDV CO-isolate (GenBank KF272920), and PRRSV 1-7-4 (GenBank: PP239061) were used for feed inoculation. The original stock contained 1 × 108 50% tissue culture infectious dose/mL (TCID50/mL) SVV1, 1 × 107 TCID50/mL PEDV, and 1 × 108 TCID50/mL PRRSV. Each individual virus was packaged into 25 mL aliquots, shipped from South Dakota State University to KSU on dry ice, and stored at -

80°C until used in the FSRC. One aliquot of each virus was removed from storage on the day of inoculation, transported to the FSRC, and allowed to thaw at room temperature.

Swine Diet

The diet was formulated similar to Elijah et al. (2022), using a 79.0% corn and 17.3% soybean meal mash gestation diet manufactured at Hubbard Feeds (Beloit, Kansas). Feed samples were collected from multiple bags after feed delivery and submitted for qRT-PCR analysis at the KSU-VDL to confirm SVV1, PEDV, and PRRSV negative status prior to entering the FSRC.

Feed Inoculation

Viruses were combined (23 mL each) in 613 mL of phosphate buffer solution (PBS) to create an adequate volume for mixing in 22.7 kg of feed. Aliquots of each virus prior to dilution and the combined viruses with PBS inoculum were retained for analysis. The 682 mL total inoculum was first added to 2.2 kg of feed and mixed by hand for 5 minutes in a 5 kg benchtop stainless steel paddle mixer (Cabela's Inc., Sidney, NE) creating 2.8 kg of feed. The inoculated feed was added to an additional 19.9 kg of feed and mixed for another 5 minutes. In total, 23 mL of each virus with an initial concentration of 1 × 108 TCID50/mL (PEDV at 1 × 107 TCID50/mL) was evenly distrusted in 22.1 of feed which provides an approximate concentration of 1 × 105 TCID50/g feed for SVV1 and PRRSV and 1 × 104 TCID50/g feed for PEDV.

Feed Manufacturing

The feed manufacturing equipment was primed with 45.4 kg of virus free feed, utilizing the full capacity of the mixer (model #SS-LI, H.C. Davis Sons Manufacturing Co., Bonner Springs, KS).

Feed was mixed for 5 minutes before discharging at a rate of 4.5 kg/min into a feed bin. Feed was then poured into the hopper of a double-shaft bucket elevator (model B3, Universal Industries, Inc., Cedar Falls, IA) with 722 cm3 sized buckets at an equal discharge rate (4.5 kg/min) which dispensed feed into a corn cleaner (Standard Gentle Roll single drum, EBM Manufacturing, Norfolk, NE) before being deposited into a feed bin. Large particles separated in the corn cleaner were discarded. Feed was transported to the drag conveyor ("RB" Round-Bottom Drag-Flite Standard Direct Inlet, Essmueller Co., St. Louis, MO) and discharged at the same rate of 4.5 kg/min before being carried up two flights of stairs and poured into the distributor (10 outlets, Model E, Hayes & Stolz Industrial Manufacturing Co., Burleson, TX) and subsequent down spout which discharged feed into a bin one floor below. The previously described inoculated batch of feed was manufactured in the same manner, but due to the limited quantity of virus stock only 22.7 kg of feed was used for the inoculated batch of feed. Due to the set-up of the FSRC the mixer, bucket elevator, corn cleaner, and distributor were segregated from the rest of the room by a cloth tarp creating their own micro-environment. The distributor was located on the third floor with the connected down spout discharging onto the second floor.

Decontamination treatments

Five decontamination treatments were included in the study, with two replicates of each treatment. Decontamination treatments followed the inoculation batch and were as follows:

1. Complete FSRC decontamination – removal of organic matter with heated pressure washing, disinfection with 1% peroxygen (Virkon S, Lanxess, Cologne Germany), disinfection with 5% bleach solution (7.5% sodium hypochlorite; Clorox, Oakland, CA),

environmental heat held at 60°C for 48 hours. The 48 hour heat up period was started once temperature on all floors reached and maintained the 60°C minimum threshold.

2. ClO2 – application of a commercial chlorine dioxide product (approximate concentration = 500 ppm; ProOxine AH, Bio-Cide International, Inc., Norman, OK) via a stationary (Automated Activation Non-Electric (AANE) modified for hand mixing product, Bio-Cide International Inc. Norman, OK) and a portable fogger (BCI Atomizer, Bio-Cide International, Inc., Norman, OK). Both foggers operated until empty (approximately 15-20 minutes). Samples were collected one hour after fogger shut-off to allow time for product contact time.
3. Organic matter removal + ClO2 – organic matter was removed with portable vacuums (4-gallon Ridgid portable wet/dry vacuum, Ridge Tool Company, Elyria, OH) from surfaces were feed build-up occurred (bottom of bucket elevator, drag conveyor, mixer, and corn cleaner, flooring, etc.). Commercial chlorine dioxide was applied via a stationary and a portable fogger. Both foggers operated until empty (approximately 15-20 minutes). Samples were collected one hour after fogger shut-off to allow time for product contact time.
4. Heat – two portable electric heaters operated at as high temperature as possible for 48 hours following the inoculation batch of feed. The 48-hour heat period began immediately once the heaters were turned on. The first portable heater (SDRA Series, Chromalox, Inc., Pittsburgh, PA) was in the micro-environment on the first floor. To trap as much heat within the micro-environment, rubber mats were laid on the slated flooring on the second floor to create a ceiling like structure. The second heater (FES Series, Fostoria Industries, Gray, TN) was located on the second floor and aimed up the down

spout into the distributor. Data loggers (HOBO MX temp/RH logger, Onset, Bourne, MA) were placed on all pieces of equipment during heat up near the sampling area.

5. Organic matter removal + heat - organic matter was removed with portable vacuums from surfaces were feed build-up occurred (bottom of bucket elevator, drag conveyor, mixer, and corn cleaner, flooring, etc.). Two portable electric heaters operated at as high temperature as possible for 48 hours following the inoculation batch of feed. The 48-hour heat period began immediately once the heaters were turned on. The first portable heater was in the micro-environment on the first floor. To trap as much heat within the micro-environment, rubber mats were on the slated flooring on the second floor to create a ceiling like structure. The second heater was located on the second floor and aimed up the down spout into the distributor. Data loggers were placed on all pieces of equipment during heat up near the sampling area.

Safety precautions

For the researchers' safety, full face respirators (6800 Series, 3M, St. Paul, MN) with disposable cartridges (Multi Gas/Vapor Cartridge/Filter 60926, P100, 3M, St. Paul, MN) were used once chlorine dioxide decontamination was implemented.

Environmental Sample Collection

Environmental samples were collected from the ribbon of the mixer, the boot and bucket of the bucket elevator, the wall near the bucket elevator (< 1 m from the hopper) the internal side of the corn cleaner, bottom of the drag conveyor, inside of the distributor, bottom of the down spout, the flooring around the down spout where feed was discharged, and the worker's boot.

Samples were taken following each feed manufacturing batch (primer and inoculation) and after each decontamination step. Briefly, a 10 cm × 10 cm cotton surgical gauze pre-moistened with 5 mL of PBS and stored in a 50 mL conical tube prior to sampling. The designated area was swabbed, and the gauze was returned to the conical tube.

Sample processing

Upon leaving the FSRC, samples were double bagged and underwent disinfection protocols before being placed on ice and transported to a biosafety level-2 laboratory in the KSU-VDL for further processing. In the laboratory, 20 mL of PBS was added to each environmental sample and vortexed for 10 sec. Environmental samples were allowed to incubate at room temperature for one hour. After one hour, a 20 mL aliquot was transferred to a fresh 50 mL conical tube and centrifuged at 4,000 × g for 10 minutes at 8°C. After centrifuging, 2-300 µL aliquots were retained for PCR and the remaining supernatant was transferred to a new conical tube and retained for the bioassay.

Quantitative polymerase chain reaction analysis

Samples were analyzed for detection of SVV1, PEDV, and PRRSV using qRT-PCR at the KSU-VDL. First, 50 µL of supernatant was placed in a deep well plate and RNA extracted using a Kingfisher Flex magnetic particle processor (Fisher Scientific, Pittsburgh, PA) and a MagMAX-96 Viral Isolation Kit (Life Technologies, Grand Island, NY). The final elution volume was reduced to 60 µL, and extracted RNA was stored at -80°C until analyzed for SVV1, PEDV, or PRRSV using a qRT-PCR tri-plex assay with a maximum cycle threshold of 45. Results were

reported as the number of samples considered positive and the cycle threshold (Ct) below 45 at which either SVV1, PEDV, or PRRSV RNA was detected.

Bioassay

The experimental bioassay included 9 treatment rooms with 3 mixed sex 10-day old piglets in each room. The day 0 inoculation treatments included 1) negative control, 2) pure virus positive control with an equal volume SVV1, PEDV, and PRRSV diluted to the approximate concentration of the inoculation batch of feed, 3) environmental samples from the inoculation batch, 4) environmental samples after organic matter removal with the vacuums, 5) environmental samples following the 48 hour heat up at 60°C from the complete FSRC decontamination, 6) environmental samples taken after ClO2 application, 7) environmental samples taken after ClO2 application in the organic matter removal treatment, 8) samples taken after using portable heaters for 48 hours, and 9) samples taken after using portable heaters for 48 hours in the organic matter removal treatment. Each pig was inoculated with 2 mL intramuscularly, 2 mL intranasally (1 mL/nostril) and a 10 mL oral gavage. Prior to piglets arriving at ISU-VDL, sow serum was tested for PRRSV with an enzyme-linked immunosorbent assay and SVA and PEDV with an indirect fluorescent antibody assay. Additionally, sow serum and piglet rectal swabs were tested for PRRSV, SVV1, and enteric coronaviruses via PCR. Prior to inoculation, individual piglet serum and feces were also tested for PRRSV, SVV1, and enteric coronaviruses via PCR. All sows and piglets tested negative for SVV1, PEDV, and PRRSV prior to inoculation. Rectal swabs were collected day 1-7 post-inoculation, blood samples were collected -1, 4, and 7 dpi. Tonsils, lung tissue, jejunal and cecal tissue and cecal contents were

collected at necropsy on d 7 dpi. Day 0 inoculum, rectal (SVV1 and PEDV), and serum (PRRSV) samples were analyzed via PCR at the ISU-VDL.

Statistical analysis

Results were analyzed as a split plot design with the inoculation cycle as the experimental unit for decontamination treatment, and the environmental samples following the inoculated batch of feed and the final decontamination step as the experimental unit for location and decontamination step using the GLIMMIX procedure of SAS version 9.4 (SAS Institute Inc., Cary, NC). Fixed effects included decontamination treatment, location, decontamination step (either inoculated batch of feed or final decontamination step), and their associated interactions. Inoculation cycle was included in the model as a random effect. Data were separated and individually analyzed based on virus (SVV1, PEDV, and PRRSV). Contrast statements were included to compare the final decontamination step of the complete FSRC plus heat decontamination to the final decontamination step of each individual treatment (ClO2, organic matter removal + ClO2, heat, or organic matter removal + heat). Contrasts statements were also used to compare ClO2 to the organic matter removal + ClO2 and heat to organic matter removal + heat. Additionally, the four inoculation cycles which included organic matter removal via vacuums were analyzed individually to compare the inoculation batch of feed to the removal of organic matter creating a decontamination step × location interaction and their associated main effects. For all analysis, two response criteria were considered: the number of PCR positive samples and the quantity of detectable viral RNA. Data were analyzed by fitting to a binary distribution, logit link, Laplace approximation, and ridge-stabilized Newton-Raphson algorithm. As a binary distribution model, data were fit by each individual interaction, starting with the decontamination treatment × decontamination step × location interaction, and their subsequent

main effects. To estimate the quantity of detectable viral RNA, the Ct of each sample was used. If no viral RNA was detected, samples were assigned a Ct value of 45. A Kenward-Roger denominator degree of freedom adjustment was used, as well as a Tukey-Kramer multiple comparison adjustment. Results were considered significant at $P \leq 0.05$.

Results

As expected, viral RNA was not detected on environmental surfaces following the primer batch of feed for the ten inoculation cycles. The average temperature and relative humidity from the environment around each piece of equipment between the four inoculations cycles using portable heaters can be found in Table 1, with temperatures ranging between 43.8-51.8°C and relative humidity between 13.9-20.9%. The average temperature was reached approximately 8-9 hours after the heaters were turned on for the 48-hour period. A decontamination treatment × decontamination step × location interaction was observed for SVV1 ($P < 0.05$) where less RNA was on environmental surfaces post-treatment compared to post-inoculation following complete facility decontamination on surfaces including the mixer, corn cleaner, drag conveyor, and floor around the feed discharge (Table 2). No differences were observed ($P > 0.05$) for the bucket elevator, wall, distributor, down spout, or boot between the inoculation step and the final complete facility decontamination step. Interactions were not noted for either PEDV or PRRSV ($P > 0.05$), but greater quantities of RNA ($P < 0.05$) were detected on surfaces following inoculation than after completion of the decontamination treatment for all viruses (Table 3). Subsequently, fewer PCR positive samples ($P < 0.05$) were detected after the decontamination treatments than after inoculation for SVV1, PEDV, and PRRSV.

Location also affected the quantity of RNA detected, where the wall near the bucket elevator, down spout, and the worker boot had less detectable SVV1 RNA ($P < 0.05$) than the mixer, bucket of the bucket elevator, corn cleaner, drag conveyor, and the flooring around the discharge (Table 4). Fewer PCR positive samples were detected ($P < 0.05$) from the locations with the least detectable SVV1 RNA. Greater quantities of PRRSV RNA ($P < 0.05$) were detected from the corn cleaner, drag conveyor, and the floor around the discharge than the wall near the bucket elevator and the worker boot. Similarly, the wall had less detectable PEDV RNA ($P < 0.05$) than the floor around the discharge; however, no differences were observed ($P > 0.05$) for the proportion of PCR positive PRRSV or PEDV samples.

Following the removal of organic matter using vacuums, a decontamination step × location effect was not observed ($P > 0.05$) for any virus. Removing organic matter (Table 5) decreased the quantity of detectable SVV1 and PEDV RNA ($P < 0.05$), decreased the number of PCR positive PEDV samples ($P < 0.05$), and tended to decrease the quantity of PRRSV RNA ($P < 0.10$). The summary of contrasts statements are shown in Table 6. Complete FSRC decontamination plus heat had less detectable SVV1 RNA ($P < 0.05$) than any other treatments, which was similar for PEDV with the exception of organic matter removal + ClO2 which did not differ from complete FSRC decontamination plus heat treatment ($P > 0.05$). The heat treatment for PRRSV was the only decontamination strategy which significantly differed ($P < 0.05$) from the complete FSRC decontamination treatment with greater quantities of PRRSV RNA found after using portable heaters for 48 hours. Removing organic matter decreased detectable SVV1 and PEDV RNA ($P < 0.05$) when comparing ClO2 to its respective organic matter removal counterparts and SVV1 and PRRSV RNA ($P < 0.05$) when comparing the two heat treatments.

The results of the swine bioassay are shown in Table 7. As expected, pigs inoculated with samples from the primer batch of feed (virus negative) showed no signs of viral infection. Signs of SVV1 and PRRSV infection were noticed from pigs inoculated with the pure virus, but interestingly, the pure virus failed to cause PEDV infection in pigs. Only SVV1 infection and replication was observed when pigs were inoculated with samples following the inoculated batch of feed. Samples from the decontamination treatments and the removal of organic matter failed to cause SVV1 and PEDV infection in any pigs. Although the inoculums were not consistently PCR positive for PRRSV, infection was observed in every decontamination treatment room except the complete FSRC decontamination and heat alone treatment. It is unknown why PRRSV RNA was not detected via PCR but was present in great enough quantities to cause infection, but subsequent analysis showed cross-contamination between rooms or infection from a different PRRSV strain to be unlikely.

Discussion

The study by Huss et al. (2017) served as the proof of concept used to decontaminate the FSRC after any pathogen-based research, so it is unsurprising no viral RNA was detected following complete facility decontamination. Individually, peroxygen (Dee et al., 2006; Hole et al., 2017; Baker et al., 2018) and sodium hypochlorite (Dee et al., 2004; Bowman et al., 2015; Singh et al., 2017) have effectively reduced SVV1, PEDV, and PRRSV concentrations. The dual application of disinfectants followed by the facility heat up allows for overlapping decontamination strategies increasing the likelihood of decontaminating hard to reach areas within mills or equipment not suitable for pressure washing. Fumigation with ClO2 and the use of portable

heaters were chosen based off their relative portability, ability to access hard to reach areas, and the lack of water needed in comparison to disinfectant application.

Viral RNA was still detectable following fumigation with ClO2¬, but these samples failed to cause SVV1 or PEDV infection when pigs were inoculated in the bioassay. Application of ClO2 is suspected to degrade viral amino acids (specifically cysteine, tryptophan, and tyrosine), thus reducing the ability of the virus to bind to specific cells (Wigginton et al., 2012). Therefore, detecting intact RNA may not signify a lack of efficacy as the genome is not the target for viral inactivation (Sigstam et al., 2013). In fact, Xue et al. (2013) found little correlation between the degree of viral inactivation in a virus isolation assay vs. the PCR results of the same samples and concluded that ClO2 had a limited effect on the genome itself. Although the viral genome is minimally affected following ClO2 fumigation, reduced infectivity is largely observed. Chlorine dioxide fumigation reduced infectious viral concentrations on wet surfaces (Morino et al., 2011), steel surfaces (Yeap et al., 2016), and hard to reach areas (Montazeri et al., 2017). Similar to this study, Houston et al. (2024) saw little impact on the quantity viral RNA following application of ClO2, but PCR positive samples failed to cause infection during a swine bioassay. The consistent presence of PEDV and especially SVV1 RNA following ClO2 fumigation in this study confirms the minimal effect ClO2 has on the genome; furthermore, the lack of infectivity from the bioassay supports viral inactivation beyond infectious levels when ClO2 fumigation is used.

Similar to ClO2, the use of heat is not likely to disrupt the viral genome, but instead will structurally damage the viral proteins, limiting its ability to bind to cells (Wigginton et al., 2012). Although the damage to proteins is able to inactivate viruses, a PCR sample is unlikely to reflect

the degree of viral degradation as evidenced by the number of PCR positive samples remaining following the 48-hour heat cycle with portable heaters in this study. Nonetheless, bioassay samples from these heat cycles were considered non-infectious for both PEDV and SVV-1. Thomas et al. (2015) found feces held at 71°C for at 10 minutes sufficiently inactivated PEDV during a bioassay, but infection still occurred when feces was only incubated at 54 or 63°C for 10 minutes. At 50°C, similar to the maximum temperature reached by the portable heaters, PEDV and PRRSV were still infectious after one hour (van Kessel et al., 2021). Gamble et al. (2021) noted that surfaces exposed to heat, similar to feed manufacturing equipment in a mill, would need longer time for viral inactivation than surfaces or containers capable of trapping heat. Given the extended time the portable heaters were used, and the microenvironments utilized to trap as much heat as possible, viral inactivation is likely as seen with PEDV and SVV-1, but to the authors' knowledge no other studies held inoculated surfaces at this temperature for such a long time period.

Removing organic matter is a common practice for livestock transport trailers before power washing and disinfection (Dee et al., 2006; Baker et al., 2017), but these studies have not evaluated the impact of removing organic matter prior to wet washing. The dry removal of organic matter utilizing vacuums is not an inherent viral inactivation strategy as removing the material does not change or destroy the virus itself. Instead removing organic matter from feed manufacturing equipment increases the likelihood the surfaces are exposed to the respective decontamination treatment. This study demonstrates this theory as less PCR positive samples were seen in the ClO2 and heat treatments with organic matter removal than their counterparts utilizing only ClO2 or portable heaters.

Prior to the bioassay, PRRSV RNA was notably less detectable than either SVV1 or PEDV on surfaces following both the inoculated batch of feed and the decontamination treatments. Although fewer PRRSV PCR positive samples were observed, those that were followed similar patterns as SVV1 and PEDV and could be expected to have similar inactivation of the viral binding proteins, but limited degradation of the viral genome as previously discussed. In fact, liquid ClO2 suppressed PRRSV replication in cell culture (Zhu et al., 2019) and van Kessel et al. (2021) considered PRRSV non-infectious when incubated at 55°C for one hour. However, the results of the bioassay dispute these previous findings as inoculum with no detectable PRRSV RNA via PCR were still capable of causing infection in all decontamination treatment rooms except the complete FSRC decontamination and the heat alone treatment. Cross-contamination between rooms could have introduced PRRSV into these rooms, but as this was part of a larger bioassay project (20 rooms total) and PRRSV was seen in multiple other rooms it is unlikely a single point of cross-contamination could spread the virus to so many rooms so quickly. Furthermore, the circulating PRRSV virus from the pigs was sequenced and matched the positive control pure virus, thus limiting the chance an outside PRRSV strain was introduced to the pigs. At this time, whether cross-contamination occurred or whether these PCR negative samples truly contained infectious PRRSV particles remains largely unanswered.

Conclusion

Complete feed mill decontamination using power washing, disinfectants, and extended heat periods is an effective, but impractical decontamination strategy. The use of chlorine dioxide and portable heaters had limited effect on the quantity of detectable viral RNA but did decrease the risk of infectivity for SVV1 and PEDV; however, PRRSV infection was still observed following

these more practical strategies. Overall, chlorine dioxide and portable heaters show promise at reducing the risk of infection when utilized in feed manufacturing facilities, but viral RNA may still be detectable regardless of the decontamination strategy utilized.

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Table 6.1 The temperature and relative humidity of the environment around each piece of feed manufacturing equipment following a 48-hour heat period using portable heaters.[1,2]

Equipment	Average Temperature, °C (Minimum-Maximum, °C)	Average Relative humidity, % (Minimum-Maximum, %)
Mixer	51.8 (22.6-61.2)	13.9 (5.0-77.7)
Bucket elevator	43.8 (24.1-53.4)	19.7 (6.8-59.7)
Corn cleaner	50.1 (20.5-65.1)	20.9 (4.9-98.3)
Drag conveyor	48.0 (22.6-57.2)	16.1 (5.2-68.3)
Distributor	47.2 (34.5-54.8)	15.6 (4.6-34.2)
Down spout	49.4 (35.6-65.6)	14.4 (2.3-35.0)

[1] The average temperature and relative humidity are from four total inoculations cycles: two from the heat treatment alone and two from the organic matter removal + heat treatment. The mixer, bucket elevator, corn cleaner, and drag conveyor were all heated by one electric heater within a closed-off microenvironment. The distributor and down spout were heated by a smaller, separate heater positioned at the bottom of the down spout and aimed upward.
[2] The average temperature and relative humidity were reached after approximately 8-9 hours once the heaters were started for the 48-hour heat cycle.

Table 6.2 Effect of decontamination treatment, decontamination step (inoculation vs post-treatment), and location on the relative quantification of Seneca Valley virus 1 (SVV1) on environmental surfaces following inoculation and the final step of the decontamination protocol.[1]

	Location									
	Mixer	Bucket elevator – boot	Bucket elevator – bucket	Wall near bucket elevator	Corn cleaner	Drag conveyor	Distributor	Down spout	Floor around discharge	Boot
ClO_2[2]										
Inoculation	31.4^{bc}	33.7^{abc}	34.8^{abc}	41.6^{abc}	30.0^{bc}	30.2^{bc}	32.2^{abc}	36.6^{abc}	29.4^{bc}	37.9^{abc}
Post-treatment	31.0^{bc}	36.1^{abc}	35.1^{abc}	45.0^{a}	30.4^{bc}	31.0^{bc}	36.3^{abc}	37.4^{abc}	31.9^{bc}	36.3^{abc}
Organic matter removal + ClO_2 [3]										
Inoculation	31.4^{bc}	40.1^{abc}	34.6^{abc}	45.0^{a}	29.9^{bc}	29.9^{bc}	35.3^{abc}	41.0^{abc}	29.6^{bc}	41.1^{abc}
Post-treatment	36.2^{abc}	37.7^{abc}	35.4^{abc}	45.0^{a}	36.1^{abc}	36.2^{abc}	40.6^{abc}	41.8^{abc}	41.2^{abc}	45.0^{a}
Heat[4]										
Inoculation	30.7^{bc}	35.8^{abc}	34.4^{abc}	45.0^{a}	30.0^{bc}	29.7^{bc}	34.2^{abc}	40.2^{abc}	30.2^{bc}	37.8^{abc}
Post-treatment	36.2^{abc}	41.1^{abc}	35.7^{abc}	42.1^{abc}	33.3^{abc}	33.3^{abc}	37.2^{abc}	41.6^{abc}	33.5^{abc}	41.6^{abc}
Organic matter removal + heat[5]										
Inoculation	29.8^{bc}	37.0^{abc}	39.1^{abc}	45.0^{a}	29.8^{bc}	30.3^{bc}	36.4^{abc}	41.0^{abc}	30.2^{bc}	41.2^{abc}
Post-treatment	41.7^{abc}	41.3^{abc}	36.7^{abc}	45.0^{a}	42.1^{abc}	41.7^{abc}	41.7^{abc}	45.0^{a}	38.1^{abc}	45.0^{a}
Complete FSRC decontamination[6]										
Inoculation	29.8^{bc}	36.7^{abc}	36.1^{abc}	45.0^{a}	30.4^{bc}	31.0^{bc}	33.2^{abc}	40.8^{abc}	30.6^{bc}	42.5^{ab}
Post-treatment	45.0^{a}	45.0^{a}	45.0^{a}	45.0^{a}	45.0^{a}	45.0^{a}	45.0^{a}	45.0^{a}	45.0^{a}	45.0^{a}

[1] A 22.7 kg batch of feed was inoculated with SVV1, porcine epidemic diarrhea virus, and porcine reproductive and respiratory syndrome virus, followed by a decontamination protocol. Environmental samples were taken following each decontamination step, but only the samples following inoculation and the final stage in each decontamination protocol are displayed. Values reported are cycle threshold (Ct) and a Ct value of 45.0 is considered negative with no detectable viral RNA. Treatment × decontamination step × location, $P = 0.04$, SEM = 2.92

[2] ProOxine AH (Bio-Cide International, Inc., Norman, OK)

[3] Organic matter removal with portable vacuums (Ridge Tool Company, Elyria, OH); ProOxine AH (Bio-Cide International, Inc., Norman, OK)

[4] Portable heaters ran for exactly 48 hours

[5] Organic matter removal with portable vacuums (Ridge Tool Company, Elyria, OH); portable heaters ran for exactly 48 hours

[6] Removal of organic matter with heated pressure washing, disinfection with 1% peroxygen (Virkon S, Lanxess, Cologne, Germany), disinfection with 5% bleach solution (7.5% sodium hypochlorite; Clorox, Oakland, CA), environmental heat held at 60°C for 48 hours. The 48 hour heat up period was started once temperature on all floors reached and maintained the 60°C minimum threshold.

[abc] means with differing superscripts within matrix differ significantly, $P < 0.05$

Table 6.3 Effect of decontamination step on the relative quantification of Seneca Valley virus 1 (SVV1), porcine epidemic diarrhea virus (PEDV), and porcine reproductive and respiratory syndrome virus (PRRSV) and the proportion of PCR positive samples on environmental surfaces following the decontamination protocol.[1,2]

	Inoculation	Post-treatment	SEM	*P* =
SVV1				
Ct	35.2[b]	39.8[a]	0.68	< 0.0001
Proportion	82/100[a]	56/100[b]	—	< 0.0001
PEDV				
Ct	41.1[b]	43.2[a]	0.47	< 0.0001
Proportion	46/100[a]	23/100[b]	—	< 0.0001
PRRSV				
Ct	44.3[b]	44.8[a]	0.22	0.022
Proportion	12/100[a]	4/100[b]	—	0.044

[1] A 22.7 kg batch of feed was inoculated with SVV1, PEDV, and PRRSV, followed by a decontamination protocol. Environmental samples were taken following each decontamination step, but only the samples following inoculation and the final stage in each decontamination protocol are displayed. A cycle threshold (Ct) value of 45.0 is considered negative with no detectable viral RNA.
[2] Means are the average Ct across all decontamination treatments (ClO_2, organic matter removal + ClO_2, heat, organic matter removal + heat, and the complete FSRC decontamination).
[ab] means with differing superscripts within row differ significantly, $P < 0.05$

Table 6.4 Effect of location on the relative quantification of Seneca Valley virus 1 (SVV1), porcine epidemic diarrhea virus (PEDV), and porcine reproductive and respiratory syndrome virus (PRRSV) and the proportion of PCR positive samples on environmental surfaces following the decontamination protocol.[1,2]

	Location											
	Mixer	Bucket elevator – boot	Bucket elevator – bucket	Wall near bucket elevator	Corn cleaner	Drag conveyor	Distributor	Down spout	Floor around discharge	Worker boot	SEM	*P* =
SVV1												
Ct	34.3^{e}	38.5^{bcd}	36.7^{de}	44.4^{a}	33.7^{e}	33.8^{e}	37.2^{cde}	41.0 ^{abc}	34.0^{e}	41.3^{ab}	1.28	< 0.0001
Proportion	17/20^{a}	15/20^{a}	17/20^{a}	2/20^{b}	17/20^{a}	17/20^{a}	16/20^{a}	10/20^{ab}	17/20^{a}	10/20^{ab}	—	0.0003
PEDV												
Ct	42.4^{abc}	41.9^{abc}	41.0^{bc}	44.5^{a}	42.1^{abc}	41.1^{abc}	43.0^{abc}	42.5^{abc}	39.8^{c}	43.6^{ab}	1.05	0.001
Proportion	6/20	7/20	10/20	2/20	8/20	8/20	6/20	7/20	11/20	4/20	—	0.054
PRRSV												
Ct	44.7^{ab}	45.0^{a}	44.4^{ab}	45.0^{a}	43.0^{b}	43.9^{b}	44.7^{ab}	45.0^{a}	43.7^{b}	45.0^{a}	0.49	0.026
Proportion	1/20	0/20	2/20	0/20	4/20	4/20	1/20	0/20	4/20	0/20	—	0.862

[1] A 22.7 kg batch of feed was inoculated with SVV1, PEDV, and PRRSV, followed by a decontamination protocol. Environmental samples were taken following each decontamination step, but only the samples following inoculation and the final stage in each decontamination protocol are displayed. A cycle threshold (Ct) value of 45.0 is considered negative with no detectable viral RNA.

[2] Means are the average Ct across all decontamination treatments (ClO_2, organic matter removal + ClO_2, heat, organic matter removal + heat, and the complete FSRC decontamination) and decontamination steps (inoculation and post-treatment).

^{abcde} means with differing superscripts within row differ significantly, $P < 0.05$

Table 6.5 Effect of decontamination step on the relative quantification of Seneca Valley virus 1 (SVV1), porcine epidemic diarrhea virus (PEDV), and porcine reproductive and respiratory syndrome virus (PRRSV) and the proportion of PCR positive samples on environmental surfaces following the inoculation step and the removal of organic matter.[1,2]

	Inoculation	Post-organic removal	SEM	*P* =
SVV1				
Ct	35.9[b]	39.5[a]	1.08	0.001
Proportion	30/40	27/40	—	0.431
PEDV				
Ct	41.0[b]	43.1[a]	0.72	0.004
Proportion	17/40[a]	9/40[b]	—	0.021
PRRSV				
Ct	44.2	44.8	0.37	0.071
Proportion	6/40	1/40	—	0.085

[1] A 22.7 kg batch of feed was inoculated with SVV1, PEDV, and PRRSV, followed by a decontamination protocol. Environmental samples were taken following each decontamination step, but only the samples following inoculation and the organic matter removal step are displayed. A cycle threshold (Ct) value of 45.0 is considered negative with no detectable viral RNA.

[2] Means are the average Ct across all decontamination treatments (ClO_2, organic matter removal + ClO_2, heat, organic matter removal + heat, and the complete FSRC decontamination)

[ab] means with differing superscripts within row differ significantly, $P < 0.05$

Table 6.6 Summary of treatment contrasts statements on the relative quantification of Seneca valley virus 1 (SVV1), porcine epidemic diarrhea virus (PEDV), and porcine reproductive and respiratory syndrome virus (PRRSV) and proportion of PCR positive on environmental surfaces following the final step of the decontamination protocol.[1,2]

	Treatment					*P* =	
	ClO_2[3]	Organic matter removal + ClO_2[4]	Heat[5]	Organic matter removal + heat[6]	Complete FSRC decontamination[7]	Ct	Proportion PCR positive
SVV1							
Complete vs ClO_2	35.0 (18/20)	–	–	–	45.0 (0/20)	< 0.0001	0.959
Complete vs Organic removal + ClO_2	–	39.5 (13/20)	–	–	45.0 (0/20)	< 0.0001	0.963
Complete vs Heat	–	–	37.6 (16/20)	–	45.0 (0/20)	< 0.0001	0.961
Complete vs organic removal + heat	–	–	–	41.8 (9/20)	45.0 (0/20)	0.009	0.965
ClO_2 vs Organic removal + ClO_2	35.0 (18/20)	39.5 (13/20)	–	–	–	0.0003	0.076
Heat vs Organic removal + heat	–	–	37.6 (16/20)	41.8 (9/20)	–	0.0005	0.029
PEDV							
Complete vs ClO_2	41.3 (9/20)	–	–	–	45.0 (0/20)	0.0004	0.965
Complete vs Organic removal + ClO_2	–	44.8 (1/20)	–	–	45.0 (0/20)	0.809	0.972
Complete vs Heat	–	–	42.8 (6/20)	–	45.0 (0/20)	0.031	0.967
Complete vs organic removal + heat	–	–	–	42.2 (7/20)	45.0 (0/20)	0.006	0.966
ClO_2 vs Organic removal + ClO_2	41.3 (9/20)	44.8 (1/20)	–	–	–	0.0009	0.016
Heat vs Organic removal + heat	–	–	42.8 (6/20)	42.2 (7/20)	–	0.538	0.737
PRRSV							
Complete vs ClO_2	44.7 (1/20)	–	–	–	45.0 (0/20)	0.404	0.988
Complete vs Organic removal + ClO_2	–	45.0 (0/20)	–	–	45.0 (0/20)	1.00	1.00
Complete vs Heat	–	–	44.2 (3/20)	–	45.0 (0/20)	0.021	0.987
Complete vs organic removal + heat	–	–	–	45.0 (0/20)	45.0 (0/20)	1.000	1.00
ClO_2 vs Organic removal + ClO_2	44.7 (1/20)	45.0 (0/20)	–	–	–	0.404	0.988
Heat vs Organic removal + heat	–	–	44.2 (3/20)	45.0 (0/20)	–	0.021	0.987

[1] A 22.7 kg batch of feed was inoculated with SVV1, PEDV, and PRRSV, followed by a decontamination protocol. Environmental samples were taken following each decontamination step, but only the samples following the final stage in each decontamination protocol are displayed. A cycle threshold (Ct) value of 45.0 is considered negative with no detectable viral RNA.

[2] The Ct represents the value averaged across all ten surfaces (mixer, boot of bucket elevator, bucket of bucket elevator, corn cleaner, drag conveyor, distributor, down spout, wall, flooring, and boots) for each decontamination step.

[3] ProOxine AH (Bio-Cide International, Inc., Norman, OK)

[4] Organic matter removal with portable vacuums (Ridge Tool Company, Elyria, OH); ProOxine AH (Bio-Cide International, Inc., Norman, OK)
[5] Portable heaters ran for exactly 48 hours
[6] Organic matter removal with portable vacuums (Ridge Tool Company, Elyria, OH); portable heaters ran for exactly 48 hours
[7] Removal of organic matter with heated pressure washing, disinfection with 1% peroxygen (Virkon S, Lanxess, Cologne Germany), disinfection with 5% bleach solution (7.5% sodium hypochlorite; Clorox, Oakland, CA), environmental heat held at 60°C for 48 hours. The 48 hour heat up period was started once temperature on all floors reached and maintained the 60°C minimum threshold.

Table 6.7 Effects of treatment on Seneca Valley virus 1 (SVV1), porcine epidemic diarrhea virus (PEDV), and porcine reproductive and respiratory syndrome virus (PRRSV) decontamination as evaluated by a swine bioassay.[1]

	SVV1			PEDV			PRRSV		
	Inoculum Ct (PCR positive inoculum)[2]	4 dpi	7 dpi	Inoculum Ct (PCR positive inoculum)[2]	4 dpi	7 dpi	Inoculum Ct (PCR positive inoculum)[2]	4 dpi	7 dpi
Control treatments									
Negative	(0/3)	– – –	– – –	(0/3)	– – –	– – –	(0/3)	– – –	– – –
Pure virus	23.5 (3/3)	+ + +	+ – –	25.9 (3/3)	– – –	– – –	28.4 (3/3)	+ + +	+ + +
Environmental	26.7 (3/3)	– – –	+ – –	36.8 (3/3)	– – –	– – –	37.9 (3/3)	– – –	– – –
Decontamination treatments[3]									
ClO_2[4]	28.5 (3/3)	– – –	– – –	38.6 (2/3)	– – –	– – –	37.1 (1/3)	+ – –	+ – –
Organic matter removal + ClO_2[5]	37.1 (3/3)	– – –	– – –	(0/3)	– – –	– – –	(0/3)	+ – –	+ – –
Heat[6]	33.2 (3/3)	– – –	– – –	36.2 (2/3)	– – –	– – –	38.3 (2/3)	– – –	– – –
Organic matter removal + heat[7]	38.0 (3/3)	– – –	– – –	39.5 (2/3)	– – –	– – –	(0/3)	– – –	+ – –
Complete FSRC decontamination[8]	(0/3)	– – –	– – –	(0/3)	– – –	– – –	(0/3)	– – –	– – –
Organic matter removal alone[9]	34.9 (3/3)	– – –	– – –	39.2 (1/3)	– – –	– – –	(0/3)	+ + –	+ – –

[1] Three pigs per each treatment were inoculated on day 0 via intramuscular, intranasal, and oral gavage and evaluated for 7 days. Rectal samples were taken daily to evaluate SVV1 and PEDV presence and blood was collected on 4 and 7 dpi to assess PRRSV presence.

[2] The cycle threshold (Ct) value is the average Ct of all the PCR positive inoculums within each room as analyzed at Iowa State University Veterinary Diagnostic Laboratory following the d 0 inoculation. If no detectable RNA was present in the inoculum, then it was not included in the average Ct. The numerator represents the number of PCR positive inoculums with the denominator representing the total number of inoculums.

[3] Pigs were inoculated with environmental samples only from the final step of decontamination, no intermediary steps were utilized.

[4] ProOxine AH (Bio-Cide International, Inc., Norman, OK)

[5] Organic matter removal with portable vacuums (Ridge Tool Company, Elyria, OH); ProOxine AH (Bio-Cide International, Inc., Norman, OK)

[6] Portable heaters ran for exactly 48 hours

[7] Organic matter removal with portable vacuums (Ridge Tool Company, Elyria, OH); portable heaters ran for exactly 48 hours

[8] Removal of organic matter with heated pressure washing, disinfection with 1% peroxygen (Virkon S, Lanxess, Germany), disinfection with with 5% bleach solution (7.5% sodium hypochlorite; Clorox, Oakland, CA), environmental heat held at 60°C for 48 hours. The 48 hour heat up period was started once temperature on all floors reached and maintained the 60°C minimum threshold.

[9] Pigs were inoculated with the environmental samples following organic matter removal using vacuums decontamination step regardless of the treatment of origin (organic matter removal + ClO_2 or heat) as no treatment was applied when organic matter was removed.
+/– corresponds to the viral status of each pig in the treatment room where + pigs signify viral RNA was present for the respective virus and – pigs had no detectable viral RNA.