PERSISTENCE OF PORCINE CORONAVIRUSES IN FEED AND FEED INGREDIENTS

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Dedication

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Abstract

Porcine Epidemic Diarrhea Virus (PEDV), Porcine Delta Corona Virus (PDCoV), and Transmissible Gastroenteritis Virus (TGEV) are threats to commercial swine farms and contaminated feed is a risk factor in transmission. The objective of this thesis was to compare the inactivation kinetics of these coronaviruses among feed ingredients, after feed treatments, and on different surfaces. For the feed experiments, ingredients were weighed into containers, inoculated with virus, and exposed to room temperature, 60-90°C, 120-145°C, irradiation treatments, or feed additives. We also determined survival of coronaviruses on surfaces. After each treatment, virus concentration was calculated. The Weibull predictive model was used to characterize inactivation kinetics. Overall, the viruses survived longer in soybean meal than other ingredients. The feed processing treatments varied in their effectiveness, but all provided some virus inactivation. There was no difference in survival among surfaces. In conclusion, this information is useful for improving feed safety to minimize coronavirus transmission.

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Chapter 1: Literature Review

Coronaviruses

Introduction

Coronaviruses are responsible for a variety of illnesses and health problems in humans and animals, ranging from people experiencing the common cold to pigs with a high incidence of diarrhea and mortality in commercial swine herds. The *coronaviridae* family includes an assortment of viruses capable of infecting different of hosts (1). Two common examples include severe acute respiratory syndrome (SARS), which is responsible for upper respiratory infections in humans, and Porcine Epidemic Diarrhea Virus (PEDV), which causes high mortality in young pigs resulting from its detrimental effects on the gastrointestinal health in swine (2). Other than SARS, coronaviruses are a relatively low health risk for humans because they are typically only associated with the common cold (1). For other hosts, most of our scientific knowledge of coronaviruses has been obtained from research in domestic animals, where infections are much more detrimental to health, productivity, and welfare. The genera *coronavirus* contains virus species that infect swine, cats, dogs, mice, chickens, turkeys, and other species (2). Depending on the specific virus, these infections can affect the central nervous system, respiratory tract, or gastrointestinal tract (2). Therefore, due to the wide range of health and physiological effects caused by coronavirus infections in many mammalian species, a general understanding of the evolution and characteristics of these viruses is important

for understanding their health implications in veterinary medicine, human medicine, and animal science fields of study.

History of Coronaviruses

Coronavirus is a genus that is included in the order *Nidovirales* and the family *Coronaviridae*. Within the family *Coronaviridae* are the genera *Coronavirus* and *Torovirus* (3). Within the coronavirus genus, there are four genera including *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and the more recently discovered *Deltacoronavirus* (4). These groups are characterized based on their nucleic acid sequences. *Alphacoronaviruses*, previously known as group 1 coronaviruses, are capable of infecting mammalian species and include Transmissible Gastroenteritis Virus, Porcine Epidemic Diarrhea Virus, Canine Enteric Coronavirus, and Human Coronavirus 229E (2). The group of *Betacoronaviruses* include SARS-coronavirus and Middle East Respiratory Syndrome (MERS) coronavirus which are both capable of infecting humans (2). The *Gammacoronavirus* group only infects birds, and includes Turkey Coronavirus, Pheasant Coronavirus, and Infectious Bronchitis Coronavirus in chickens (2). The most recently discovered group are the *deltacoronaviruses* which include the Porcine Delta Coronavirus (PDCoV) (5). In general, coronaviruses are species specific and require a specific receptor in the host for each virus (1). Although it is not common for coronaviruses to be zoonotic, the history and evolution of Severe Acute Respiratory Syndrome (SARS-Cov) suggests that it is possible for coronaviruses to mutate and be transmitted across species (6).

In 1968, coronaviruses were first recognized as a unique group based on morphological differences compared with other viruses (7). Although they were not recognized until 1968, a molecular time analysis, based on the genomic region of these viruses, indicates that the four genera of corona viruses had a common ancestor over 10,000 years ago (8). Using SARS as an example of coronavirus evolution, recent research has suggested that the last common ancestor for coronaviruses even preceded this evolutionary time estimate, and infers that their origins could have occurred over 300 million years ago (9). Much of the research on coronavirus evolution is based on the presence of SARS in the bat population because changes in SARS coronavirus evolution provide an interesting story of how the virus has mutated to infect multiple host species (9,10). It is believed that SARS may have infected bat species since their divergence as a species in the carboniferous period (9). This indicates a long-term history of the relationship between bats and coronaviruses, and the presence of a co-evolutionary relationship (9). As a result, it is no surprise that there is great diversity in bat coronaviruses that are widely distributed within the population, and that bats are considered the natural reservoir for SARS(10). This is especially apparent when taking into account the population dynamics that demonstrate consistent prevalence of SARS in bat populations, and increased, epidemic-like growth observed in other host populations (10). This epidemic-like growth is an indication of inter-species transmission (11). When considering the evolutionary history between bats and coronaviruses, and the identified interspecies transmission, it has been suggested that bats are the original natural reservoir for all coronaviruses that exist today (10).

Properties of Coronaviruses

Coronaviruses are a family of plus strand RNA viruses, and their distinguishing characteristic is their large genome size. The genome size ranges from 27,000 to 30,000 nucleotides, which is considerably larger than picornaviruses (with an average size of 7,200 to 8,400 nucleotides) and togaviruses that contain 12,000 to 13,000 nucleotides (1). After translation, coronaviruses are released into the cytoplasm, which requires that the RNA genome be capped and polyadenylated to prevent degradation (1). The RNA genome of these viruses is packaged in a unique, distinguishable morphology (12). When viewed under an electron microscope, the shape resembles a crown with a circular shape and surface spike layer (2). An additional structural component unique to coronaviruses is the helical nucleocapsid (1). This structure is a long, flexible helix that is comprised of RNA and N proteins. The purpose of the helical packaging of this segment is not well understood. Surrounding the nucleocapsid is a lipid envelope that contains other proteins including envelope (E), membrane (M), and spike (S) proteins (1). The M protein interacts with the cytoplasmic and internal domain along with the helical nucleocapsid (13). The S proteins have the appearance of the spikes on a crown when viewed with an electron microscope, and differs from the M protein because it only has a short internal domain and is instead, primarily external. This large external domain is essential for its function of binding and fusing to cellular membranes (14). The S protein plays a role in virus entry into cells and pathogenesis because it provides specificity and contributes to

membrane fusion activity (14). The E protein interacts with M proteins and is essential for the budding process to form more virus particles (2).

One of the main distinguishing characteristics of coronaviruses is their unique method of genome expression. In replication, multiple copies of mRNA are produced with the same 3' end. These copies have unique 5' ends and produce a "nested set," where only the new gene on the 5' end will change between each copy. During translation, only the unique gene from the 5' end is translated (1). A potential consequence of this unique method of expression is the high frequency of recombination observed in coronaviruses. This frequent recombination (mutation) makes coronaviruses more capable of thriving in nature by adapting to new hosts and niches (1). This results in the potential of these viruses to be transmitted between species (6).

Porcine Coronaviruses

There are five coronaviruses that can infect swine including: Transmissible Gastroenteritis Virus, Porcine Epidemic Diarrhea Virus, Porcine Delta Coronavirus, Porcine Hemagglutinating Encephalomyelitis Coronavirus, and Porcine Respiratory Coronavirus. Of these viruses, Transmissible Gastroenteritis Virus (TGEV), Porcine Epidemic Diarrhea Virus (PEDV), and Porcine Delta Coronavirus (PDCoV) cause gastrointestinal damage in pigs, leads to diarrhea, reduction in growth performance, and high mortalities in young pigs.

Transmissible Gastroenteritis

Transmissible Gastroenteritis Virus was the first coronavirus to cause an infection in pigs. Clinical disease was first observed in 1946 on a swine farm in Indiana (15). After its initial identification, TGEV has been identified in multiple countries and has caused significant economic loss in the global pork industry. The primary countries impacted included the United States, France, England, Asia, and the Netherlands (16,17). During the TGEV outbreak in 1987-1988, the Iowa pork industry lost an estimated $10 million dollars from neonatal mortalities and reduced growth performance (18). Because Iowa is the largest producer of pork in the United States, this loss was significant to the entire United States pork industry as a whole. Today, there are still isolated cases of TGEV in the United States and Europe, but there has not been a major outbreak since the 1980's, and the devastating economic losses have decreased.

Signs of TGEV infection include vomiting, diarrhea, dehydration, and high mortality in suckling piglets (16). Upon infection, TGEV can be shed through the feces and nasal secretions for 10 to 11 days (19). The virus can then be transmitted through mechanical vectors such as contaminated surfaces, employees, or equipment. After the virus enters a barn, it is capable of causing an infection once it enters the oral cavity of a pig either through inhalation or ingestion (20). Once the virus is ingested, it infects the villous epithelial cells of the small intestine. During this time, the S protein binds to its receptor, amino peptidase N, to start the infection (21). After infection, the virus begins to destroy the intestinal epithelium, which reduces the digestion of nutrients and causes

diarrhea. With intestinal epithelium destruction, digestive enzyme production and activity is decreased, cellular transport is interrupted, and the osmotic force is changed in the small intestine. In addition to causing diarrhea, the destruction of intestinal epithelium causes morphological changes including a reduction and blunting of the villi (22). To diagnose TGEV, a variety of laboratory analyses can be conducted to identify the virus, its genome, antibody response, or virus antigens (23–26).

Porcine Epidemic Diarrhea Virus

Porcine Epidemic Diarrhea Virus (PEDV) was first identified in 1971 in the United Kingdom, and has since been transmitted through large areas of Europe and Asia (27). Specific countries impacted by PEDV include Canada, Korea, China, Thailand, Italy, Hungary, Germany, France and Vietnam (28). When comparing the significance of the detrimental effects of these outbreaks, PEDV is more of a concern in Asian countries because the outbreaks tend to be more acute and severe with a higher mortality than those experienced in European countries (28). The PEDV was first identified in the United States in May 2013, and it rapidly spread to 17 states (29). The devastating economic consequences of the PEDV outbreak in the United States affected all segments of the pork chain, including the packing industry, which experienced an annual revenue reduction of $481 million dollars due to a 3% decrease in market hog supply (30). This widespread outbreak also impacted consumers who experienced an increase in retail pork prices. Although this PEDV outbreak had devastating economic effects on the entire pork industry, the shortage of pigs created an opportunity for huge profits for those farms not

infected with PEDV because they had the supply necessary to fill this shortage and market prices for slaughter hogs was near a record high (30). Since this initial outbreak, the number of PEDV cases in the United States has decreased, with only a few isolated cases reported each subsequent year (31).

Signs of a PEDV infection are similar to those for TGEV and include diarrhea, vomiting, and a high mortality in nursing piglets. When PEDV is orally ingested by pigs, it enters the small intestine and binds to its receptor, aminopeptidase N, to initiate an infection (32). Once PEDV infects the enterocytes of the small intestine, it causes similar villous degeneration as described for TGEV, along with reductions in digestive enzymes and compromised nutrient absorptive capacity (33,34). Morphological changes in the infected villi may be observed as early as 12 to 18 hours after exposure (35). Because of the many similarities between the clinical presentation of PEDV and TGEV, laboratory analysis is necessary to confirm a PEDV infection. Common diagnostic tests include immunofluorescence, immunohistochemical techniques, direct electron microscope, and ELISA (28). The use of RT-PCR allows confirmation of the presence of PEDV, but it has the limitation of only confirming the presence of viral RNA. Consequently, results from RT-PCR cannot be used to distinguish between an active infection and a previous infection. In lab experiments, PEDV can be propagated in cell culture and the virus can be inoculated into Vero cells with the presence of trypsin (36). The cells can then be observed for cytopathic effects that consist of vacuolation and formation of syncytia (36). Currently, the use of cell culture to detect PEDV is the least costly way of measuring viable virus concentration in a sample.

Porcine Delta Coronavirus

The most recently identified swine coronavirus is Porcine Delta Coronavirus (PDCoV). The virus was first identified in China in 2012, and was found 2 years later in the United States in 2014 (8,37). The PDCoV belongs to the subfamily of *Deltacoronsviruses*, and differs from PEDV and TGEV, which are in the subfamily of *Alphacoronaviruses.* The virus was initially discovered during a large-scale animal antigen experiment that sampled multiple hosts and used RT-PCR to identify conserved regions of *Deltcoronavirus* genes (8). The results showed that 10.6% of the pig samples were positive for this gene, indicating that the presence of a deltacoronavirus may infect domestic swine (8). The first confirmed case of an PDCoV infection in the United States came from an investigation of 5 Ohio swine farms (38). When completing a retrospective analysis from archived samples, PDCoV was identified in samples collected in 2013 (39). This indicates that the PDCoV outbreak in the United States may have followed a similar timeline as for the PEDV outbreak in 2013. The route of the sudden introduction of PEDV and PDCoV into the United States is still unclear. Recently, PDCoV was detected in mainland China and Thailand (40,41), suggesting that the PDCoV will continue to be a challenge in global pork production systems in the foreseeable future.

Signs of PDCoV infection in pigs include vomiting, dehydration, and diarrhea similar to PEDV and TGEV. Generally, the signs of PDCoV infection are less severe than for PEDV and TGEV, and PDCoV causes lower mortality. The primary route of transmission for PDCoV is fecal-oral and contaminated fomites, including contaminated

feed and livestock trailers, and are being investigated because of their ability to transmit other coronaviruses like PEDV (42,43). The pathology of PDCoV is similar to that of PEDV and TGEV, with the virus causing massive destruction of intestinal enterocytes resulting in malabsorption of nutrients (44). Diagnostic tests are necessary to distinguish PDCoV from other gastrointestinal porcine coronaviruses. Many of the same methods for PEDV and TGEV identification can be used to confirm a PDCoV infection, and include electron microscopy, various PCR assays, immune florescence, and virus isolation (44).

Coronavirus Summary

Overall, coronaviruses pose a threat to the health of multiple species, and understanding their modes of transmission is important to develop mitigation strategies to minimize their devastating effects on animal health and the economic losses they cause. In swine production systems, TGEV, PEDV, and PDCoV all cause diarrhea, dehydration, and a loss of intestinal digestion and absorption capacity, which leads to reduced growth performance, and high mortality in piglets. The source and route of PEDV introduction into the United States has not been confirmed, but improved biosecurity methods and potential mitigation strategies are being developed to prevent future corona virus outbreaks in the U.S. swine population.

Feed Safety

Delivering safe animal feed is essential when raising animals for human consumption. Consumers of animal-derived food products expect them to be free of

biological, chemical, and physical contaminants pose a threat to human health and are wholesome. Achieving this expectation starts with the quality and safety of feed ingredients used to manufacture diets to feed animals. Although ensuring safe animal feed is essential, assessing and achieving safe feed is a very complex and challenging task that requires knowledge in multiple areas including: microbiology, animal nutrition, veterinary medicine, toxicology, crop production, and feed manufacturing (45). This was exemplified in an adulterated pet food case, where contaminated pet food caused widespread illness in humans. In 2006-2008, there was a multi-state outbreak of *Salmonella enterica* that infected 79 people, and led to the recall of 23,109 tons of dry pet foods (46). The relatively large number of people infected and amount of product that was wasted emphasizes the importance of producing safe food for animals.

The increasing number of cases of food borne illnesses in the U.S. led to the development and enactment of a new law by the U.S. Food and Drug Administration (FDA) called the Food Safety Modernization Act (FSMA). This ruling implemented increased requirements for feed safety in the feed industry. The first requirement is to develop and implement good manufacturing practices (GMPs) for animal feed production (47), which were established by the FDA. In addition, feed ingredient and feed production facilities must complete a hazard analysis and the identification of the critical control points (HAACP) in their operation (47). To do this, potential feed and food safety hazards must be identified, and can include, mycotoxins, antibiotic residues, microbiological contaminants, chemical contaminants, or other feed safety risk factors that may be intentionally or unintentionally introduced. After the potential hazards are

identified, controls need to be put in place to ensure that the risk from these potential hazards is minimized. Lastly, a recall plan must be in place in the event that contamination does occur in the final product (47).

There are a variety of different components of feed safety that need to be addressed. The first of these is the need to comply with genetically modified organisms (GMO)-free label claims of some animal-derived food products. Although a comprehensive review of the scientific literature showed that feeding animal diets containing GMOs did not cause any differences in food safety and the nutritional profile of animal-derived food products (48), some food manufacturers are providing GMO-free foods for niche market segments of consumers desiring these attributes. In fact, new legislation in some states requires the labeling of food products derived from animals fed GMO ingredients (49). Another feed safety risk factor is mycotoxins. Mycotoxins are toxic metabolites that are produced by certain fungi species and are commonly found in grains that are used in animal feed (50). Aflatoxins are one type of mycotoxins that can be passed through animals to contaminate food products (50). Because of this contamination risk, aflatoxins are the only group of mycotoxins that are regulated by the FDA (51). Antibiotic residues are another feed safety risk factor because residues of some of these compounds can be deposited in meat, milk, and eggs if proper dosage and withdrawal times are not followed. In addition, use of antibiotics as growth promoters has led to the development of bacterial pathogen resistance, particularly for those used in human medicine, which has multiple negative health implications for humans, animals, and the environment (52–54). As a result, ample scientific evidence and increasing

societal concerns have led to government regulations to reduce or eliminate the use of growth promotion levels of antibiotics in animal feeds (55). Other chemical contaminants, such as melamine and heavy metals, also pose a risk to humans and the animals consuming the feed. In a 2007 recall of pet food, animals that consumed the food contaminated with melamine developed acute renal failure (56). This concern of toxicity is also present with other chemical contaminants including cyanuric acid, and monensin. Physical contaminants are also a potential risk factor in animal feed, and include nails, bolts, plastic, rubber, or glass that inadvertently be mixed into the feed.

In addition to these general categories of feed safety risk factors, other biological contaminants such as pathogenic bacteria, prions, and viruses are a concern. Perhaps the most widely studied bacterial pathogen has been *Salmonella* and its risk of transmission through animal feed and possible contamination of animal-derived food products (57). However, a new feed safety concern has emerged with the epidemic transmission of porcine coronaviruses such as PEDV. Unlike *Salmonella*, which is both an animal health concern and a human food borne pathogen, corona virus contamination is not a human food safety risk, but rather, an swine health risk that can lead to devastating economic losses for infected commercial swine farms (58). There are multiple critical control points for bacteria and virus pathogen contamination of feed and feed ingredients in the feed supply chain, beginning with production processes and exposure to contamination through storage, transport, and complete feed manufacturing. Contamination can occur in feed storage facilities or transport trucks that are not adequately cleaned after exposure to these biological hazards (59). Contamination can also be introduced by pests including

rats, insects, and wild birds, where feral pigeons have been shown to contaminate poultry feed with excreta containing Newcastle Disease (60). An additional concern is that animal by-products derived from infected animals may be at risk for transmitting these viruses. This concern was especially apparent after a report from the United Kingdom showed that Bovine spongiform encephalopathy, a neurodegenerative disease in cattle caused by prions, was linked to contaminated meat and bone meal (61). With the potential of risk of these biological hazards contaminating feed early in the production chain, it is important to understand how each feed processing step may impact the risk of infectivity of each potential contaminant.

Feed Processing

Introduction

The various steps involved in production, transport, storage and mixing of feed ingredients into complete feed is complex (Figure 1). It is important to note that many processing steps involve heating or drying an ingredient. Therefore, steps that involve heat are potential inactivation steps for reducing the concentration of pathogens in the various feed ingredients. These potential inactivation steps are shown in red in the figure. It is also important to note the location of the last virus inactivation step before feed reaches the susceptible animals. Any contamination that occurs after this final kill step will be a risk of contaminated feed reaching a farm.

This process is not made any simpler by the wide range of ingredients used in commercial animal feeds that are constantly changing based on supply and market price.

When a process has multiple steps, there are more opportunities for a contaminant to be introduced. Feed contamination has been an especially pertinent topic with the implementation of the new standards for the Food Safety and Modernization Act (47). Animal feed contamination can vary based on the particular contaminant, but biological contaminants of animal feed has been especially concerning after the 2013 outbreak of Porcine Epidemic Diarrhea Virus in the United States, where feed was identified the cause of virus transmission (62,63). Therefore, it is important to understand the stages in the animal feed manufacturing chain that may serve as inactivation steps for most biological hazards and what points may be at risk for contamination. As a result, determining the relative risk of transmission needs to be considered when choosing ingredients and determining how to handle these ingredients to minimize transmission risk.

There are many considerations when choosing feed ingredients including availability, cost, digestibility, nutrient density, palatability, and handling characteristics. Energy is the most costly nutritional components of animal feeds, and corn is the most common energy source in swine diets (64). With the recent use of corn for ethanol production, the price of corn has generally increased, creating a need for using alternative energy sources, such as distillers dried grain with solubles which is a by-product from ethanol production. The second most expensive component in the animal diet is protein. Protein can come from plant sources, such as soybean meal, or various animal by-products such as meat and bone meal, meat meal, and blood meal. The remainder of the diet is comprised of macro mineral supplements, such as ground limestone, monocalcium

phosphate, and salt, along with the appropriate premixes containing required vitamins and trace minerals. Finally, starter diets for nursery pigs require that energy and protein sources from animal origin be used because they are more digestible than the typical plant sources. Spray dried porcine plasma is an example of the commonly used highly digestible protein sources in starter diets because it also provides some immune system benefits. Each of these ingredients have different chemical composition and physical characteristics (e.g. bulk density, particle size) which may affect the survival of bacterial and viral pathogens.

Feed Ingredient Processing

Corn

Corn is an important feed ingredient in swine diets because it serves as an economical and major source of energy. One of the main problems with handling corn is its susceptibility to mycotoxins and molds. The best way to manage this risk is to properly dry it to contain less than 15% moisture, minimize kernel cracks and breakage, and store it under dry conditions (65).

During the harvesting process, it is important that the grain experiences minimal cracking or damage during the harvest process because damage exposes the sensitive portions of the kernel which encourages mold growth and may lead to mycotoxin production (65). After harvest, corn is dried to reduce moisture content, which minimizes mold growth and potential mycotoxin production. Typically, depending on weather conditions, corn is not harvested until the moisture is reduced to 20-25% (66). It is further

in storage bins on the farm or at the feed mill until contains 12-15% moisture which is considered optimal for preventing spoilage (67). Natural air-drying can be done when air temperatures are between 4 and 15°C with 55-75% relative humidity, but this method can only be used when the original moisture content is below 23% (68). Natural air-drying uses multiple electric fans to push air through the corn and reduce moisture. Depending on the fan speed and the initial moisture of the corn, this process can take 4 to 6 weeks (68). To speed the process of drying, increased temperatures can be used as long as the temperatures used remain below 110°C. If the temperature in the drying corn bin is above 110°C the nutrient value of the grain will be decreased (67). When using heat to dry corn, the maximum temperature of 110°C is recommended to minimize energy costs of drying. However, exposing corn to temperatures over 94°C for more than 2 hours increases the rate of Maillard reactions (browning) which reduces amino acid digestibility. Therefore, temperatures greater than 94°C should not be used for long periods of time (69). After the shelled corn is properly dried it can be stored for extended periods of time. The amount of time corn is stored is highly variable and depends on prices, demand, season, and current supply.

Dried Distillers Grains with Solubles

One of the major by-products used in swine diets is dried distillers grains with solubles (DDGS), which is produced from a dry-grind process in the ethanol industry. This by-product contains about 3 times more protein, lipid, fiber, and minerals than corn because the starch is fermented by yeast to produce ethanol. After the removal of starch,

DDGS contains greater gross energy content (5,434 kcal/kg DM) than corn, but the metabolizable energy content is similar to corn (70). One of the challenges of using DDGS in animal feed is the variability in nutrient content among different sources and over time (70). However, because it is usually a more economical source of energy and protein than corn and soybean meal, respectively, nutritionists have used it extensively in swine diets.

During the ethanol and DDGS production process, corn enters the ethanol plant and is cleaned and ground with a hammer mill to a medium-coarse to fine (600-1000 microns) particle size. The meal is then mixed with water at a known concentration to create a slurry for liquefaction. In this step, the pH of the slurry is adjusted about 5 to 6 and the temperature is adjusted to 82-90°C to maximize the activity of the enzyme amylase, which is added to aid in the hydrolysis of cornstarch. Once this is complete, the mash is heated to kill unwanted bacteria, then cooled to 33°C and sent to a large vessel for fermentation. In the fermentation vessel, a glucoamylase enzyme is added to convert the long chain sugars into simple sugars. These simple sugars are then used by yeast (*Saccromyces cerviseae),* which is also added to the fermentation, to produce ethanol and carbon dioxide. The fermentation process continues for 40-60 hours and then the fermented mash, now referred to as the beer, is piped to distillation columns to distill the ethanol. During this process, water and solids are collected and centrifuged to separate liquids and solids. In addition, most ethanol plants have added another centrifuge step to partially extract some of the oil before removing water to produce condensed distillers

solubles. The coarse solids that are gathered after centrifugation are referred to as the wet cake. This wet cake is then combined with solubles and dried to produce DDGS (71).

Soybean Meal

Protein is the second most expensive portion of the swine diet after energy. Soybeans are the most common source of plant protein in swine diets because of their abundant supply, high concentrations of essential amino acids, digestibility, and balance relative to animal requirements. Soybeans have been valued as a food and feed source for decades but heating raw soybeans under moist conditions increases their biological value (72). Heating soybeans helps reduce some of the anti-nutritional factors such as trypsin inhibitors can be inactivated and this enhance the amino acid digestibility. However, although heat can be used to reduce trypsin inhibitor activity, excessive temperatures also reduce protein digestibility because of the formation of Maillard products. These products are products of reactions between reducing sugars and amino groups (73). As a consequence of this reaction, the amino acids are less available to be utilized by the animal and protein digestibility is decreased (73). There are multiple methods used to process soybeans including extrusion, and solvent extraction of oil, in addition to heating, which is the most common method to produce soybean meal (74).

The first step in solvent extraction is drying the raw soybeans to a moisture content of below 13%. This can be done by using forced air circulation dryers, or high temperature (55-60°C) dryers for no longer than 30 min (75) to ensures maximum stability for long-term storage. After soybeans are dried, they require 2 to 5 days to allow

moisture equilibrium, which is a tempering phase that allows a constant and continuous supply of soybeans for the remainder of the processing steps (76). Soybeans are then cleaned using cleaner-separator machines that remove soil, plant waste, and other contaminants (74), and then are subsequently cracked into 8 to 16 pieces using a hammer mill. The hulls are then separated and blown away from the cracked seeds by a stream of air in an aspiration process (74). The dehulled portion is then conditioned by heating to approximately 65-75^{o}C to soften the seed. Following conditioning, the softened seed is passed through a roller mill that creates one large flake about 0.2to 0.35mm in thickness (76), which provides enough pressure to rupture the cells in the seed and allow efficient oil extraction. Additional pressure is applied in the expansion process, and when that pressure is released and steam is applied, the product is more porous. This increase in the number of pores also increases extraction efficiency (74). The next step is the addition of hexane to begin the solvent extraction. After the oil has been extracted, the remaining "spent flakes" enter the desolventizer and toaster where the flakes are heated (100 ^{o}C for 10 seconds) to evaporate any remaining hexane (74), and then cooled for approximately 10 min so the temperature of the flakes decreases to 50^{o}C (77). After the hexane is removed, the soybean meal is dried to 12% moisture and ground in a hammer mill for storage and transport to feed mills.

Rendered Animal By-Products

Rendering is the process of collecting raw materials from on-farm mortalities and slaughterhouses and removing moisture, separating fats and proteins, and inactivating

microbes in these materials. A wide variety of these rendered animal by-products can be used in swine diets as a protein source. Because of the variability in the chemical composition of the raw materials, they have more variable nutrient content and digestibility than many high protein ingredients derived from plant sources.

In general, about 55 to 60% of animal carcasses is used for human consumption, with the inedible components such as fat, offal, bones, skin, hair, blood, and horns rendering into animal feed ingredients (78). Other materials including trimmings from meat processers or waste restaurant grease may also be used to produce rendered animal by-products such as blood meal, feather meal, meat meal, meat and bone meal that have different nutritional profiles

The first step in the rendering process is to collect and transport raw materials to the site where they will be processed. To prevent cross contamination from raw materials, internal biosecurity protocols are used with third party audits, and are handled following the established regulations (79). After receiving, the pieces of raw material go through a sizing process and are then cut into uniform size pieces to facilitate uniform thermal processing (78). The target size of these pieces of animal tissues is 40 mm, but should not be larger than 70 mm (80). Additional processes vary based on the type of tissue being processed, but all methods include the heating, removal of moisture, and extraction of fat (78). In the United States, the most common method of rendering is a continuous flow process by heating at 115º to 145ºC for 40 to 90 minutes. After thermal treatment is complete, the slurry is separated into liquid fats and solids through a screw press system

that uses pressure to separate these components. The protein fraction is referred to as "cracklings", and is dried further and ground into a meal for shipment to feed mills (78).

Spray Dried Porcine Plasma

Spray dried porcine plasma (SDPP) is a high protein, highly digestible by-product that has been shown to increase growth performance of weaned pigs. This ingredient is derived from blood collected at slaughter plants where the plasma portion is separated and spray dried. In addition to the high concentrations of amino acids in SDPP, it also appears to contain immunoglobulins that are biologically active and prevent the binding of pathogens in the intestine (81). For this reason, spray drying is used to avoid excessive drying temperature to process plasma because it minimizes damage to these functional proteins.

The blood used to manufacture SDPP is only collected from USDA inspected slaughter plants in the United States, where an approved anticoagulant is added to prevent clotting. The whole blood is centrifuged to separate the plasma portion, which is then chilled (4 to 10ºC) for storage and transport to the plasma processing facility (79). Water is evaporated by altering the inlet air temperature and humidity, which alters the water activity to a final value of less than 0.6, but ideally a value of 0.4 (79). This is accomplished using inlet temperatures of hot, dry air (170-310ºC) that are exposed to the wet particles for 2 to 5 seconds, and cooling with the outlet air temperature of 80 to 84ºC. This process rapidly exposes the wet particles to high temperatures and changes the water

activity to achieve a powder consistency of the final product that prevents growth of bacteria and allows for easy handling (79).

Vitamin and Trace Mineral Premix

The premix manufacturing process is fairly similar to that used to mix complete feed. Concentrated vitamin and trace mineral sources are weighed to match the required amounts specified in the formulation, and are added to a mixer along with a carrier (e.g. rice hulls) and a source of oil for dust control. Once all ingredients are added, it is mixed for a specified period of time to ensure a uniform dispersion of all ingredients throughout the mix. After mixing, the premix is often placed in plastic-lined bags to prevent moisture accumulation before transporting to feed mills for use in manufacturing complete feeds. Perhaps one of the greatest concerns about preserving nutritional value of vitamin-trace mineral premixes involves preventing loss of vitamin potency during storage. Vitamins are sensitive to light, heat, moisture, and pro-oxidant trace minerals such as copper, iron, and zinc (82). Minimizing vitamin losses can be managed by selecting protected chemical forms of vitamins that are more stable, as well as replacing inorganic sources of trace minerals with metal specific amino acid complexes that are less destructive of vitamin activity (83). Furthermore, it is recommended that feed mills use premixes within 2 months of being manufactured to minimize extensive degradation of vitamins (82).

Feed Mixing

After all feed ingredients are individually manufactured, they are mixed in various proportions to produced nutritionally balanced complete feeds for various animal species in various stages of production. Nutritionists formulate diets to meet the nutrient requirements of pigs by selecting appropriate feed ingredients, determining the amount of each ingredient to add to the diet, and then mixing to produce a uniformly dispersed complete feed that can be transported to farms.

There are many types of feed mixers including horizontal, vertical, and rotating drum mixers. It is recommended that mixer tests be conducted as part of the feed mill quality control program to determine uniformity of mix at different mixing times. Factors such as overfilling mixers, worn equipment, and buildup of ingredients on surfaces can all lead to problems in mix uniformity. Mixer tests typically use the coefficient of variation (CV) of analyzing a nutrient marker, such as salt, to determine mix uniformity based on variation between samples. In general, a CV of 5% or less is suggested as the standard (84). If a diet is to be fed as a mash, mixing is the last step in the feed manufacturing process before the diet is transported to the farm.

Pelleting

The benefits of pelleting a diet include decreased segregation of particles of various ingredients, reduced dust, increased bulk density, and improved handling characteristics (85). Pelleting has been shown to increase average daily gain and improve feed efficiency (86). In the process of pelleting, the mixed meal is combined with steam

and then forced through a pellet die by an extrusion process where it reaches a final temperature of 85°C (85). After pellets are formed, they are passed through a stream of air to cool and reduce their moisture content to 10-12%, and subsequently sending them through a crumble roll if desired, to breaks the pellets into smaller pieces. Once pelleting is complete, diets can be packaged and transported to the farm.

Feed processing for decontamination

Common feed ingredient and complete feed processing procedures include reducing particle size and using heat to dry or inactivate pathogens prior to storage or use. A summary of processing steps for various ingredients is outlined in Table 1. The three major categories are storage time, thermal treatments, and moisture changes. It is well known that virus and bacteria survival is affected by changes in temperature and moisture (87–89). Therefore, a key consideration for developing biosecurity protocols for feed mills is determining the extent that various processing methods have on reducing the concentration or infectivity of various pathogens.

Salmonella

Because of its heavy presence in the environment, *Salmonella* is of particular concern and a potential risk factor for feed safety and transmission through animal feed (57,90,91). For example, 18.8% of feed samples collected prior to thermal exposure in a poultry feed mill in Ireland were positive for *Salmonella* (92). *Salmonella* concentration can be reduced 10,000 fold (4 logs) when heated for 90 seconds at 90°C (93). This indicates that the current processing steps including drying, rendering, and pelleting may

be sufficient to reduce *Salmonella* concentration in complete feed and feed ingredients. Studies have also shown that the high temperatures used during the pelleting process are sufficient to reduce the bacterial load in feed (94). However, *Salmonella* contamination of complete feed can still occur because the same feed mill in Ireland measured *Salmonella* in 22.6% of feed samples in a post-heat treatment area (92). This indicates that cross contamination occurs after heat treatment is applied, and alternative risk mitigation strategies should be considered to control *Salmonella* concentration in feed. One of these strategies is to add specific feed additives to reduce the concentration of *Salmonella* in feed (95–97). However, little is known about the effectiveness of various commercial feed additives on reducing other pathogens in feed, which needs to be investigated.

In addition to *Salmonella*, initial investigations of causes of the 2013 outbreaks of Porcine Epidemic Diarrhea Virus suggested that this virus may also be transmitted through contaminated feed (62,63). A similar mode of transmission has been suggested for other coronaviruses including Porcine Deltacoronavirus (98). Though feed has never been thought to serve as a mode of transmission for TGEV, its similar characteristics with PEDV and PDCoV suggest that contaminated feed might also be a risk factor for this virus. Therefore, it is important to determine if the processes used during feed ingredient and complete feed processing are sufficient to reduce corona virus concentrations.

Porcine Epidemic Diarrhea Virus

Length of storage of feed and feed ingredients can be effective in reducing the concentration of PEDV in certain feed ingredients. When PEDV was inoculated into 18 feed ingredients, there was no viable PEDV detected isolation after 1 day of storage in corn and spray dried porcine plasma (99), suggesting that storing these ingredients for an extended period of time may be an effective method to decrease virus concentration. This is an important observation for corn, because it is typically stored for many months before being used to manufacture animal feed, however, this low virus survival has not been observed for all the feed ingredients. For example, in the same experiment, PEDV was detected after 180 days of storage of soybean meal (99). This indicates that relying on extended storage time for soybean meal to decrease virus survival is not an effective strategy. Even though extended storage time may not completely inactivate PEDV in soybean meal, it does decrease in virus concentration to some extent in all ingredients. For example, vitamin and mineral premixes should not be stored longer than 2 months to avoid significant vitamin potency losses, and that other methods need be used to reduce virus concentration for some ingredients.

Thermal processing can decrease virus concentration because PEDV is sensitive to heat treatments (89,98). Heat treatments of 120°C for 16 min in complete swine feed caused a 1 log reduction in virus concentration (89), which can also achieved after 3 min at temperatures greater than 130°C (89). These temperatures are in the range (115-145°C for 40 to 90 min) used in the rendering of animal by-products to make meat meal, meat and bone meal, and blood meal. Depending on the starting virus titers, it appears that the

rendering process is effective in reducing virus concentration. High inlet temperatures are also used during the spray drying process (170 to 310ºC) used to produce SDPP, although though for a much shorter length of time (2 to 5 seconds). However, particles only reach 60 to 90 ºC during spray drying, which is a relatively short-term exposure to high temperatures, suggesting that heat alone may not be sufficient to completely inactivate PEDV during spray drying. Lower temperatures of 55 to 60°C are used to dry grains such as corn and soybeans, and prior to solvent extraction of oil from soybeans to produce soybean meal (65 to 75^{o}C). Heat treatments (70^{o}C for 10 min) of trailers used to transport pigs has been shown to completely inactivate PEDV (100). Therefore, PEDV is susceptible to inactivation, even at lower temperatures used in drying and processing of some feed ingredients.

In addition to temperature, the moisture content plays a role in reducing virus concentration. Previous research has shown that PEDV survives longer in wet feed compared with dry feed (101). Currently, data on PEDV survival in feed ingredients with varying moisture content is limited, but needs to be evaluated. Most of the research that is available for inactivation of PEDV during processing is limited to the spray drying process of porcine plasma. Studies have shown that after infected porcine plasma is spray-dried, it is no longer able to cause an infection of PEDV in pigs (102,103). Although these results are convincing, there is still debate among researchers and veterinarians whether the spray drying process is sufficient to completely inactivate PEDV because positive batches of SDPP were identified on farms that had outbreaks of PEDV (63). To understand the extent of PEDV inactivation in drying processes, more

research is needed to characterize the interaction between moisture, water activity, and virus survival.

In contrast to processing steps that may decrease PEDV survival, some stages of feed processing may increase the risk of contamination. Feed mixing may cause wide spread contamination because a small dose of PEDV present in one ingredient may be distributed to the entire batch of complete feed. The infectious dose of PEDV in feed has been determined to be 5.6×10^{1} $TCID_{50}$/g. In fact, 1 gram of infected feces has been reported to be capable of contaminating 500 tons of feed (104). There is also potential risk that surfaces in feed mills can be contaminated and facilitate PEDV transmission. Results of one study showed that a batch of contaminated feed was capable of causing widespread contamination of feed mill surfaces (105). Therefore, sequencing feed batches by alternating feed mixing for other animal species (e.g. dairy, poultry) can decrease the risk of transmission of PEDV from contaminated feed to other batches of feed, but it does not completely eliminate it (106). Virus contamination of surfaces poses a greater risk of widespread contamination because feed ingredients that arrive at feed mills that are not contaminated with PEDV, can quickly become contaminated.

Transmissible Gastroenteritis

There is limited research on the survival of TGEV in feed and feed ingredients. However, because TGEV is a corona virus like PEDV and has similar characteristics and disease signs, it has been hypothesized that TGEV will respond similarly to feed processing in activation conditions as for PEDV. One study showed that TGEV survival

is decreased at 40^{o}C compared to 20^{o}C (107). At 40^{o}C there was complete inactivation (4 to 5 log reduction) between 6 hours to 5 days, depending on the humidity (107). However, when TGEV survival was determined at 20^{o}C and 20% relative humidity, only a 2 log reduction was observed after 28 days. The maximum log reduction (5 logs) in this study occurred when stored at 20^{o}C for 11 days at 80% relative humidity (105). Though the temperatures used in this experiment do not coincide with any commonly used feed ingredient processing conditions, the decreased survival at increasing temperatures indicates that TGEV is sensitive to heat treatments. This study also showed that relative humidity affects survival because TGEV survived longer (5 days) at low 20% RH compared to survival at 80% RH (< 6 hours) (107). Although research on TGEV survival in feed and feed ingredients is limited, it appears that thermal feed processing treatments may be effective in reducing TGEV concentration.

Porcine Deltacoronavirus

Similar to TGEV, very little research has been conducted on PDCoV survival in feed ingredients, but it appears that PDCoV survival varies among ingredients (98). Survival of PDCoV was evaluated in feces and slurry and results showed that survival was decreased at 60^{o}C compared to 25^{o}C (108), suggesting that PDCoV appears to be sensitive to the heat treatments used in feed processing, but more research is necessary to confirm this.

Feed Mill Biosecurity

After a review of the current literature, there are multiple steps used in processing feed ingredients and complete feed that decrease PEDV, PDCoV, and TGEV survival including drying, spray drying, rendering, solvent extraction of oil, and extended storage. Most of these processing treatments occur during feed ingredient manufacturing, pelleting is the only potential inactivation process that can affect corona virus survival when manufacturing complete feeds. Unlike contamination of drug residue being diluted when mixed in large batches of feed, a small dose of virus can contaminate large batches of feed (1 gram of PEDV infected feces is capable of contaminating 500 tons of feed) (104). If that feed is then able to cause an active infection in just one animal on the farm, that animal can then spread the virus to all of the animals in the facility. A single batch of contaminated feed can also cause widespread contamination of feed mill surfaces and potentially contaminate future batches of feed being manufactured. Because of all of these risk factors, development of a feed mill biosecurity plan is essential to identify critical control points and potential mitigation strategies to reduce the risk of corona virus contamination similar to those used to manage risk of drug residue contamination.

To do this, Cochrane and collaborators outlined a systematic approach for creating a biosecurity plan to decreasing virus transmission among feed ingredients and diets produced in a feed mill (109). In this approach, the carriers of virus include contaminated ingredients and contaminated people entering the feed mill. For contaminated ingredients, they suggested that high-risk ingredients be routinely tested for target pathogens such as *Salmonella*, PEDV, or PDCoV. In addition, protocols limiting

personnel movement in the receiving area can help prevent contamination of ingredients. All feed mill visitors should wear disposable boots and follow common biosecurity measures such as no recent contact with livestock for a specified period of time, and to shower and use clean clothes provided by the feed mill prior to entering the feed mill. Delivery drivers should stay in their truck as much as possible when unloading ingredients. In addition to preventing introduction of corona viruses to feed mills, there should also be procedures in place to prevent cross contamination of surfaces feed ingredients and batches of complete feed. These include cleaning the mill after a known contaminant enters, utilizing feed batch sequencing to dilute the virus concentration t and mix higher risk ingredients into diets for other animal species. Both prevention and effective mitigation strategies are necessary to reduce the risk of coronavirus contamination in feed ingredients and complete feed and increase the chance that the feed delivered to the farm will not be a source of transmission.

Conclusions

Overall, there are multiple factors that need to be monitored and used to minimize the risk of coronavirus contamination and transmission in animal feed

Data on survival of viruses in the environment and common feed ingredients are necessary to develop meaningful and useful biosecurity measures. However, there are limited data on survival of PEDV, TGEV, and PDCoV in feed and feed ingredients. Determining individual corona virus survival at different temperatures, moisture concentrations, and relative humidity during storage are critical factors that need to be

evaluated, along with comparison of difference in survival among these 3 viruses. Various types of surfaces commonly found in feed mills need to be evaluated for coronavirus survival to determine relative risk of transmission. The effectiveness of common feed additives must also be determined to develop potential prevention strategies to prevent virus transmission to farms. Collectively, understanding these factors will enable the development of improved biosecurity plans for feeds mills to prevent the transmission of these devastating virus to commercial swine farms.

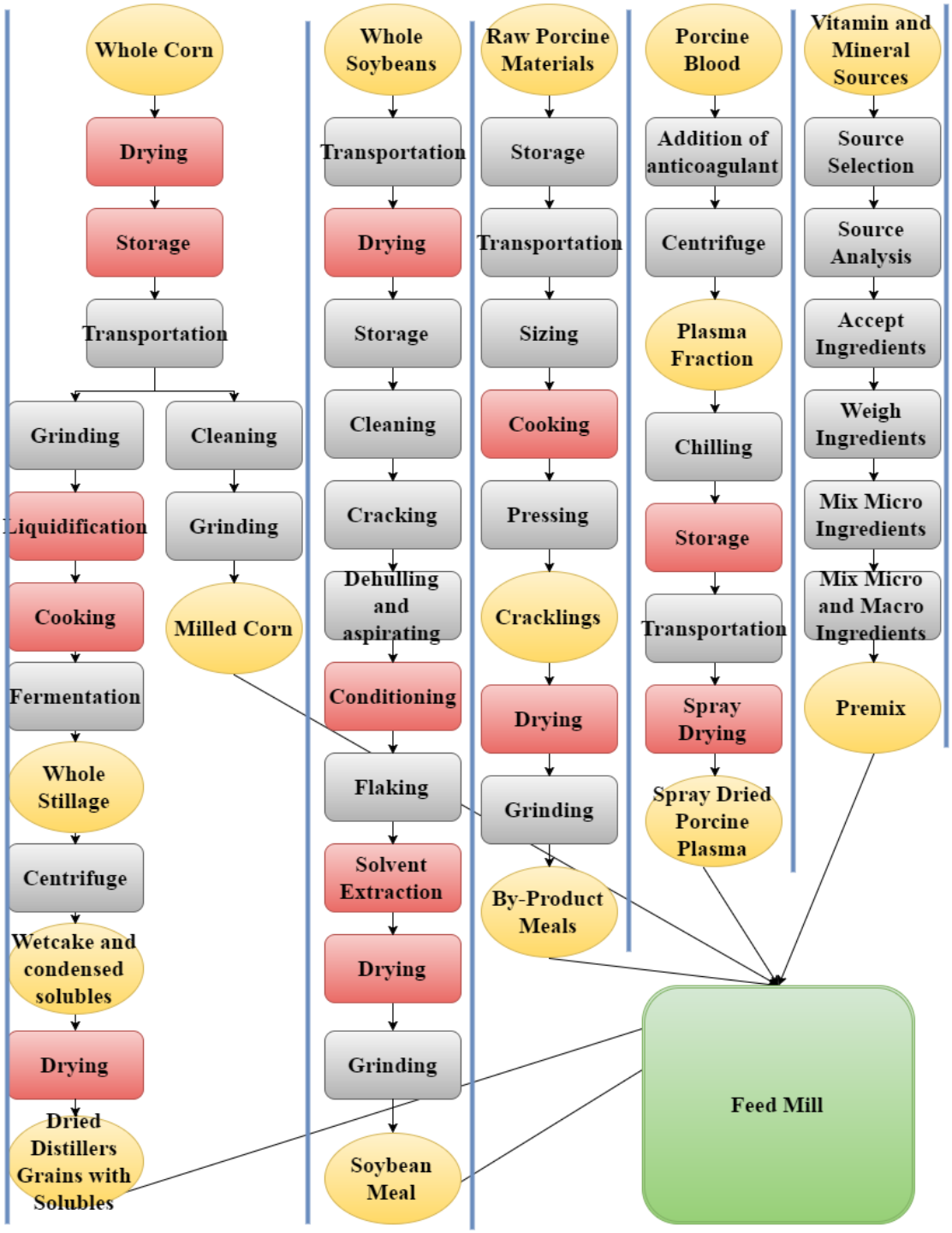

Figure 1. Processing steps of various feed ingredients prior to transport to a feed mill. Red boxes indicate potential inactivation steps for coronaviruses.

Table 1. Summary of treatment conditions for feed ingredients that may reduce coronavirus concentration

Ingredient	Temperature	Moisture	Storage	Other
Corn	4-15 ºC for 4-6 weeks 94 ºC for < 2 hours	12-15% final moisture	varies greatly depending on prices	none
Dried distillers grains with solubles	slurry cooked at 82-90ºC then cooled to 33ºC	10% final moisture		pH adjusted to 5-6 40-60 hour fermentation
Soybean meal	55-60 ºC for < 30 min for drying 65-75 ºC for conditioning	< 13% in raw soybeans 12% in soybean meal	2-5 days after drying	exposure to hexane
Meat meal, Meat and bone meal, and Blood meal	115-145ºC for 40 to 90 min	final moisture 4-11%		none
Spray dried porcine plasma	chilled 4-10ºC for transport dried 170-310 ºC for 2-5 seconds final temperature of 80-84ºC	rapid decrease in water activity to a final value of 0.4-0.6		none
Vitamin Trace mineral premix	none	none	< 30 days	none
Complete feed	reaches 85 ºC during pelleting	reduced to 10-12% if pelleted 12-14% if mash		none

Chapter 2: Persistence of Porcine Coronaviruses in Feed and Feed Ingredients

Summary

Porcine Epidemic Diarrhea Virus (PEDV), Porcine Delta Corona Virus (PDCoV), and Transmissible Gastroenteritis Virus (TGEV) are major threats to herd health and profitability of commercial pork production operations and contaminated feed plays a role in virus transmission. The objective of our study was to characterize the inactivation kinetics of PEDV, PDCoV, and TGEV in various feed ingredient matrices. Samples of complete feed, spray dried porcine plasma, meat meal, meat and bone meal, blood meal, corn, soybean meal, and corn dried distillers grains with solubles (with 3 different oil concentrations) were weighed (5 g/sample) into scintillation vials and inoculated with 1 mL of PEDV, PDCoV, or TGEV. Samples were incubated at room temperature for up to 56 days and at each time point surviving virus was inoculated into cells to calculate a median tissue culture infective dose. Inactivation kinetics were determined using the Weibull model, which estimates a delta value indicating the time necessary to reduce virus concentration by 1 log. Delta values were compared among ingredients and to the analyzed ingredient nutrient composition. There was a trend for soybean meal to have the greatest delta value (7.50 days) for PEDV ($P < 0.06$) compared with all other ingredients. Likewise, the greatest ($P < 0.01$) delta values were also observed in soybean meal for PDCoV (42.04 days) and TGEV (42.00 days). There was a moderate correlation between moisture content and the delta value for PDCoV ($r = 0.49$, $P = 0.01$) and TGEV ($r = 0.41$,

P = 0.02). There was also a moderate negative correlation between TGEV survival and ether extract content (r = -0.51, P = 0.01). In conclusion, these results indicate that the first log reduction of PDCoV and TGEV takes the greatest amount of time in soybean meal. In addition, the content of moisture and crude fat appear to be an important determinant of corona virus survival in feed ingredients.
Keywords: inactivation kinetics, virus transmission, feed, ingredients, PDCoV, PEDV, TGEV

Introduction

Coronaviruses belong to the order *Nidovirales*, family *Coronavirdae*, and subfamily *Coronavirinae* (4), and are characterized by their large genome, helical nucleocapsids, and unique method of gene expression(1). The subfamily includes four genera: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and the more recently discovered *Deltacoronavirus* (37). Corona viruses are typically species specific and can infect a variety of birds and mammals (1). In humans, coronaviruses are typically responsible for upper respiratory infections and are the second leading cause of the common cold (1). In swine, there are 3 coronaviruses (Transmissible Gastroenteritis Virus, TGEV; Porcine Epidemic Diarrhea Virus, PEDV; Porcine Delta Corona Virus, PDCoV) that affect gastrointestinal health(2,5).

Transmissible Gastroenteritis Virus has been identified as a cause of severe diarrhea, dehydration, vomiting, and high mortality in neonatal pigs since the 1960's (110). The first case of PEDV occurred in the United States in 2013, that led to a

devastating outbreak with a high mortality in piglets observed in 17 different states (29). Shortly after the PEDV outbreak, cases of Porcine Delta Corona Virus (PDCoV) were identified in the United States in 2014 (37). The signs of disease in PDCoV infected pigs are similar but less severe than in pigs infected with PEDV and TGEV (7).

Until recent years, the main concern involving feed safety has been the risk of contamination with *Salmonella*. Several research studies have evaluated the survivability of *Salmonella* in feed and feed ingredients, along with the effect of processing on reducing its concentration in feed (87,88,91,92,95,97). During the initial PEDV outbreak, batches of feed containing spray-dried porcine plasma were found to be PCR positive for the virus, indicating that viruses may be transmitted through feed (62,63). Subsequent research confirmed that feed contaminated with PEDV was capable of causing an active infection in pigs after consuming it (42). With the high concentrations of PEDV excreted in feces of infected pigs, and the low infectious dose of PEDV, only 1 gram of infected feces is necessary to contaminate 500 tons of feed (104). Different heat treatments, such as spray drying, and some feed additives are capable of reducing PEDV concentration in feed and feed ingredients (89,102,103,111). The results from these studies suggest that virus contamination likely occurs post-processing in order to cause an active infection in pigs. If contamination does occur after processing, multiple feed ingredients may be at risk to post-processing contamination. This risk was confirmed by a risk assessment analysis was conducted by researchers at the University of Minnesota that showed the risk of PEDV surviving thermal processing of porcine by-products was negligible, but in some facilities, the risk of post-processing contamination was low to moderate (79).

With the potential risk of post-processing contamination, feed mills are developing biosecurity plans to minimize the risk of corona virus transmission from feed, feed ingredients, and feed transport. To do this, identifying ingredients that are of greater risk of prolonging the survival of these corona viruses is essential for developing these biosecurity procedures. Previous research studies have shown that PEDV survival in feed varies depending on the specific ingredient, with soybean meal appearing to promote extended survival time (99). However, there has been limited research published that has determined survival of TGEV and PDCoV in feed and feed ingredients (112,113). Therefore, the objective of this study was to characterize the survival of PEDV, TGEV, and PDCoV in complete feed and feed ingredients. We hypothesized that the 3 corona viruses would have a similar inactivation pattern in all the ingredients evaluated, and chemical composition may be a contributing factor to virus survival.

Materials and Methods

Virus Propagation

The National Veterinary Swine Laboratory (NSVL; Ames, IA) strain of PEDV was propagated in Vero-81 (African green monkey kidney, ATCC, CCL-81^{TM}) cells. The PDCoV (NVSL) and TGEV (Purdue University, West Lafayette, IN) strains were propagated in ST (swine testicular) cells. The cells were grown in Minimum Essential Medium (MEM) with Earle's salts supplemented with L-glutamine (Mediatech, Herndon, VA), 8% fetal bovine serum (FBS; Hyclone, South Logan, UT), 50 μg/mL gentamicin (Mediatech), 150 μg/mL neomycin sulfate (Sigma, St. Louis, MO), 1.5 μg/mL fungizone

(Sigma), and 455 µg/mL streptomycin (Sigma). The maintenance medium for PEDV included Dulbecco's Modified Eagle Medium (DMEM, Mediatech) with antibiotics, and 10 µg/mL of trypsin (Gibco, Life Technologies, Grand Island, NY). For PDCoV, the maintenance media were MEM with antibiotics and 5 µg/mL trypsin (Gibco). Maintence Medium for TGEV consisted of MEM with antibiotics and 4% donor horse serum (DHS, Hyclone). The cells were washed 3 times with phosphate buffered saline (PBS, pH 7.2) before virus inoculation. After virus inoculation, the cells were incubated at 37^0C for 1 h for virus adsorption using appropriate maintenance medium. Inoculated cells were incubated at 37°C under 5% CO_2 for up to 8 days. The inoculated cells were observed for the appearance of virus-induced cytopathic effects (CPE). The CPE appeared at about 8 to 10 days post-infection for PEDV, and 5 to 6 days post-infection for PDCoV and TGEV. The cells were subjected to 3 freeze-thaw cycles (-80°C/25°C) followed by centrifugation at 2500 × g for 15 min at 4 °C. The supernatant was collected and aliquoted into 50 mL centrifuge tubes (Corning, Life Sciences, NY) followed by storage at -80°C until used in each experiment.

Feed and feed ingredient samples and composition

The complete feed used in the experiment was obtained from the University of Minnesota College of Veterinary Medicine swine isolation barns, and was obtained from Vita-Plus (CGI enhanced phase II starter feed; Madison, Wisconsin). This diet did not contain any animal by-products (batch no. 831458). The feed ingredients including spray dried porcine plasma (SDPP), meat meal, meat and bone meal, blood meal, corn, soybean

meal, as well as low, medium and high oil corn dried distillers grains with solubles, were obtained from the University of Minnesota feed mill at the Southern Research and Outreach Center (Waseca, Minnesota). A sample of each feed and feed ingredient was sent to the Minnesota Valley Testing Laboratory (New Ulm, Minnesota) chemical composition analysis (Table 2). Standard AOAC procedures (2005) were used to determine moisture (method 930.15), ash (method 942.05), ether extract (method 2003.05), crude fiber (method 930.39), and crude protein (method 990.03) content (114). The pH of each feed or ingredient source was measured by mixing 50 mL of distilled water with 5g of sample. The mixture was then stirred with a magnetic stirrer for 20 min to allow the feed to be suspended in the liquid. The pH of the suspension was then measured using a pH probe (Fisher Scientific, Waltham, MA) and the value was recorded. The pH measurements were performed in triplicate, while the chemical composition values were determined from a single measurement.

Virus survival in feed and feed ingredients

All feed samples were tested negative for PEDV, PDCoV and TGEV using real time RT-PCR. Five gram aliquots of complete feed and feed ingredients (plasma, meat meal, meat and bone meal, blood meal, corn, soybean meal, as well as low, medium and high oil corn dried distillers grains with solubles) were prepared in scintillation vials in triplicate. One mL of each virus (PEDV, PDCoV and TGEV) with initial titers of 3.2×10^4, 1.5×10^6, 6.8×10^6, respectively, was added to the vials and mixed thoroughly. All samples were stored at room temperature (approximately 25°C) for 0, 1, 3, 7, 14, 21, 28,

35, 42, 49, and 56 days. The samples were stored uncovered and the humidity in the room was not controlled.

Virus elution

In all experiments, the surviving virus was recovered in an eluent consisting of a 3% solution of beef extract in 0.05M glycine. After each incubation period, 10 mL of eluent was added to each sample and mixed thoroughly. Following elution, the eluate was lightly centrifuged to remove organic matter/debris. The supernatants were serially diluted10-fold in maintenance medium to determine the amount of surviving virus, if any. For titration, supernatant dilutions were inoculated into Vero-81and ST monolayers in 96-well microtiter plates (Nunc, Rochester, NY) using 100µL/well. Three wells were used per dilution. Inoculated cells were incubated at 37°C under 5% CO_2 until the CPE appeared. Virus titers were calculated as $TCID_{50}$/mL using the Karber method (Karber, 1931). The highest dilution showing CPE was considered the end point.

Statistical analysis

Virus concentration data (log $TCID_{50}$/mL) was modeled by GInaFiT (115). Weibull distribution model was used to describe inactivation patterns because it provided a better fit of our data, which show non-linear rates of inactivation. Because the survival data of the viruses is more accurately matches a Weibull distribution, Mafart et al. (2001) developed the following Weibullian equation (116):

$$Log(N) = Log(N_o) - \left(\frac{t}{\delta}\right)^n \quad \text{Eq. 3}$$

In this equation, *N* is the surviving virus after the treatment expressed as (log $TCID_{50}$/mL), N_0 is the initial virus titer (log $TCID_{50}$/mL), Delta (δ) is the time of the first logarithm decline for the virus titer population (days), and *n* is the shape parameter.

Three valid replicates were used to evaluate how well the model fit with the experimental data by calculating the adjusted R^2 (Adj. R^2) as follows:

$$Adj.R^2 = \left[1 - \frac{(m-1)(1-\frac{SSQ\ regression}{SSQ\ total}}{m-j}\right] \quad \text{Eq. 4}$$

where *m* is the number of observations, *j* is the number of model parameters, and *SSQ* is the sum of squares.

An ANOVA test using the mixed procedure of SAS (SAS Inst., Cary, NC) was used to compare differences among delta values. Least squared means with Tukey adjustment were used to determine differences among treatment means, with $P < 0.05$ considered to be significantly different. The fixed effect was the feed ingredient being analyzed, and the results for each virus were analyzed independently. A correlation analysis was also conducted using the CORR procedure of SAS (SAS Inst., Cary, NC) to determine potential associations between feed and feed ingredient chemical composition and the delta values for complete feed or each ingredient.

Results

The day 0 concentration of PEDV ranged from 2.06 to 4.28 log $TCID_{50}$/mL. At the conclusion of the 56-day incubation period, the titers for all samples were 0.50 log

$TCID_{50}$/mL, except for soybean meal, which was greater at 0.83 log $TCID_{50}$/mL (Table 3). When comparing the overall log reduction in virus concentration during the storage period, the greatest reduction was observed in complete feed (3.78 log reduction) and high oil DDGS (3.44 log reduction). The ingredient with the least log reduction was SDPP (1.56) and medium oil DDGS (2.01). For PDCoV, day 0 titers were greater than those for PEDV, and ranged from 3.72 to 6.61 log $TCID_{50}$/mL (Table 4). After the 56-day storage period, the greatest log reduction for PDCoV was observed in low oil DDGS (5.78) and high oil DDGS (5.45), and greatest virus survival occurred in soybean meal, with only 1.44 log reduction. A similar trend was observed for TGEV survival, where samples inoculated with TGEV had the greatest initial virus titers ranging from 3.51 to 7.17 log $TCID_{50}$/mL (Table 5). After the 56-day storage, the greatest log reduction was achieved in high oil DDGS (6.56) and medium oil DDGS (5.67) samples. Similar to PDCoV, the least log reduction was observed in soybean meal, with only 2.22 log reduction in the initial virus concentration.

In general, all virus inactivation data over time were non-linear, with tails and shoulders in the curves. Because of this reason, the Weibull model was generally a better representation of the data, resulting in greater Adj. R^2 values compared with those obtained with the log linear model. Delta values from the Weibull model (time necessary to reduce virus concentration in 1 log) were compared among the feed and feed ingredient samples for PEDV, PDCoV and TGEV to characterize the virus inactivation kinetics (Table 6). There were no differences in delta values for PEDV among the different feed or ingredient samples. Delta values were greater for PDCoV in soybean

meal (42.04 days) and corn (25.60 days) samples compared with the other ingredients, indicating lower inactivation kinetics. A similar trend was observed for TGEV, where soybean meal had a greater delta value (41.94 days) compared that observed for the other ingredients.

A Pearson correlation was performed to examine the associations between the ingredient chemical composition and kinetics of virus survival. There was a moderate positive correlation for TGEV ($r = 0.41$, $P = 0.02$) and PDCoV ($r = 0.49$, $P = 0.01$) survival with moisture content. This suggests that increased moisture content may lead to increased virus survival, but this correlation was not observed for PEDV (Table 7). Furthermore, there was a moderate negative correlation between TGEV survival and ether extract content ($r = -0.51$, $P = 0.01$), suggesting increased ether extract content may reduce virus survival time, but this correlation was not observed for PEDV or PDCoV.

Discussion

Previous research demonstrated that the survival of PEDV varies between different feed ingredients, and it appears to survive for the longest time in soybean meal (99). No studies have been conducted to evaluate PDCoV and TGEV survival among various types of feed ingredients. The hypothesis for our experiment was that virus survival would be associated with the chemical composition of each ingredient. One of these components is the pH of the feed ingredients. Porcine epidemic diarrhea virus is stable at a pH between 6.5 to 7.5 (117), and results from a previous experiment showed that the virus is more sensitive to heat treatments at a greater pH (7.2 vs. 10.2) (118). These

findings suggest that virus survival in ingredients that have a pH outside of this favorable range may be lower. When comparing the moisture content of chemical composition, previous experiments have demonstrated that PEDV survives up to 28 days in wet complete feed, but only 7 days in dry complete feed (119). The TGEV was used as a control in this experiment and demonstrated the same behavior, with higher survival in wet feed than dry feed (119). These data lead to the hypothesis that higher moisture ingredients will also have a higher survival of PEDV, PDCoV, and TGEV.

In our experiment, a variation in virus survival was observed among ingredient in all three viruses right away at day 0. The titer of the virus inoculated into the sample was identical for all ingredients with an average titer of 3.2×10^4 for PEDV, 1.5×10^6 for PDCoV, and 6.8×10^6 for TGEV. Though the initial inoculum had identical titers, there is an immediate difference observed in the virus concentration among ingredients in all 3 viruses. For example, in the PEDV experiment, the virus titer dropped to 2.06 log $TCID_{50}$/mL in SDPP from the initial 4 logs of virus that was inoculated into the sample. This indicates that some ingredients had an immediate effect on virus survival that caused a decrease before the incubation period even started. This immediate variation in virus survival was also observed in another experiment measuring the survival of PEDV in various feed ingredients, with CT values ranging from 16 to 36 on the first day of incubation despite being inoculated with the same virus inoculum of a CT value of 16.3 (99).

In addition to comparing initial virus concentration, there is also a variation in overall log reduction among feed ingredients for each of the three viruses. Previous

research suggests that a dose of 5.6 x 10^1 $TCID_{50}$/mL, or 1.7 log $TCID_{50}$/mL, of PEDV in a feed matrix is sufficient to cause an active infection in young pigs (104). Using our starting virus titer of 4.5 log $TCID_{50}$/mL, this threshold of 1.7 log $TCID_{50}$/mL was reached at 3 days in all of the ingredients except meat and bone meal, blood meal, soybean meal, and corn. At 14 days, the virus concentration was below this limit in all of the ingredients except soybean meal. It wasn't until 21 days until the virus concentration in soybean meal dropped below the proposed minimum infectious dose 1.7 log $TCID_{50}$/mL threshold. An infectious dose for PDCoV and TGEV has not yet been determined in a feed matrix, but soybean meal also had the highest survival for both viruses with a virus concentration of 5.17 log $TCID_{50}$/mL of PDCoV and 4.95 log $TCID_{50}$/mL of TGEV even after 56 days of incubation.

When comparing the inactivation kinetics of each virus, there is a similar trend for soybean meal to have the greatest virus survival. With such a high survival of each virus in soybean meal, a correlation was done to determine what feed characteristic might cause this a high survival. As stated in our hypothesis, moisture was one of the variables believed to have an impact on virus survival. This was somewhat confirmed in the correlation which determined a moderate positive relationship between PDCoV ($r = 0.4823$, $P < 0.05$) and TGEV ($r = 0.4128$, $P < 0.05$). This means that as predicted, as moisture content increases, so does virus survival. The same relationship, however, was not observed in PEDV survival. Contradicting our hypothesis, there was no correlation between pH and virus survival. This is potentially because our experiment was done in dry feed ingredients, and pH is related to the activity of hydrogen in an aqueous solution.

Other experiments evaluating the impact of pH on virus survival that have shown an effect were performed in liquid samples (118). In addition to moisture, there was also a significant negative correlation between ether content and TGEV survival (-0.5067, $P<0.05$). This correlation was unexpected and only observed in TGEV survival.

One of the major limitations to this experiment was the addition of liquid media necessary to inoculate the feed samples with virus. 1mL of liquid media was added to 5 grams of feed or ingredient, greatly increasing the moisture content of the sample. As indicated by our results, moisture plays a significant role in virus survival and so changing the moisture content will alter the survival that would be observed in completely dry feed. Though the moisture content was altered, the same amount of moisture was added to each feed ingredient making it possible to still compare the survival among the ingredients. A second limitation to our experiment was the low virus titers used in the PEDV experiment. These low titers limited the amount of reduction that could occur in virus concentration, which then limited the variation we could observe among feed ingredients. The second problem with the low virus titers is that they may not represent the virus titers observed in the field. A study measuring the residual amount of PEDV in a contaminated feed bin estimated these CT values between 19.5 and 22.2 (42). This amount of virus is equivalent to 8.9 to 9.2 log copies of RNA/g based on a calibration curve obtained from the University of Minnesota (120). With our low titers of virus, it was impossible for us to show an 8 to 9 log reduction in virus concentration that would be necessary to inactivate PEDV in a realistic scenario.

Conclusion

Overall, it can be concluded that the survival of PEDV, PDCoV, and TGEV varies based on feed ingredient. Out of the feed ingredients analyzed, soybean meal had greatest delta value in all three viruses, along with the highest concentration of remaining virus after 56 days of incubation. These results suggest soybean meal may be a risk factor for virus transmission and should be considered when developing mitigation strategies for virus transmission.

Table 2 Chemical composition of feed ingredients

Ingredient[1]	Moisture (%)	Ash (%)	Ether extract (%)	Crude fiber (%)	Crude Protein[2] (%)	pH[3]
CF	8.57	9.45	4.47	2.02	24.20	5.82
SBM	12.12	6.42	0.71	3.26	45.40	6.73
C	14.90	1.55	3.86	1.55	7.03	6.21
LDDGS	10.40	30.70	5.87	5.77	4.56	4.31
MDDGS	10.38	29.91	9.85	5.16	4.03	3.81
HDDGS	9.66	28.57	14.23	5.41	4.62	4.17
SDPP	11.60	7.44	0.15	< 0.01	77.79	7.15
BM	11.58	1.79	0.16	0.05	92.60	8.40
MM	4.80	24.26	13.54	1.83	54.90	6.64
MBM	5.74	24.77	10.77	1.16	55.70	6.50

[1]CF: complete feed, SDPP: spray dried porcine plasma, MM: meat meal, MBM: meat and bone meal, BM: blood meal, SBM: soybean meal, C: corn, LDDGS: low oil dried distillers grains with solubles, MDDGS: medium oil dried distillers grains with solubles, HDDGS: high oil dried distillers grains with solubles
[2]Crude protein is calculated from nitrogen content × 6.25
[3]Average of 3 replicates

Table 3 Porcine Epidemic Diarrhea Virus (PEDV) titer during the 56 day incubation period in feed ingredients

	PEDV Virus titer (log $TCID_{50}$/mL) in feed ingredients[1]									
Time (days)	CF	SDPP	MM	MBM	BM	SBM	C	LDDGS	MDDGS	HDDGS
0	4.28	2.06	2.83	3.06	3.84	3.50	2.84	3.51	2.51	3.94
1	2.51	1.51	2.17	2.28	2.95	3.84	2.51	1.73	1.51	2.18
3	1.51	0.50	1.17	1.94	2.39	3.28	2.40	1.62	1.28	1.62
7	1.40	0.50	1.51	1.72	1.61	2.83	1.51	1.72	1.51	1.51
14	0.61	1.06	1.50	1.51	1.51	2.06	0.50	1.51	1.51	1.51
21	0.50	0.83	1.62	1.51	1.51	1.62	0.50	1.40	1.17	1.51
28	1.17	0.50	1.51	1.51	1.51	1.51	0.50	0.61	0.72	1.51
35	0.50	0.50	1.51	1.51	1.51	1.40	0.50	0.50	0.50	1.51
42	0.50	0.50	1.51	1.51	0.50	0.50	0.50	0.50	0.50	1.51
49	0.50	0.50	0.50	0.50	0.50	0.72	0.50	0.50	0.50	1.51
56	0.50	0.50	0.50	0.50	0.50	0.83	0.50	0.50	0.50	0.50
Log Reduction (TCID50/mL)										
7	2.89	1.56	1.32	1.33	2.23	0.67	1.33	1.79	1.00	2.43
14	3.67	1.01	1.33	1.55	2.33	1.45	2.34	2.00	1.00	2.43
28	3.11	1.56	1.32	1.55	2.33	1.99	2.34	2.90	1.79	2.43
42	3.78	1.56	1.32	1.55	3.34	3.00	2.34	3.01	2.01	2.43
56	3.78	1.56	2.33	2.56	3.34	2.67	2.34	3.01	2.01	3.44

[1] CF: complete feed, SDPP: spray dried porcine plasma, MM: meat meal, MBM: meat and bone meal, BM: blood meal, SBM: soybean meal, C: corn, LDDGS: low oil dried distillers grains with solubles, MDDGS: medium oil dried distillers grains with solubles, HDDGS: high oil dried distillers grains with solubles

Table 4 Porcine Delta Coronavirus (PDCoV) titer during 56 day incubation in feed ingredients

	PDCoV Virus Titer (Log TCID50/mL) in feed ingredients[1]									
Time (days)	CF	SDPP	MM	MBM	BM	SBM	C	LDDGS	MDDGS	HDDGS
0	5.51	5.62	5.51	5.72	5.51	6.61	3.72	6.28	5.51	5.95
1	5.28	5.06	5.17	6.17	5.28	4.51	4.73	6.06	5.28	5.61
3	4.40	3.73	4.61	4.29	4.40	5.17	3.51	4.51	4.40	4.73
7	3.17	3.95	3.61	4.95	3.17	5.50	3.61	5.39	3.17	5.28
14	2.73	2.51	2.40	4.06	2.73	5.40	4.40	3.84	2.73	3.73
21	2.51	2.51	3.17	3.51	2.51	5.40	3.51	3.95	2.51	3.84
28	2.51	2.51	3.51	3.51	2.51	5.29	2.84	2.51	2.51	2.51
35	2.62	2.51	2.84	2.61	2.62	5.51	2.40	2.40	2.62	3.29
42	1.28	0.95	2.17	2.51	1.28	5.06	1.51	0.51	1.28	1.06
49	0.50	0.51	1.95	1.95	0.50	5.40	0.51	0.51	0.50	0.51
56	0.50	0.51	1.51	1.51	0.50	5.17	0.51	0.51	0.50	0.51
Log Reduction (TCID50/mL)										
7	2.34	1.67	1.89	0.77	2.34	1.11	0.11	0.89	2.34	0.67
14	2.78	3.11	3.11	1.67	2.78	1.22	0.00	2.44	2.78	2.22
28	3.00	3.11	2.00	2.22	3.00	1.33	0.89	3.78	3.00	3.45
42	4.23	4.67	3.33	3.22	4.23	1.55	2.22	5.78	4.23	4.89
56	5.01	5.11	4.00	4.22	5.01	1.44	3.22	5.78	5.01	5.45

[1] CF: complete feed, SDPP: spray dried porcine plasma, MM: meat meal, MBM: meat and bone meal, BM: blood meal, SBM: soybean meal, C: corn, LDDGS: low oil dried distillers grains with solubles, MDDGS: medium oil dried distillers grains with solubles, HDDGS: high oil dried distillers grains with solubles

Table 5 Transmissible Gastroenteritis virus (TGEV) titer during 56 day incubation in feed ingredients

	TGEV Virus titer (Log TCID50/mL) in feed ingredients[1]									
Time (days)	CF	SDPP	MM	MBM	BM	SBM	C	LDDGS	MDDGS	HDDGS
0	5.61	3.51	6.06	5.28	5.83	7.17	4.51	6.62	6.17	7.06
1	4.50	3.95	4.62	3.51	4.95	4.61	3.17	4.06	3.84	4.06
3	3.40	3.51	3.17	3.29	4.06	5.40	2.06	3.84	3.06	3.06
7	2.72	3.40	2.51	2.51	3.06	4.95	2.51	2.95	2.61	2.51
14	2.51	3.51	2.51	2.40	2.29	4.51	3.61	2.51	2.40	2.51
21	2.51	2.73	1.51	1.51	1.40	5.51	2.18	1.61	2.51	2.40
28	2.51	2.51	1.51	1.51	1.51	4.73	2.29	2.28	2.17	2.51
35	2.51	1.51	1.28	1.51	1.51	5.29	1.95	2.06	1.51	1.84
42	2.51	0.51	0.95	0.61	1.51	4.50	1.61	0.51	0.51	1.72
49	1.06	0.51	0.51	0.51	0.51	5.17	1.51	0.51	0.51	0.51
56	0.51	0.51	0.51	0.51	0.51	4.95	0.51	0.51	0.51	0.51
Log Reduction (TCID50/mL)										
7	2.89	0.11	3.56	2.78	2.77	2.22	2.00	3.67	3.56	4.56
14	3.11	0.00	3.56	2.88	3.55	2.67	0.89	4.11	3.78	4.56
28	3.11	1.00	4.56	3.78	4.33	2.44	2.22	4.34	4.00	4.56
42	3.11	3.00	5.11	4.67	4.33	2.67	2.89	6.11	5.67	5.34
56	5.11	3.00	5.56	4.78	5.33	2.22	4.00	6.11	5.67	6.56

[1] CF: complete feed, SDPP: spray dried porcine plasma, MM: meat meal, MBM: meat and bone meal, BM: blood meal, SBM: soybean meal, C: corn, LDDGS: low oil dried distillers grains with solubles, MDDGS: medium oil dried distillers grains with solubles, HDDGS: high oil dried distillers grains with solubles

Table 6 Comparison between delta values in feed ingredients for Porcine Epidemic Diarrhea Virus (PEDV), Porcine Delta Coronavirus (PDCoV), and Transmissible Gastroenteritis Virus (TGEV)

Ingredient[1]	PEDV		PDCoV		TGEV	
	Delta (days)	Adj. R^2	Delta (days)	Adj. R^2	Delta (days)	Adj. R^2
CF	1.1 ± 0.8	0.76	2.3 ± 0.6[a]	0.91	3.2 ± 3.0[a]	0.79
SDPP	1.1 ± 1.3	0.72	3.3 ± 2.3[a]	0.89	19.2 ± 4.3[a]	0.90
MM	3.9 ± 2.2	0.57	2.8 ± 1.4[a]	0.79	1.0 ± 1.7[a]	0.94
MBM	4.9 ± 3.9	0.80	6.2 ± 0.9[a]	0.89	0.9 ± 0.9[a]	0.95
BM	2.8 ± 0.7	0.87	1.2 ± 1.3[a]	0.69	2.1 ± 0.9[a]	0.91
C	2.3 ± 1.6	0.83	25.6 ± 1.4[b]	0.88	11.8 ± 16.8[a]	0.59
SBM	7.5 ± 4.6	0.90	42.0 ± 14.0[c]	0.50	41.9 ± 19.8[b]	0.41
LDDGS	0.7 ± 1.1	0.88	6.2 ± 2.2[a]	0.92	1.0 ± 1.4[a]	0.88
MDDGS	7.3 ± 6.0	0.77	3.8 ± 2.4[a]	0.93	1.7 ± 1.2[a]	0.85
HDDGS	0.6 ± 0.5	0.72	8.8 ± 4.4[a]	0.90	0.8 ± 0.9[a]	0.81
SEM	1.70		2.82		4.24	
P value	0.06		< 0.01		< 0.01	

[1] CF: complete feed, SDPP: spray dried porcine plasma, MM: meat meal, MBM: meat and bone meal, BM: blood meal, SBM: soybean meal, C: corn, LDDGS: low oil dried distillers grains with solubles, MDDGS: medium oil dried distillers grains with solubles, HDDGS: high oil dried distillers grains with solubles

Table 7 Pearsons correlation coefficients (r), among feed ingredient composition and time necessary for first log reduction in transmissible gastroenteritis virus (TGEV), Porcine Epidemic Diarrhea Virus (PEDV), Porcine Delta Coronavirus (PDCoV) concentration

	Virus		
Item	TGEV	PEDV	PDCoV
Moisture			
r	0.41	-0.05	0.48
P-Value	0.03	0.81	0.01
Ether extract			
r	-0.51	-0.01	-0.31
P-Value	0.01	0.99	0.10
Crude Protein			
r	0.07	0.08	-0.31
P-Value	0.71	0.69	0.10
Ash			
r	-0.19	0.14	-0.23
P-Value	0.34	0.46	0.22
Fiber			
r	-0.17	0.02	0.11
P-Value	0.38	0.90	0.58

Chapter 3: Survival of Porcine Epidemic Diarrhea Virus (PEDV) in swine feed ingredients after thermal processing

Summary

Infection with Porcine Epidemic Diarrhea Virus (PEDV) causes vomiting, diarrhea, and dehydration in pigs. The virus made its first appearance in the U.S. in 2013, where it caused substantial neonatal mortality and economic losses in the U.S. pork industry. Based on outbreak investigations, it is hypothesized that the virus could be transmitted through contaminated feed and feed ingredients. This potential risk created a demand for research on the inactivation kinetics of PEDV in various feed ingredients. The objective of this study was to evaluate the survival of PEDV in 9 different feed ingredients when exposed to 60, 70, 80, and 90°C. Overall, there were no differences ($P > 0.05$) in virus survival among the different feed matrices studied when thermally processed at 60 to 90°C for up to 30 min. However, the time necessary to achieve the first log reduction in virus concentration occurred was less ($P < 0.05$) when ingredients were exposed to temperatures from 70 to 90°C compared with 60°C. The maximum inactivation level (3.9 log) was achieved when heating all ingredients to 90°C for 30 min. About a 3 log reduction was achieved by thermal exposure at 90°C for 10 min, at 80°C for 15 min, or 70°C for 30 min. The results of this study showed that PEDV survival among the 9 feed ingredients tested was not different when exposed to thermal treatments for up to 30 min. However, different combinations of temperature and time resulted in achieving a 3 to 4 log reduction of PEDV in all feed ingredients evaluated.

Introduction

Upon infection with Porcine Epidemic Diarrhea Virus (PEDV), pigs experience vomiting, diarrhea, and dehydration leading to high mortality in suckling pigs (121). The virus is excreted in large amounts in the feces of infected pigs, making it highly contagious and difficult to control (27). After the virus was identified in Belgium in 1978, it slowly spread to multiple countries including Canada, Korea, and China (28). In the United States, the virus was first detected in May of 2013, and the mode of introduction has not yet been confirmed, although contaminated feed has been suspected (62).

Recent research on PEDV survival in feed ingredients has shown that it appears to survive longer in soybean meal (over 180 days) compared with other commonly used feed ingredients (99). These authors also showed that PEDV can survive up to 30 days in blood meal, corn dried distillers grains with solubles, meat and bone meal, red blood cells, lysine HCl, L methionine, choice white grease, choline chloride, and complete feed. However, these researchers determined virus survival when samples were stored at low, uncontrolled temperatures (varying between -15 to 20°C) and did not investigate the impact of any thermal processing treatment. Results from other studies suggest that specific thermal processing treatments, such as spray drying (a process using dry hot air to reduce the moisture of a particle) can reduce the survival of PEDV in porcine and bovine plasma by 5 log (102,103). However, the impact of thermal processing on the inactivation kinetics of PEDV in feed is still unknown. The objective of this study was to determine the effect of thermal treatment on the survival of PEDV in nine commonly

used feed ingredients. We hypothesized that different chemical characteristics of the feed ingredients would show differences in virus survivability when subjected to thermal treatment.

Materials and Methods

Virus propagation

The NVSL strain of PEDV was grown in Vero-81 cells, which were grown in Dulbecco's Modified Eagle Medium (DMEM), (Mediatech, Herndon, VA), 8% fetal bovine serum (FBS; Hyclone, South Logan, UT), 50 μg/mL gentamicin (Mediatech, Herndon, VA), 150 μg/mL neomycin sulfate (Sigma, St. Louis, MO), 1.5 μg/mL fungizone (Sigma, St. Louis, MO), and 455 μg/mL streptomycin (Sigma, St. Louis, MO). Before inoculation, the cells were washed 3 times with phosphate buffered saline (pH 7.2). After inoculation, the cells were incubated at 37°C allowing virus absorption using maintenance medium (DMEM, antibiotics, and 10.0 μg/mL trypsin; Gibco, Life Technologies, Grand Island, NY). After 1 hour, new media were added to the flask and the cells were placed in an incubator at 37°C under 5% CO_2. The cells were examined daily for the appearance of cytopathic effects (CPE), usually appearing 4 to 5 days post-infection. After CPE was observed, the cells underwent 3 freeze-thaw cycles (-80°C to 25°C) and were then centrifugation at 2500 x g for 15 min at 4°C. After centrifugation, the supernatant was collected, aliquoted in small amounts, and stored at -80°C until used.

Feed ingredients composition

Feed ingredients (soybean meal, grow-finish vitamin and trace mineral premix, spray dried porcine plasma, meat meal, meat and bone meal, blood meal, corn, and corn distillers dried grains with solubles) were obtained from the feed mill at the Southern Research and Outreach Center of the University of Minnesota (Waseca, MN). The sample of complete feed evaluated was a phase II starter diet that did not contain any animal by-products (Vita-Plus CGI, enhanced NP-NT, batch no. 831458). All feed and feed ingredients were tested for PEDV by real time RT-PCR. Samples were sent to Minnesota Valley Testing Laboratory (New Ulm, MN) to analyze the nutrient composition of each ingredient (Table 8). Standard procedures established by AOAC International were used to measure moisture content (method 930.15), ash content (method 942.05), ether extract (method 2003.05), crude fiber (method 930.39), and crude protein (method 990.03) (114). The proximate analysis values were obtained from a single sample. The pH was measured by mixing 50 mL of distilled water with 5 g of each feed ingredient, premix, and complete feed. The mixture was then stirred with a magnetic stirrer for 20 min. The pH of the suspended feed was measured using a pH probe (Fisher Scientific, Waltham, MA) and recorded. All of the composition values for the ingredients were determined from one replicate. The pH samples were measured in triplicate.

Virus survival after thermal processing

Five gram aliquots of each ingredient were weighed into plastic scintillation vials (Fisher Scientific, Pittsburgh, PA) and placed into sealed, airtight, and water proof

containers. The containers were then placed in a water bath at 60, 70, 80 and 90°C for 1 hour to reach water bath temperature. Once the samples reached the desired temperature, they were removed and 1 mL of PEDV (passage 19, titer $3.2x10^4$ $TCID_{50}$/mL) was added to the samples. During this time, the samples were out of the water bath for about 5 min to complete the inoculation procedure. The inoculated samples were then immediately placed back into the water bath for 0, 5, 10, 15, or 30 min.

Virus elution

To elute the surviving virus from the samples of feed and feed ingredients, an eluent solution, 3% beef extract (Lab Scientific, Highlands, NJ) -0.05M glycine (Sigma), pH 7.2 was used. After various time points, this solution was added to the sample aliquot and mixed well. After light centrifugation to remove organic debris, the supernatant was collected. To determine the concentration of surviving virus, a titration was performed by preparing serial 10-fold dilutions of the supernatant in maintenance medium. These dilutions were inoculated into monolayers of Vero-81 cells grown in 96 microtiter well plates (Nunc, Rochester, NY) at 100µL/well using three wells per dilution. The inoculated cells were incubated at 37°C under 5% CO_2 for 4 to 5 days and observed for CPE. The virus titer was then calculated as 50% tissue culture infective dose ($TCID_{50}$/mL) (122). The virus titers of the supernatants were compared to those of starting virus titer to determine the amount of virus inactivation.

Statistical analyses

Inactivation kinetics data on virus survival were analyzed using the Weibull model (123). The fitting of the model to the experimental data was performed by using the GINAFiT add-in software on Microsoft excel (115). Assuming that the temperature resistance for PEDV follows a Weibull distribution, an equation was used to predict the log concentration of surviving virus after the thermal treatment (Equation 1):

$$Log(N) = Log(N_0) - (^{t}/_{\delta})^{n} \quad \text{Eq. 1}$$

In equation 1, N is the surviving virus expressed as $TCID_{50}$/mL, N_0 is the initial virus titer at the start of the experiment, t is time (min), δ is the time of the first log reduction of virus concentration (min), and n is the shape parameter. The shape parameter (n) indicates the shape of the curve with a value $n > 1$ representing the formation of a shoulder-shaped curve and being convex, $n < 1$ representing the formation of a tail-shaped curve and being concave, and $n = 1$ being linear. The adjusted R^2 value (Adj. R^2) was used to evaluate how well the model fit the experimental data.

The delta values obtained from the Weibull model indicated the amount of time necessary to reduce the virus concentration by 1 log. The delta values were compared across treatments. An ANOVA statistical analysis using the PROC-MIXED procedure of SAS was performed to determine statistically significant differences between feed ingredients and between temperature treatments. Least squared means with a Tukey adjustment were used to determine differences between each treatment if $P < 0.05$. The experimental unit was a single vial.

Results

There were no differences ($P > 0.05$) observed in the survival of PEDV after the thermal treatment among all of the 9 feed materials evaluated (Table 9). This observation was consistent at each of the 4 temperatures evaluated, indicating that the virus resistance to thermal treatment was not affected by the different chemical composition of the feed matrices. There were differences ($P < 0.05$) between the delta values at 60°C compared with those from 70 to 90°C (Table 10). Delta values at 70 to 90°C were lower than 60°C, indicating higher virus inactivation kinetics. The shape parameter of the virus inactivation curves was similar among all of the treatments (0.45-0.60), indicating that at each temperature, curves were concave and formed tails to a similar extent. This behavior corresponds to a rapid decrease of virus concentration after short treatment times followed by a plateau where the virus survives for a long period.

When comparing the log reduction achieved after 10, 15, or 30 min, no differences ($P > 0.05$) were observed between 60 and 70°C, but greater reductions ($P < 0.05$) were achieved at 80 and 90°C. A reduction of 1.9 to 2.0 log was achieved at 60°C for 15 min or 70°C for 10 min, and a 2.2 to 2.4 log reduction occurred after treatment at 60°C for 30 min, 70°C for 15 min, or 80°C for 10 min. Greater than a 3 log reduction was observed when applying 80°C for 30 min (3.4 log), or 90°C for 15 min (3.3 log). The maximum log reduction (3.9 log) was achieved at 90°C for 30 min.

Discussion

Recent epidemiological investigations have shown that feed contaminated with PEDV is capable of infecting pigs (42). Therefore, it is important to develop mitigation strategies to reduce the risk of virus transmission to swine farms through contaminated feed. Previous research has suggested that contaminated feed ingredients can be a risk factor for PEDV transmission among swine farms. For example, spray dried porcine plasma (SDPP) has been of particular concern after the first PEDV outbreak on a Canadian farm, which was found to have originated from an infected lot (63). However, after further investigation, the contaminated SDPP was found to not be capable of producing an active infection in piglets when fed, which was likely due to a dilution affect or extended periods between sample collection and bioassay resulting in partial or complete virus inactivation (63). In a similar experiment, feeding a diet that contained 5% SDPP that tested positive for PEDV RNA was also unable to cause an infection in piglets (124). Results from both of these experiments suggest that PEDV contamination of plasma was likely a post-spray drying event and that the conditions present in the spray drying process may be sufficient to reduce virus concentration if it exists. This hypothesis was confirmed when porcine plasma was experimentally inoculated with PEDV, spray dried, and fed to piglets, resulting in no infection even though PEDV RNA was present in the sample (103). When thermal treatment was evaluated at higher temperatures, heating complete feed at 120°C for 25 min resulted in a 3 log reduction in PEDV (89). However, the latter study was performed using complete feed, and there has been limited information about PEDV inactivation among other feed ingredients

commonly used by the feed industry. Therefore, first step to understand the role of feed in PED virus transmission is to understand how the virus behaves in feed matrices with different chemical composition, and determine if any differences in virus survival among feed ingredients requires different thermal processing conditions.

The hypothesis for this study was that PEDV survives differently depending on the feed or feed ingredient chemical composition, and that some ingredients may require higher processing temperatures to achieve an adequate virus inactivation. Neutral pH values in soybean meal (6.73) and spray dried porcine plasma (7.15) were theorized to provide a more favorable environment for virus survival. The interaction between pH, time, and temperature has been previously investigated by testing PEDV survival in different media (118). Results from this study showed heat treatment at 48°C and a pH of 10.2 for 4.6 min was the most effective method in reducing virus survival in spray dried porcine plasma (118). Furthermore, as the pH became more basic, the virus was more sensitive to the heat treatment being applied (15).

Our study evaluated feed ingredients, premix, and complete feed with different chemical composition and pH values, however, no differences were observed among the virus inactivation kinetics (delta values). This result may suggest that under the conditions evaluated in this study (high temperatures and long exposure times), rapid virus inactivation may occur independently of chemical composition of ingredients, and thus, similar processing conditions can be applied to all ingredients to achieve a similar reduction in virus. Our results were unexpected because of the dramatic differences in the pH values of the feed matrices evaluated (3.49 to 8.40). Quist-Rybachuk et al. (2015)

found that PEDV was more heat sensitive when the pH increased from 7.2 to 10.2 (15). The lack of differences in PEDV inactivation among ingredients despite the pH differences may also be due to the use of dry ingredients instead of liquid media. Because pH is only a characteristic of solutions, the impact of the pH on virus survival in a dry ingredient with a small amount of liquid (1 mL) is likely to be minimal.

Although there were no differences on virus survival between the feed materials evaluated between 60 and 70°C, higher inactivation was achieved at 80 and 90°C. In order to optimize the thermal processing conditions (high temperature and short time) to inactivate PEDV, our data suggest that a combination of 80°C for 15 min was suitable to achieve a 3-log reduction. This could also be achieved by thermal processing at 90°C for 10 min or heating at 70°C for 30 min. These findings and parameters agreed with those of Hoffman and Wyler (1989) who found that PEDV was relatively stable at 50°C, but at temperatures higher than 60°C, the virus lost total infectivity within 30 min (36). These results were also comparable to the survival of PEDV on the metal surface of hog transport trailers, where heating at 71°C for 10 min was capable of reducing virus titer low enough to not cause infection in any of the 4 inoculated pigs, but the log reduction of PEDV was not measured in this experiment (100).

One of the limitations for applying this knowledge into practice is the addition of 1 mL of media containing the virus into the feed sample. The addition of liquid media necessarily increases the moisture content of the sample, and this may affect the virus survival. More research is necessary to compare the effect of moisture content and water

activity on PEDV survival to determine the extent that this factor plays in virus inactivation.

The initial virus titer for each experiment was limited to a maximum of 4.8 log $TCID_{50}$/mL. Typically, a higher virus titer is desirable to achieve a 5 log reduction after a treatment. It is highly likely that the amount of virus excreted by an infected pig and potentially transmitted via feed would be much greater than the titer used in the present study. In a study that evaluated residual material in a suspected PEDV contaminated feed bin, CT values between 19.5 and 22.2 were determined (42). When using a calibration curve obtained from the University of Minnesota and published by Alonso et al. (2014), this amount of virus is equivalent to 8.9 to 9.2 log copies of RNA/g (120). In this potential scenario, the maximum log reduction (3.9 log) achieved by thermal processing only, would not be enough to completely inactivate the virus found in the feces of infected animals and have the potential to be transmitted via feed during an outbreak. If this scenario represents the reality, a new approach is needed. Multiple processing steps may need to be combined to achieve the desired virus reduction. The use of eBeam irradiation, antimicrobials, and organic acids has been effective in reducing PEDV concentration in feed (14). If these treatments are combined with a thermal processing as described in this study, an overall increase on virus inactivation is expected.

Conclusion

Feed, premix, and feed ingredients are potential biosecurity risk factors in the wide spread of PEDV in the U.S. and possibly around the world. The results of this study

indicate that there are no differences in virus survival among complete feed, premix, and ingredients with different chemical composition when thermally treated, suggesting that similar processing conditions will be effective to inactivate PEDV across types of feed materials. A maximum of 4-log reduction was achieved when applying 90°C for 30 min.

Table 8 Chemical composition of feed ingredients

Ingredient[1]	Moisture (%)	Ash (%)	Ether extract (%)	Crude fiber (%)	Crude Protein[2] (%)	pH[3]
CF	8.57	9.45	4.47	2.02	24.20	5.82
SBM	12.12	6.42	0.71	3.26	45.40	6.73
C	14.90	1.55	3.86	1.55	7.03	6.21
DDGS	10.31	4.56	5.86	6.50	30.10	4.39
PM	2.41	73.77	1.42	1.62	1.91	3.49
SDPP	11.60	7.44	0.15	<0.01	77.79	7.15
BM	11.58	1.79	0.16	0.05	92.60	8.40
MM	4.80	24.26	13.54	1.83	54.90	6.64
MBM	5.74	24.77	10.77	1.16	55.70	6.50

[1] CF: complete feed, SDPP: spray dried porcine plasma, MM: meat meal, MBM: meat and bone meal, BM: blood meal, SBM: soybean meal, C: corn, PM: vitamin-trace mineral premix, DDGS: Corn distillers dried grains with solubles
[2]Crude protein is calculated from nitrogen content × 6.25
[3]Average of 3 replicates

Table 9 Kinetic parameters and correlation coefficients corresponding to the Weibull model fitted to Porcine Epidemic Diarrhea Virus (PEDV) survival curves on feed ingredients after thermal treatment

Temperature	60°C		70°C		80°C		90°C	
Ingredient[1]	Delta (min)[2]	Adj. R^2	Delta (min)[2]	Adj. R^2	Delta (min)[2]	Adj. R^2	Delta (min)[2]	Adj. R^2
CF	3.8 ± 1.2	0.72	1.1 ± 1.3	0.88	1.5 ± 1.6	0.84	2.0 ± 2.2	0.84
SBM	3.3 ± 2.3	0.83	1.3 ± 5.0	0.83	1.7 ±1.8	0.68	2.0 ±2.1	0.85
C	3.4 ±3.2	0.85	3.3 ±4.5	0.75	2.2 ±1.5	0.90	1.7 ±1.6	0.89
DDGS	2.5 ± 1.7	0.84	2.2 ± 2.4	0.87	1.3 ± 1.4	0.87	2.1 ± 1.7	0.87
PM	4.9 ± 4.3	0.89	1.4 ± 5.0	0.76	2.0 ± 2.4	0.85	2.0 ± 1.7	0.83
SDPP	3.6 ± 3.4	0.86	2.1 ± 1.8	0.86	2.3 ± 1.6	0.85	2.1 ± 3.1	0.87
BM	2.0 ± 6.0	0.83	3.5 ± 4.4	0.81	1.5 ± 2.8	0.84	0.64 ± 0.3	0.84
MM	3.0 ± 2.6	0.85	2.0 ± 1.6	0.89	2.1 ± 1.2	0.90	2.1 ± 0.9	0.84
MBM	6.0 ± 2.5	0.88	2.4 ± 1.4	0.84	2.3 ± 1.0	0.85	0.9 ± 0.9	0.75
P-Value	0.75		0.50		0.98		0.78	

[1]CF: complete feed, SDPP: spray dried porcine plasma, MM: meat meal, MBM: meat and bone meal, BM: blood meal, SBM: soybean meal, C: corn, PM: vitamin-trace mineral premix, DDGS: Corn distillers dried grains with solubles

[2]Average of 6 replicates, Delta values indicates the time to achieve 1 log reduction

Table 10 Survival of Porcine Epidemic Diarrhea Virus (PEDV) in all ingredients when thermally treated

Temp	Average δ [1,2] (min)	Shape Paremeter[3]	Adj. R^2	Log reduction at 10 min[1]	Log reduction at 15 min[1]	Log reduction at 30 min[1]
60° C	4.4[a] ± 3.5	0.50	0.83	1.7[a] ± 0.4	2.0[a] ± 0.4	2.4[a] ± 0.4
70° C	3.7[b] ± 3.7	0.45	0.84	1.9[a] ± 0.5	2.3[a] ± 0.4	2.7[a] ± 0.7
80° C	2.4[b] ± 1.8	0.50	0.85	2.2[b] ± 0.3	2.8[b] ± 0.8	3.4[b] ± 0.9
90° C	2.3[b] ± 1.9	0.60	0.84	2.6[c] ± 0.9	3.3[c] ± 1.1	3.9[c] ± 0.8
P-Value	0.0002			0.0001	0.0001	0.0001

[1] Different letters in the same column differ at $P < 0.05$.

[2] δ is the time of the first log reduction of virus concentration.

[3] The shape parameter (n) indicates the shape of the curve with a value n > 1 forming shoulders and being convex, n < 1 forming tails and being concave, and n = 1 being linear.

Chapter 4: Comparison of thermal and non-thermal processing of swine feed and the use of selected feed additives on inactivation of Porcine Epidemic Diarrhea Virus (PEDV)

Summary

Infection with porcine epidemic diarrhea virus (PEDV) causes diarrhea, vomiting, and high mortality in suckling pigs. Contaminated feed has been suggested as a vehicle of transmission for PEDV. The objective of this study was to compare thermal and electron beam processing, and the inclusion of feed additives on the inactivation of PEDV in feed. Feed samples were spiked with PEDV and then heated to 120-145°C for up to 30 min or irradiated at 0-50 kGy. Another set of feed samples spiked with PEDV and mixed with Ultracid P (Nutriad), Activate DA (Novus International), KEM-GEST (Kemin Agrifood), Acid Booster (Agri-Nutrition), sugar or salt was incubated at room temperature (~25°C) for up to 21 days. At the end of incubation, the virus titers were determined by inoculation of Vero-81 cells and the virus inactivation kinetics were modeled using the Weibull distribution model. The Weibull kinetic parameter delta represented the time or eBeam dose required to reduce virus concentration by 1 log. For thermal processing, delta values ranged from 16.52 min at 120°C to 1.30 min at 145°C. For eBeam processing, a target dose of 50 kGy reduced PEDV concentration by 3 log. All additives tested were effective in reducing the survival of PEDV when compared with the control sample (delta = 17.23 days). Activate DA (0.81) and KEM-GEST (3.28) produced the fastest inactivation. In conclusion, heating swine feed at temperatures over 130°C or

eBeam processing of feed with a dose over 50 kGy are effective processing steps to reduce PEDV survival. Additionally, the inclusion of selected additives can decrease PEDV survivability.

Introduction

Porcine Epidemic Diarrhea Virus (PEDV) is a pleomorphic, enveloped RNA virus, classified as a coronavirus under the family *Coronaviridae* (36). The virus was first identified during an outbreak of diarrhea on a Belgian swine breeding farm in 1978 (27). Upon infection with PEDV, suckling pigs experience diarrhea, vomiting, and high mortality (121). Since the initial identification of PEDV, it has been reported in Canada, Korea, China, Thailand, Italy, Hungary, and Vietnam (28). It is important to note that China and Vietnam are two of the top swine producing countries in Asia and that they were both impacted by PEDV (125).

In the United States, the virus was first detected in April 2013 and has since caused high piglet mortality in over 17 states (29). Some have suggested that PEDV is transmitted via contaminated feed (62). Although research has been conducted to show the survival of PEDV in feed and feed ingredients (42,63,79) there is limited data comparing feed processing treatments and their efficacy in inactivating the virus in complete swine feed.

In the feed and ingredient industry, temperature and time conditions vary with the processing method used e.g., pelleting, rendering, pasteurization, or spray drying. Moisture content of feed and ingredients is also impacted by the type of processing method used. The interactions between temperature, time, and moisture play a role in

virus inactivation in feed. Ingredients of porcine origin have been thought to have the greatest risk for disease transmission (63). When processing rendered ingredients for animal feed, the processing conditions vary based on the composition of the raw material. In general, the National Renderers Association suggests temperatures between 115°C and 145°C for 40 to 90 min for most rendering systems. Such temperatures are deemed effective in inactivating pathogens such as Salmonella spp. (126). Similarly, the enveloped classical swine fever RNA virus is readily inactivated in pasteurization processes after only 1 min at 71°C (127). High temperatures (around 100°C) are also used in other processes including micronization to prepare cereal grains (128,129). The high temperatures used in processing rendered products and cereal grains may also be effective in inactivating PEDV in contaminated feed, but no research has been conducted to compare PEDV survival at temperatures greater than 80°C.

Other processing procedures such as ionizing irradiation have been shown to reduce the survivability of pathogens in feed, specifically *Listeria monocytogenes* in poultry feed (130). More recent applications of ionizing radiation technology such as electron beam (eBeam) are now used to routinely pasteurize foods and decontaminate animal feeds from pathogens (131,132). The main characteristics of eBeam technology include its non-thermal nature (thereby reducing potential nutrient losses), utilization of commercial electricity (does not rely on radioactive isotopes), high speed processing, and the ability to precisely control the dose delivered (132,133). The US Food and Drug Administration (FDA) has recognized this process as a treatment method for food and animal feed for doses up to 50 kGy (134). All of these attributes make the eBeam

technology attractive for a wide variety of applications, including pasteurization of animal feed.

In addition to thermal and eBeam treatments, certain organic acid feed additives including propionic, formic, and butyric acid have been used in the past for their antimicrobial properties. These additives have previously been used as a method of controlling pathogens such as Salmonella spp. and *Escherichia coli* in poultry feed and other matrices (95,96). However, the effects of organic acids and other additives including sugar and salt in feed have not been investigated. Therefore, the objective of this study was to determine if thermal and non-thermal methods of microbial inactivation, as well as the use of selected feed additives, are effective in reducing the survival of PEDV in experimentally contaminated swine feed.

Materials and Methods

Virus and virus recovery

The NVSL (National Veterinary Service Laboratory, Ames, IA) strain of PEDV was propagated and titrated in Vero-81 (African Green monkey kidney, ATCC® CCL-81™) cells. We have found that both Vero-76 and Vero-81 cells are susceptible to PEDV. We chose to use vero-81 in this study. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM; Mediatech, Herndon, VA, USA) containing 8% fetal bovine serum (FBS; Gibco, Life Technologies, Grand Island, NY, USA), 50 μg/mL gentamicin (Mediatech), 150 μg/mL neomycin sulfate (Sigma, St. Louis, MO, USA), 1.5 μg/mL fungizone (Sigma), and 455 μg/mL streptomycin (Sigma). The cells were washed

three times with phosphate buffered saline (PBS; pH 7.2). After virus inoculation, the cells were incubated at 37°C for 1 h, allowing virus adsorption using maintenance medium (DMEM with gentamicin, neomycin sulfate, fungizone, streptomycin and 10 µg/mL trypsin). The cells were washed again 60 minutes after inoculation. The washing medium was Hanks' balanced salt solution containing no trypsin. However, the maintenance medium did contain trypsin. Inoculated cells were incubated at 37°C under 5% CO_2. Cells were examined daily under an inverted microscope for the appearance of virus-induced cytopathic effects (CPE) for up to five days post-infection. The virus was harvested by subjecting the infected cells to three freeze-thaw cycles (-80°C/25°C) followed by centrifugation at 2500 × g for 15 min at 4°C. The supernatant was collected and aliquoted into 50 mL tubes followed by storage at -80°C until used.

In all experiments, the surviving virus after a certain treatment was recovered in an eluent consisting of a 3% solution of beef extract solution (Lab Scientific, Highlands, NJ) in 0.05M glycine (Sigma, St. Louis, MO), pH 7.5. Following elution, the eluate was centrifuged for 10 min at 2500 x g to remove organic matter/debris. The supernatants were used to determine the amount of surviving virus, if any. For titration of PEDV in virus stock and other samples, serial ten-fold dilutions of the eluates prepared in DMEM (maintenance medium) were inoculated into Vero-81 monolayers contained in 96-well microtiter plates (Nunc, NY, USA) using 100 µL/well and three wells per dilution. Inoculated cells were incubated at 37°C under 5% CO_2 for up to seven days until the CPE appeared. The highest dilution showing CPE was considered the end point. Virus titers were calculated as a median tissue culture infectious dose ($TCID_{50}$/mL) by the Karber method (122). The amount of surviving virus was compared with the starting virus titer to

calculate the amount of inactivated virus and was expressed in log scale (log_{10} $TCID_{50}$/mL).

Inactivation of PEDV by thermal treatment

A complete phase II pig starter feed (CGI, enhanced NP-NT, batch no. 831458) was obtained and confirmed to be PEDV negative by RT-PCR. The proximate analysis of this feed sample is displayed in Table 11. Aliquots of feed (5 g amounts) were prepared in glass beakers, which were then placed into drying ovens set at 120, 130, 140, and 145°C. The feed aliquots remained in the ovens for 30 min to reach oven temperature. Once the feed reached the indicated temperature, the beakers were immediately removed and spiked with 1 mL of PEDV ($6.8x10^3$ $TCID_{50}$/mL) that was previously tempered at room temperature (~25° C). After mixing, beakers were placed back in the ovens at the appropriate temperatures and incubated for 0, 5, 10, 15, 20, 25, and 30 min. After the incubation period, the samples were removed from the oven, actively cooled with a fan for 15 min, the virus eluted from the feed using the 3% beef extract eluent solution, and the number of surviving viral particles titrated. Triplicate samples of each temperature were combined for a single titration and inoculation into cells. The experiment was performed in duplicate.

Inactivation of PEDV by eBeam processing

Preliminary dose mapping experiments were performed to calculate the dose uniformity within the samples. A dose-uniformity ratio was calculated by maximum

dose/minimum dose for each experiment. Under ideal circumstances, the dose-uniformity ratio (DUR) should be about 1.0. During commercial processing of animal feed using eBeam technology, the size of the final package needs to be optimized to assure dose uniformity. The optimization involves adjusting the dimensions and weight to ensure adequate penetration of the eBeam electrons as well as dose uniformity within the package. Under commercial processing conditions, the goal is to try to attain as uniform a dose as possible. At the eBeam facility on the TAMU campus, the DUR of a commercial pet food product is 1.78. Aliquots of complete phase II pig starter feed (5 g amounts) were prepared in several 50 mL plastic centrifuge tubes followed by the addition of 1 mL of PEDV to each tube. After mixing, the virus-spiked feed was placed into individual plastic bags. The bags were flattened to remove all of the air and create a thin, even layer of the feed sample. The bags were triple-sealed to meet the biosafety procedures at the eBeam irradiation facility. The sealed bags were shipped on ice via overnight shipping to the National Center for Electron Beam Research at Texas A&M University, College Station, TX.

Preliminary dose delivery trials were performed to determine the appropriate conveyor speed and other specifications to achieve the target doses. On the day of arrival, the samples were exposed to target eBeam doses of 10, 20, 30, and 50 kGy. A control sample was also shipped with the irradiated samples, but it was not exposed to eBeam irradiation at the facility. After treatment, the samples (including the controls) were shipped back to the University of Minnesota (St. Paul) on ice via overnight shipping. Upon arrival, the samples were immediately eluted using the aforesaid eluent solution. This experiment was performed only once.

Effect of feed additives on PEDV survivability in feed

Aliquots of feed in 5 g amounts were placed in plastic scintillation vials. The amount of feed additive used in the 5 g sample was determined based on the recommended dosage from the manufacturers. The following additives were added to separate aliquots of feed: 0.015 g Ultracid P (Nutriad, Dendermonde, Belgium), 0.02 g Activate DA (Novus International, St. Charles, MO), 0.01 g of Acid Booster (Agri-Nutrition, DeForest, WI), 0.01 g KEM-GEST (Kemin Agrifoods, Des Moines, IA), 0.02 g of granulated sugar (Shoppers Value, Eden Prairie, MN) and 0.02 g of commercial salt (Essential Everyday, Eden Prairie, MN). Another set of vials was used as paired controls without the addition of any feed additive. After the additives were added to the appropriate vials, 1 mL of PEDV was added to each vial followed by vortexing. All vials were incubated at room temperature (around 25°C during the season which this experiment was performed). After incubation for 0, 1, 3, 5, 7, 14, and 21 days, the surviving virus was eluted from the samples using the 3% beef extract eluent solution. The experiment was performed in duplicate. Each experiment used a set of triplicate samples that were combined and used for a single titration and inoculation into cells.

After completion of the trial, the pH of each feed sample solution with and without the additive was measured. The pH was measured by adding 50 mL of deionized water to 5 g of feed followed by mixing continuously on a magnetic stirrer for 15 min at room temperature. After mixing, a pH probe was used to measure the pH of the liquid. The pH measurement experiment was performed in triplicate. In addition to measuring

pH, the active ingredients for Activate DA (2-hydroxy-4-methylthiobutanoic acid), KEM-GEST (phosphoric, fumaric, lactic, and citric acids), sugar (sucrose), Acid Booster (phosphoric, citric, and lactic acids), salt (sodium chloride), and Ultracid P (orthophosphoric, citric, fumaric, and malic acids) were compared.

Statistical analysis

Inactivation kinetic data (log $TCID_{50}$/mL) were analyzed by GInaFiT, a freeware Add-in for Microsoft Excel developed by Geeraerd et al. (115). The traditional log-linear model that assumes a linear relationship between the virus concentration and processing time was developed by Bigelow and Esty (135) and used to characterize the survival curves of PEDV by using the following equation:

$$LogN = LogN_o - k \times t \quad \text{Eq. 1}$$

where N is the surviving virus titer after the treatment (expressed as $TCID_{50}$/mL), N_0 is the initial virus titer ($TCID_{50}$/mL), k is the kinetic parameter (min^{-1} or day^{-1}), and t is the treatment time (min or days). The kinetic parameter k is usually expressed as D, also known as the decimal reduction time (time required at a certain temperature to reduce 90% or 1 log of the initial virus titer) and was calculated as follows:

$$D = \frac{2.3}{K} \quad \text{Eq. 2}$$

The Weibull distribution function has been used to describe non-linear inactivation patterns of various microorganisms after different thermal and non-thermal processing (123). Assuming that the temperature resistance of the virus is governed by a Weibull distribution, Mafart et al. developed the following Weibullian equation:

$$Log(N) = Log(N_o) - \left(\frac{t}{\delta}\right)^n \quad \text{Eq. 3}$$

where N is the surviving virus after the treatment expressed as ($TCID_{50}$/mL), N_0 is the initial virus titer ($TCID_{50}$/mL), δ is the time of the first logarithm decline for the virus titer population (min or days), and n is the shape parameter. The n value provided a general description of the form of the curve; if n > 1, the curve is convex (it forms shoulders), if n < 1, the curve is concave (it forms tails), and if n = 1, the curve is a straight line and can be described by a linear model.

Two valid replicates were used to evaluate how well the model fit with the experimental data by calculating the adjusted R^2 (Adj. R^2) as follows:

$$Adj.R^2 = \left[1 - \frac{(m-1)(1-\frac{SSQ\ regression}{SSQ\ total}}{m-j}\right] \quad \text{Eq. 4}$$

where m is the number of observations, j is the number of model parameters, and SSQ is the sum of squares.

An ANOVA test using the mixed procedure of SAS (SAS Inst., Cary, NC) was used to determine statistical differences across treatments. Least squared means with Tukey adjustment was used to determine differences between treatment means, with $P < 0.05$ considered to be significantly different. The experimental unit was the value obtained from the combined triplicate vials. The fixed effects were temperature or feed additive used.

Results

Thermal inactivation of PEDV

Virus concentrations (log $TCID_{50}$/mL) at 120, 130, 140, and 145°C as a function of time are shown in Fig. 2. Survival curves displayed non-linear behavior (shoulder), meaning a certain exposure time was needed to be surpassed to initiate viral inactivation. The kinetic parameters of the log-linear and Weibull model (D and delta values) for each temperature are shown in Table 12. The PEDV showed a high thermal resistance in the dry feed samples and it was completely inactivated (3.0 log reduction) at each of the tested temperatures within 30 min. The original moisture content of the feed sample was 8.57% (W/V). After adding 1 mL of virus media the moisture increased to 23.8% (W/V). This change in moisture content potentially increased the ability of heat to reduce virus concentration, as heat inactivation is more efficient in higher moisture content as previously demonstrated by the higher sensitivity of bovine parvovirus to moist heat vs. dry heat (136).

Weibull model provided a better fit for the experimental data by generating greater adj. R^2 values (0.76 to 0.84) than the log-linear model (0.64 to 0.84). Delta values were reduced as the temperature increased indicating a greater inactivation rate. In order to achieve a 5 log virus reduction, predicted exposure times of 82.6, 14.2, 10.5 and 6.5 min were estimated at 120, 130, 140 and 145°C, respectively. Shape parameter (n) was greater than 1 for all temperatures except 120°C, indicating the shoulder appearance of the inactivation curves shown in Figure 1. Delta values at 130, 140, and 145°C were not statistically different ($P > 0.05$).

Inactivation of PEDV by eBeam processing

Figure 3 shows the results of PEDV inactivation after eBeam processing. The figure represents the inactivation curve predicted by the Weibull model and the points illustrate the observed values. The delta value using the Weibull model was estimated as 17.25 kGy. The survival of PEDV was dose dependent with higher inactivation being achieved with increasing doses. In order to obtain a 5 log reduction of PEDV in feed, a dose of 86.25 KGy would be needed.

Effect of feed additives on PEDV inactivation

The virus concentration in feed (control sample) and feed with additives is shown in Table 13. The addition of the additives to the feed sample decreased the initial virus titer. The D and delta values of the log-linear and Weibull models respectively, are shown in Table 14. The use of the Weibull model resulted in greater adj. R^2 values than the log-linear model, and therefore, was used to characterize the survival of PEDV with the addition of additives during storage at room temperature. Delta values for all the additive-containing samples, except Ultracid P and salt, were significantly lower than the control sample ($P < 0.05$) indicating faster inactivation kinetics (Table 14). After 21 days of incubation at room temperature (25°C), a 3 log reduction was observed in samples containing KEM-GEST, sugar, and salt. In the same 21-day incubation period, a 2 log reduction was observed in Activate DA, and <2 log reduction was observed in the control, Ultracid P, and Acid Booster.

The comparison of all feed additives in terms of active ingredient and pH value is shown in Table 15. In general, the additives containing phosphoric acid were more effective at reducing virus concentration. The only additive containing 2-hydroxy-4-methylthiobutanoic acid (Activate DA) was the most effective in reducing virus concentration. There were differences in the pH values of the feed samples after the addition of additives.

Discussion

Feed was suggested as a vehicle of transmission of PEDV when the first Canadian swine farm tested positive for the virus (63). A follow-up epidemiological investigation suggested that a batch of spray-dried porcine plasma (SDPP) used in swine nursery feed was the potential source of PEDV infection. In a bioassay experiment, a contaminated SDPP sample was able to infect pigs; however, when added to the complete feed no additional pigs were infected. This was potentially caused by a dilution effect or an extended time period between virus contamination and bioassay (63). A similar conclusion that feed or feed ingredients could be spreading PEDV between farms was made during an investigation of a PEDV outbreak in Ohio. In this situation, virus was detected in the feed through RT-PCR on a farm with infected sows. In a follow up bioassay, none of the pigs developed an infection after consuming the contaminated feed (62). In this study, the source of feed contamination was not found, although the RT-PCR test was positive for PEDV. Dee et al. (42) showed that feed experimentally contaminated with PEDV RNA could cause an active infection in pigs when feed was consumed via natural feeding behavior. The collection of references indicates that if

complete feed happens to be contaminated with PEDV, it is possible for the contaminated feed to cause an active infection on a farm. In summary, these results suggested that additional research is needed to evaluate potential mitigation strategies to control transmission of PEDV through feed processing methods.

The survival of PEDV in feed that was thermally treated at 120 to 145° C for 0 to 30 minutes was first evaluated. Previous research has focused on the survival of PEDV at temperatures below 80°C. One study found that a cell culture-adapted strain of PEDV was moderately stable at 50° C for 30 min, with only a reduction of 0.4 $\log_{10}$ PFU/mL at 50°C when compared to the control (36). A further investigation indicated that treating PEDV at temperatures between 60 to 80°C for 30 min caused a complete loss of infectivity, but the log reduction in virus concentration was not reported (36). In another study, the survival of a wild type strain of PEDV in fecal samples required only 10 min of exposure at 71°C to be inactivated to the extent that it was not able to cause infection in live pigs (100). This variability in virus inactivation could be possibly due to differences in moisture content between the different samples tested. The moisture content will ultimately have an impact on virus survivability during heat treatment.

Although the exposure times and temperatures varied in these studies, their results correspond with previous data indicating that PEDV is a low thermally stable virus (28). Our hypothesis was that the thermal treatment of feed would reduce the survival of PEDV in swine feed. This hypothesis was confirmed by our experimental results which demonstrated that a 3 log (99.9%) reduction of the PEDV concentration is achieved within 25 min of exposure at 120°C. These results also highlight the finding that temperatures over 130°C don't increase the inactivation of PEDV. This is an important

observation because excessive heating of high protein feed ingredients results in reductions of amino acid digestibility through Maillard reactions (73). In addition to the log reduction of PEDV in feed at high temperatures, the production of a shoulder in the inactivation curve produced by the Weibull model also help determine PEDV survival at high temperatures. The observed shouldering phenomenon observed in the inactivation curves may indicate that virus is resistant to a certain temperature-time level combination and a threshold needs to be surpassed to see a significant level of inactivation. Another possible reason for this shoulder in the curve could be the possibility that the feed was cooled when the room temperature inoculum was added. This would mean the first few min the feed was placed back in the oven was spent heating back up to oven temperature, which could explain the plateau in virus concentration. Overall, the results from our experiment confirm that the concentration of PEDV in complete feed can be reduced through the application of heat.

When exploring non-thermal treatment processes to reduce PEDV in feed, an eBeam irradiation dose of 50 kGy was found to be effective in reducing virus concentration by 3 log (99.9%). Currently, the Food and Drug Administration has stipulated an upper dose limit of 50 kGy of ionizing irradiation doses in animal feeds (134). The use of eBeam processing of animal feeds and diets has been shown to be an effective process for inactivating pathogens in the US (131,132). Animal feed and bags (weighing about 20 kg) are routinely treated using eBeam processing. Process controls are in place to measure the maximum and minimum doses in these sample bags to meet customer needs and adhere to regulatory limits. Though we have shown that eBeam treatment is effective in reducing the concentration of PEDV in swine feed, follow-up

studies are needed to verify the results seen in this study with different types of feed. Our experimental data, suggesting that a target dose of 50 kGy will achieve approximately 3 $\log_{10}$ reduction in PEDV, are important in that it highlights the criticality of ensuring low viral bioburden during feed formulation by adhering to Good Manufacturing Processes. These results suggest that if the initial PEDV bioburden is greater than 4 $\log_{10}$ $TCID_{50}$, the FDA mandated 50 kGy dose upper limit may not be effective in complete inactivation of virus in these feeds. Furthermore, if the maximum titers of PEDV in commercial swine feed are known, then the dose can be calibrated to theoretically achieve the total elimination of PEDV in commercial swine feed. In addition to more research investigating maximum PEDV titers, it may also be necessary to investigate the use of irradiation on individual feed ingredients. Our experiment only measured the impact of irradiation on complete swine feed, and so it cannot be confirmed if the same results would be seen in individual feed ingredients.

Finally, we evaluated the survival of PEDV in feed when various feed additives were included at manufacturers' recommended doses. The additives experiment was only performed at room temperature. On a swine farm, feed can be stored at a variety of temperatures based on season, barn layout, bagged feed vs. bulk feed, barn temperature, and other variables. Room temperature was used in this experiment as a feasible average temperature, though it may not accurately apply to every situation. Feed is suggested to be stored in a cool, dry place and so it is likely some swine feed will be stored at temperatures below the approximately 25°C tested in this experiment.

The addition of organic acid feed additives in the diet has been shown to increase nutrient digestibility by lowering pH and modifying gut microflora, which ultimately

improve growth performance and provide an alternative to antibiotic use in weaned pig diets (137). Organic acids have been used to reduce the prevalence of bacterial pathogens such as Salmonella in poultry feed (95). In an additional experiment, the antimicrobial product SalCURB, which is used to control *Salmonella* in feed, was tested for its ability to decrease the presence of viable PEDV in feed. In this experiment, a bioassay showed that the feed treated with the SalCURB product was not able to cause an infection in naïve pigs (111). A proposed method for how the SalCURB inactivated the PEDV was not reported. Differences in PEDV survival in feed has not only been observed in the presence of additives, but also in the presence of different feed ingredients. In an experiment testing 18 different feed ingredients, it was determined that the survival of PEDV in these ingredients was ingredient specific with an extended survival being observed in soybean meal (99). This indicates that variation as small as changes in ingredients could change virus survival in feed.

It has been reported that PEDV is stable at a pH of 6.5 to 7.5 at a temperature of 37° C (117). Because intestinal viruses must survive the acidic conditions of the stomach, they are generally more resistant to acidic pH. With this being said a change in pH outside of ideal conditions for an extended period could cause an increase in virus inactivation. A previous review reported that the pH of the diet was decreased from an average pH of 5.96 to 4.71 when organic acids (fumaric, propionic, formic, citric, sodium citrate, hydrochloric, sodium fumaric, or malic acid) were added to feed (138). This pH decrease of the diet was hypothesized to decrease the survival of PEDV in the presence of additives. The results from our study did not necessarily support this hypothesis. Activate DA and KEM-GEST were the most effective in reducing virus survivability and

also had the most acidic pH values when added to feed, which confirm the original hypothesis. However, Ultracid P did not result in a different diet pH compared with KEM-GEST, yet it had the highest delta value of all additives evaluated. In addition to this, the feed sample would require a type of liquid to be added to the feed for pH to even play a role, as a dry substrate will not have a pH. In the experiment, 1mL of virus media was added to the sample and mixed. This means it could be possible for the pH of the media to change because of the additive when the virus was initially added. If this single change were to explain the differences in survival for our experiment, there would be a more prominent pattern between the pH of the feed solution and the delta value observed. With a lack of a clear pattern between pH and virus survival, and in some cases no difference between the pH of the solution with the control and with some of the additives tested, there is another aspect causing the changes in virus survival between the feed additives used.

There are other components of feed additives that have the capability to reduce PEDV survivability, as suggested by the inability to explain the decrease in PEDV survivability due to change in pH alone. We hypothesized that specific active ingredients play a role in differences in virus survivability among additives. Many of the less effective additives contained citric acid and lactic acid as active ingredients. It has been previously shown that citric and lactic acids only have moderate antimicrobial activity because they are solely effective at a low pH in the environment (139). Because most swine feed samples have a neutral pH, it is not surprising that additives containing citric acid as an active ingredient may not have been as effective in inactivating PEDV. In contrast, fumaric acid has been shown to be a very effective antimicrobial, and is

extremely effective in reducing the survivability of *E. Coli* (140). No studies have been published to show that fumaric acid is an effective anti-viral compound, but its presence in KEM-GEST, which resulted in the second lowest PEDV survivability in our study, implies it may also be an effective ingredient in reducing survivability of PEDV. The active ingredient of Activate DA, the most effective additive in our study, is 2-hydroxy-4-methylthiobutanoic acid. This ingredient has been researched exclusively as a cost effective supplement in low methionine diets for swine (141). Although it has not been approved for use as an antimicrobial or antiviral agent, the results from our study suggest it could have potential in reducing virus survivability. Overall, careful selection of additives containing the most effective active ingredients is necessary for achieving reduced PEDV survivability in complete swine feeds. Further research is needed to confirm the relative effectiveness of each active ingredient and how they may interact with each other.

One limitation in this experiment is the unknown variable of water activity. By adding liquid media to the samples the moisture content was already dramatically increased. This increase in moisture potentially caused changes in the inactivation of the virus when exposed to heat that may not be observed in the low moisture conditions of animal feed. It has been demonstrated that PEDV inactivation using heat treatments and changes in pH differs in liquid media compared with a dried plasma product (118). Because our experiments investigated both change in pH through an additive and change in heat treatment, our increased moisture from inoculating the sample could cause different inactivation kinetics than what would be seen in dry feed without media. In addition to this, the humidity in the environment was not controlled and the water activity

of the samples were not measured. Without measuring these variable there is no way to tell the role they played in the inactivation of PEDV in the feed samples. In addition to unknown water activity, this experiment also had the limitation of low starting virus titers. This made it impossible to reach the standard of complete inactivation (typically 7 log reduction) because the starting virus titers were only 4 logs.

Our results show that PEDV survivability can be reduced using eBeam processing or thermal processing at temperatures greater than 130° C. The use of some feed additives was also effective in reducing virus levels, but future investigations are needed to determine the prevalence and maximum titers of PEDV in commercial swine feeds to determine the most appropriate treatment parameters (time and temperature of thermal processing, dose of eBeam processing, and feed additive selected) to be most effective in inactivating PEDV.

Conclusions

Feed has been suggested as a possible vehicle of PEDV transmission. This has created a need to evaluate new and existing feed processing methods to reduce PEDV survivability in feed. Results from this study have shown that both thermal (130°C for at least 15 min) and non-thermal (50 kGy eBeam dose) feed processing technologies can eliminate 99.9% of PEDV infectivity when working with low virus titers ($6.8x10^3$ $TCID_{50}$/mL). Additionally, the survivability of PEDV can be reduced by the use of selected acidifiers and organic acids in swine feeds.

Table 11 Proximate Analysis of the Vita-Plus CGI enhanced feed used for the experiment

Analysis	Percent (%)
Moisture	8.57
Protein	24.2
Fat	4.47
Fiber	2.02
Ash	9.45

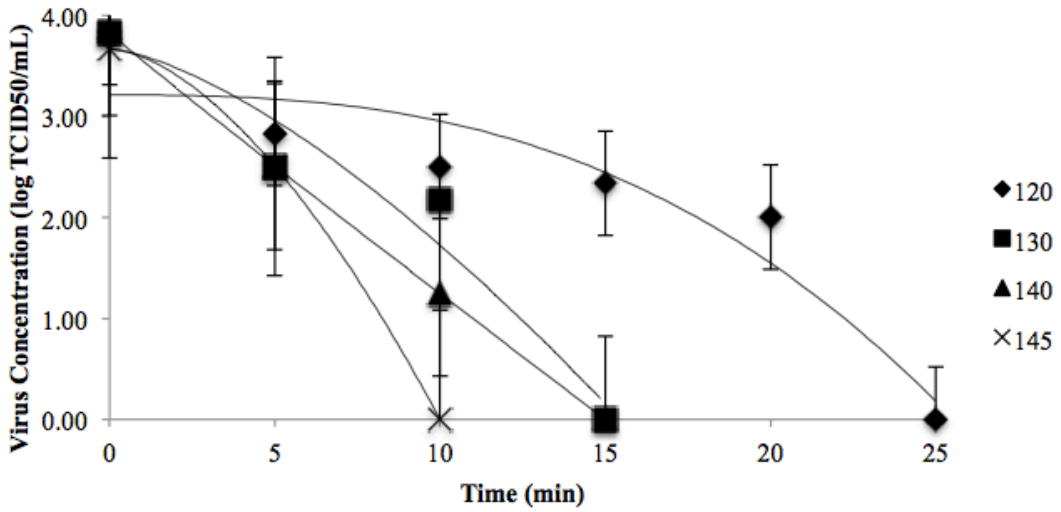

Figure 2 Inactivation of PEDV in complete feed when exposed to thermal processing. The inactivation curves determined by the Weibull model for the survival of PEDV in complete feed at 120°C, 130°C, 140°C, and 145°C.

Table 12 Comparison of Log Linear and Weibull kinetic parameters and goodness of fit for PEDV thermal inactivation kinetics in feed

Temp	Weibull model			Log Linear model	
	Delta[1] (min)	Shape Parameter[2] (n)	Adj. R^2	D[1] (min)	Adj. R^2
120°C	16.52[a] ±0.08	2.7	0.79	8.07[a] ± 0.20	0.80
130°C	2.85[b] ± 0.00	0.7	0.84	6.05[b] ± 0.00	0.84
140°C	2.10[b] ± 2.39	0.6	0.82	6.26[b] ± 0.72	0.73
145°C	1.30[b] ± 0.88	0.5	0.76	6.77[b] ± 0.28	0.64

[1] Different letters within the same column differ ($P < 0.05$)
[2]The shape parameter (n) indicates the shape of the curve with a value n > 1 forming shoulders and being convex, n < 1 forming tails and being concave, and n = 1 being linear.

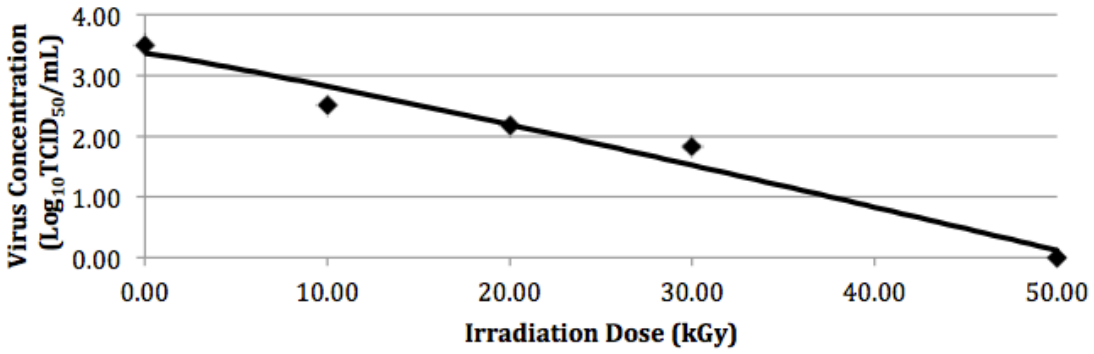

Figure 3 Inactivation of PEDV by eBeam irradiation processing. Inactivation curve modeled by the Weibull model for the survival of PEDV in complete feed when exposed to eBeam irradiation.

Table 13 Comparison of the concentration of PEDV in feed with and without feed additives

Incubation Period (days)	Average Virus Titer ($TCID_{50}$/mL)						
	Control	Ultracid P	Activate DA	Acid Booster	KEM-GEST	Sugar	Salt
0	5000	3250	6800	3250	5000	6800	3250
1	3460	6800	1500	1500	6800	6800	6800
3	4200	680	150	680	6800	1500	3200
5	8000	680	374	320	320	320	3200
7	4460	680	68	680	68	150	68
14	909	320	68	68	68	320	320
21	252	68	68	68	1	1	1

Table 14 Comparison of Log Linear and Weibull kinetic parameters and goodness of fit for PEDV survival kinetics in feed additives**.**

Additive	Weibull model			Log linear model	
	Delta[1] value (days)	Shape Parameter[2] (n)	Adj. R^2	D value (days)	Adj. R^2
Control	17.23[bc] ± 0.78	1.9	0.83	18.34± 10.01	0.79
Ultracid P	13.00[ac] ± 3.41	0.7	0.87	14.90 ± 3.95	0.86
Activate DA	0.81[a] ± 0.52	0.2	0.78	13.59± 1.13	0.39
Acid Booster	7.24[a] ± 3.71	0.5	0.90	13.25 ± 1.60	0.82
KEM-GEST	3.28[a] ± 2.05	0.8	0.83	5.68 ± 0.10	0.86
Sugar	5.66[a] ± 0.00	0.9	0.77	6.39 ± 0.00	0.81
Salt	11.42[ac] ± 4.43	2.6	0.86	6.31 ± 0.37	0.76

[1] Different letters in the same column differ ($P < 0.05$)

[2]The shape parameter (n) indicates the shape of the curve with a value n > 1 forming shoulders and being convex, n < 1 forming tails and being concave, and n = 1 being linear.

Table 15 Composition and properties of the feed additives used

Company	Additive Name	Ingredients	pH[1]	Delta[1] Value (days)
Novus	Activate DA	2-hydroxy-4-methylthio butanoic acid, fumaric and benzoic acid	$5.50^{c} \pm 0.03$	$0.81^{a} \pm 0.52$
Kemin	KEM-GEST	phosphoric, fumaric, lactic, and citric acids	$5.74^{b} \pm 0.03$	$3.28^{a} \pm 2.05$
Sugar	Sugar	Sucrose	$5.88^{e} \pm 0.03$	$5.66^{a} \pm 0.00$
AgriNutrition	Acid Booster	phosphoric, citric, and lactic acids	$5.84^{a} \pm 0.03$	$7.24^{a} \pm 3.71$
Salt	Salt	sodium chloride	$5.84^{a} \pm 0.02$	$11.42^{ac} \pm 4.43$
Nutriad	Ultracid P	orthophosphoric, citric, fumaric, and malic acids	$5.73^{b} \pm 0.01$	$13.00^{ac} \pm 3.41$
Control	None	no additive	$5.82^{a} \pm 0.02$	$17.23^{bc} \pm 0.78$

[1] Different letters in the same column differ ($P < 0.05$)

Chapter 5: Survival of porcine epidemic diarrhea virus (PEDV) and porcine delta coronavirus (PDCoV) on different surfaces

Summary

Common equipment surfaces contaminated by the feces of infected pigs are a potential route of transmission for porcine epidemic diarrhea virus (PEDV) and porcine delta coronavirus (PDCoV). The objective of this study was to determine the survival of PEDV and PDCoV on four different surfaces (i.e. galvanized steel, stainless steel, aluminum and plastic). Virus was inoculated into each surface followed by air-drying for a few minutes. After incubation at room temperature for various time periods, the surviving virus was washed with an eluent (3% beef extract-0.05M glycine solution, pH 7.2). Serial 10-fold dilutions of all eluates were inoculated in Vero-81 or ST (swine testicular) cells for titration of PEDV and PDCoV, respectively. Weibull and Log Linear models were used to characterize the virus inactivation kinetics by generating the kinetic parameters (delta and D values) to determine the amount of time needed to inactivate 1 log of the initial virus concentration. Survival kinetics values among surfaces were compared using a mixed model with time as a fixed effect and replicate as a random effect The higher virus inactivation kinetics for PEDV were found in plastic (delta value of 0.69 days). A 3.83 log reduction in PDCoV was observed on aluminum and stainless steel within 28 days, but the lowest delta value was observed for plastic (0.27 days). In

conclusion, virus survival was similar on all four equipment surfaces used commonly on swine farms.

Introduction

Coronaviruses (CoVs) infect the gastrointestinal tract, respiratory tract, peripheral nervous system, and central nervous system of different mammalian and avian species, and their effects tend to be species-specific (1). Two human CoVs that cause upper respiratory illness belong to the genus *Alphacoronavirus* under the family *Coronavirida* (4). Transmissible gastroenteritis virus (TGEV) and porcine epidemic diarrhea virus (PEDV) also belong to genus *Alphacoronavirus*, but cause diarrhea and gastrointestinal illness in swine (4). Porcine delta coronavirus (PDCoV) causes diarrhea in swine, but is classified under genus *Deltacoronavirus* (37).

Contaminated surfaces have been recognized as contributors to the transmission of CoVs (142). During 2002-2004, outbreaks of severe acute respiratory syndrome (SARS) occurred in healthcare facilities that infected workers, patients, and visitors, and resulted in 1,091 deaths (143). Although the SARS virus is mainly transmitted via the respiratory system, it can also spread through contaminated objects or surfaces (6). The PEDV was first discovered in the United States in 2013, when it rapidly spread to 17 states causing high mortality in unweaned piglets (29). Shortly thereafter in 2014, PDCoV was also discovered in the United States (37). Infection with PEDV or PDCoV causes diarrhea, vomiting, and high mortality in suckling pigs (58), but mortality rates are lower for PDCoV than PEDV infections (144). Previous research has demonstrated that PEDV can survive in feed and environmental samples (99,119,145), and also on

contaminated feed mill surfaces (105). Because of the devastating effects that these two viruses have in commercial pork production operations, it is important to study the virus survival on equipment surfaces that are commonly used on swine farms. Therefore, the objectives of this study were to determine the survival of PEDV and PDCoV at room temperature on four different surfaces (i.e. galvanized steel, stainless steel, plastic, and aluminum) which may help identify appropriate mitigation strategies.

Materials and Methods

Viruses and cells

Both PEDV and PDCoV were obtained from the National Veterinary Services Laboratory in Ames, IA. They were propagated and titrated in Vero-81 (African Green monkey kidney, ATCC® CCL-81™) and swine testicular (ST) cells, respectively. The cells were grown in Minimum Essential Medium (MEM; Mediatech, Herndon, VA, USA) supplemented with 8% fetal bovine serum (FBS; Hyclone, South Logan, UT), 50 μg/mL gentamicin (Mediatech), 150 μg/mL neomycin sulfate (Sigma, St. Louis, MO), 1.5 μg/mL fungizone (Sigma), and 455 μg/mL streptomycin (Sigma). The medium for ST cells also contained L-glutamine (Mediatech, Herndon, VA), and the maintenance medium for both cell types included 5 μg/mL of trypsin (Gibco, Life Technologies, Grand Island, NY) and the previously described antibiotics.

Virus propagation

Cells were inoculated with the stock virus followed by incubation at 37°C under 5% CO_2. The inoculated cells were examined daily for up to 7 days under a light microscope for the appearance of virus-induced cytopathic effects (CPE). The infected cells were then subjected to 3 freeze-thaw cycles (-80°C/25°C) followed by centrifugation at 2500 × g for 15 min at 4^{o}C. The supernatant was collected and aliquoted into smaller amounts and stored at -80°C until use.

Virus titration

The surviving virus was eluted from various surfaces at different points in an eluent solution (see below). Serial 10-fold dilutions of the eluate were prepared in MEM, followed by inoculation in monolayers of Vero or ST cells contained in 96-well microtiter plates, using 3 wells per dilution (100 μL/well). Inoculated cells were incubated at 37°C under 5% CO_2 for up to 7 days until CPE appeared. The highest dilution showing CPE was considered the end point. Virus titers were calculated as $TCID_{50}$/mL (122). The amount of surviving virus was compared with the starting virus titer to calculate the amount of inactivated virus, and was expressed in log scale (log_{10} $TCID_{50}$/mL).

Equipment surfaces tested

A total of 4 surfaces were evaluated and included stainless steel, aluminum, plastic, and galvanized steel. Stainless steel and aluminum sheets were purchased from

Hardware Hank (St. Paul, MN), and 6-well plastic plates (Nunc, New York, NY) were used. Galvanized steel (28 gal. Silver Galvanized Steel Hobby Sheet Sleeved; Model # 57321) was obtained from Home Depot (Roseville, MN).

Inoculating surfaces with virus and elution of surviving virus

PEDV inoculation solution (40 µL) was applied to the center of a sample of stainless steel, aluminum, plastic, and galvanized steel. The virus was allowed to dry for 30 min, and the sheets and plates were then stored at room temperature (~25°C) for up to 10 days. At 0, 1, 2, 5, and 10 days, surviving virus was eluted from three different areas of a surface using 400 µL of an elution buffer (3% beef extract in 0.05 M glycine, pH 7.2). This was done by applying the elution buffer to the surface and then sucking the elution buffer off the surface with a pipette. This process was repeated 3 times for each sample. The triplicate samples were combined in a single pool, followed by titration inVero-81 cells to determine virus concentration. This experiment was then repeated once more to provide a total of two experiments.

The experiment with PDCoV was conducted in a similar manner described for the PEDV experiments, except samples were collected at 0, 1, 3, 7, 10, 15, 21, and 28 days after inoculation of surfaces, eluates were titrated in ST cells, and the entire experiment was repeated two more times for a total of three experiments.

Mathematical models

A freeware add-in for Microsoft Excel (Microsoft, Redmond, WA), called GInaFIT software, was developed to analyze inactivation kinetic data (log $TCID_{50}$/mL) (13) . A traditional log-linear model was used to characterize the survival curves of PEDV and PDCoV by using the following equation(146):

$LogN=LogN_0-k\times t$

Eq. 1

In this equation, N is the surviving virus after treatment (expressed as $TCID_{50}$/mL), the initial virus titer ($TCID_{50}$/mL) is defined by N_0, the kinetic parameter in days is *k*, and *t* is the treatment time (days).

The kinetic parameter *k,* is commonly expressed as *D* and represents the decimal reduction of time at certain temperature to reduce the initial virus titer by 90% or 1 $\log_{10}$, and can be determined by using the following equation:

$$D = \frac{2.3}{k}$$

Eq. 2

The Weibull distribution function describes the nonlinear inactivation pattern of different microorganisms after different thermal and non-thermal processing. Mafart et al. (2002) developed an equation assuming that the temperature resistance of the virus is governed by a Weibull distribution according to the following equation(116):

$$Log(N) = \log(N_0) - \left(\frac{t}{\delta}\right)^n$$

Eq. 3

In this equation, *N* is the surviving virus titer after a given treatment and is expressed as $TCID_{50}$/mL; the initial virus titer ($TCID_{50}$/mL) is defined by N_0; δ is the time of the first logarithm decline for the virus titer population (days); and the shape parameter is identified by *n*. This *n* value is used to describe the shape of the curve. If the curve is convex (it forms shoulders) and when $n > 1$, it is concave (it forms tails) when $n < 1$. If the curve is a straight line, it can be described by a linear model when $n = 1$.

Statistical Analysis

Two replicates for PEDV, and 3 replicates for PDCoV were used to determine how well the model fit the experimental data by calculating the adjusted coefficient of correlation R^2 (Adj. R^2), which is defined as follows:

$$\text{Adj.}\, R^2 = \left[1 - \frac{(\text{m} - 1)\left(1 - \frac{SSQregression}{SSQtotal}\right)}{m - j}\right]$$

where *m* is the number of observations, *j* is the number of model parameters, and *SSQ* is the sum of squares.

The effect of different treatments on the kinetic parameters, and on survival of PEDV and PDCoV, were assessed by a mixed ANOVA test to determine if there were significant differences among treatments, using time as a fixed effect and replicate as a random effect. The experimental unit was each sample and data were analyzed for overall structure, absence of outliers, and normal distribution using the UNIVARIATE procedure of SAS (SAS Institute, Cary, NC). Tukey's test was used to calculate and separate the least squares means between treatments, and significant differences were considered to be $P < 0.05$.

Results

During the 10-day incubation period, PEDV titer was reduced by 1.3 log on all surfaces except for stainless steel in which only a 0.83 log reduction was observed. PEDV remained viable (10^2 $TCID_{50}$/g) on each of the four surfaces after 10 days of incubation (Table 16). For PDCoV, a 2.4 to 3.0 log reduction was observed on the different surfaces within 10 days. After 15 and 28 days, a 3.3-4.0 log reduction was observed (Table 17).

The Weibull model provided a better fit for the virus inactivation curves generated for all four surfaces in the PEDV experiment because of tails and shoulders in the curves. This was reflected by greater Adj. R^2 values (0.56 to 0.94) by using the Weibull model compared with the log linear model (Adj. R^2 ranged from 0.14 to 0.32). When delta values were compared, there were no statistical differences among all 4 surfaces ranging from 0.7 and 7.7 days (Table 18).

When comparing the inactivation curves for PEDV in the four surfaces tested, there was an initial rapid decrease in virus concentration on each surface, followed by a plateau (Figure 4). This plateau was more significant for the stainless steel and aluminum than the galvanized steel and plastic surface. Similar to the inactivation results for PEDV, the PDCoV inactivation curves were more accurately described by the Weibull model (Adj. R^2 ranged from 0.62 to 0.85) compared with the log linear model (Adj. R^2 values ranged from 0.43 to 0.63). The greatest initial inactivation kinetics for PDCoV occurred on the plastic surface, with a delta value of 0.27 days, which is almost 2x times faster than the rate of inactivation observed for the other 3 surfaces that ranged among 0.3-0.6

days (Table 18). In fact, the first log reduction in PDCoV occurred within less than 1 day on all four surfaces tested. Remarkably, inactivation curves for PDCoV on all surfaces over the 28-day experiment were very similar (Figure 5), indicating that the rate of PDCoV inactivation to be similar.

Discussion

The predominant route of transmission of PEDV and PDCoV is fecal-oral. However, outbreaks of PEDV have occurred on farms despite using strict biosecurity measures. In addition, PEDV has been shown to survive in livestock trailers (43), feed ingredients (63,147), and swine feed (42,99) for variable periods of time. Potential pathways linked to cross-contamination include personnel, vehicles, and airflow movement. These events can lead to contamination of surfaces or feed that may enter a swine farm. By understanding the survival of viruses on equipment and transportation surfaces, it may be possible for pork producers to implement improved biosecurity measurements to reduce the risk of virus transmission.

Data from this experiment suggest that PEDV and PDCoV survive for extended periods of time on the material surfaces evaluated, which are in agreement with other reports in the literature. In similar experiments, Casanova et al. (2010) found that TGEV, another swine coronavirus, can remain infectious on hard non-porous surfaces for up to 28 days (107). In that study, there was a 3.2 log reduction in TGEV after 28 days at room temperature with 80% relative humidity. Another human coronavirus, SARS, has been actively studied for its survival on different surfaces (21). This virus has been reported to survive for up to 36 h on stainless steel, but the initial concentration of virus in this study

was not reported (148). In a different experiment, Rabenau et al. (2005) reported a 4-log reduction in SARS virus concentration after 9 days of incubation on a polystyrene surface (149). When the survival on smooth plastic was evaluated, SARS virus was able to survive for more than 5 days at room temperatures (150).

These previous studies and others (151,152), indicate that coronaviruses may pose a risk for transmission via contaminated surfaces. Our results showed longer virus survival time (up to 28 days), which indicates that additional mitigation measures (i.e. proper cleaning and disinfection) need to be implemented to minimize risk of virus transmission to swine farms. The primary corona virus transmission risk factor between and within swine herds are contaminated surfaces in livestock trailers (27,153). Our data suggest that PEDV concentration is reduced by about 1 log after 10 days of incubation, while PDCoV concentration is reduced by more than 3 logs within 15 days on all surfaces. These results indicate that both viruses may survive long enough on stainless steel livestock trailers and other surfaces to pose a risk for transmission between farms. Therefore, the risk of coronavirus transmission among swine herds can be reduced by preventing the use of trailers and trucks for several weeks before transporting animals or other materials between herds. However, our data would suggest that the common 2 to 3 day non-use biosecurity practice for transport vehicles is not long enough to cause any significant decrease in virus inactivation, because after 2 days of incubation, there is minimal subsequent decrease in virus concentration. Therefore, the use of effective disinfectants may be a necessity to minimize risk of coronavirus transmission among swine herds via trucks, trailers, and other farm equipment. With this risk being identified, more research is necessary to determine the effectiveness of surface cleaning and

sanitation, as well as the use of commercially available disinfectants, on their ability to inactivate PEDV and PDCoV.

Conclusions

There was no difference in the PEDV and PDCoV inactivation kinetics (delta values) among all the surfaces tested in the study. Complete inactivation was not achieved with either virus, regardless of surfaces tested in this experiment. Virus survival after 10 days for PEDV, and after 28 days for PDCoV suggests the need to develop effective mitigation strategies involving sanitation and disinfection to minimize the risk of transmission of these viruses via contaminated surfaces.

Table 16 Concentration of viable Porcine Epidemic Diarrhea Virus at various time points after inoculation

	Concentration of viable PEDV (Log $TCID_{50}$/mL) on:			
Time (days)	Stainless Steel	Aluminum	Plastic	Galvanized Steel
0	3.51	3.51	3.51	3.51
1	2.51	2.51	2.51	2.51
2	2.51	2.51	2.51	2.51
5	2.45	1.70	1.51	2.51
10	2.70	2.18	2.18	2.18
Total reduction in virus titer after 10 days	0.81	1.33	1.33	1.33

Table 17 Concentration of viable Porcine Delta Coronavirus at various time points after inoculation

	Concentration of viable PDCoV (Log $TCID_{50}$/mL) on:			
Time (days)	Stainless Steel	Aluminum	Plastic	Galvanized Steel
0	5.51	5.51	5.51	5.51
1	3.32	2.55	2.64	3.25
3	3.35	3.05	3.46	3.46
7	3.24	3.46	3.21	3.17
10	2.64	3.11	3.11	2.51
14	2.98	3.42	2.58	3.07
15	1.51	1.51	1.51	1.51
21	1.58	1.51	1.58	2.00
28	1.77	1.84	2.03	2.21
Total reduction in virus titer after 10 days	2.86	2.40	2.40	3.00

Table 18 Kinetic parameter and correlation coefficient corresponding to the Weibull model and log linear model fitted to Porcine Epidemic Diarrhea Virus and Porcine Delta Coronavirus survival curves on various surfaces

Item	Stainless Steel	Aluminum	Plastic	Galvanized Steel	*P* - value
Porcine Epidemic Diarrhea Virus					
Log Linear Model					
D-value, days	31.85 ± 14.15	9.60 ± 0.40	9.20 ± 0.00	11.50 ± 0.00	>0.05
Adjusted R^2	0.14	0.20	0.16	0.32	
Weibull Model					
Delta, days	7.72 ± 7.16	0.79 ± 0.10	0.69 ± 0.00	1.74 ± 0.00	>0.05
Adjusted R^2	0.88	0.67	0.56	0.94	
Porcine Delta Coronavirus					
Log Linear Model					
D-value, days	8.99 ± 0.54	10.23 ± 1.56	10.03 ± 1.05	10.25 ± 1.37	>0.05
Adjusted R^2	0.63	0.48	0.43	0.53	
Weibull Model					
Delta, days	0.63 ± 0.62	0.61 ± 0.56	0.27 ± 0.24	0.47 ± 0.60	>0.05
Adjusted R^2	0.85	0.62	0.69	0.85	

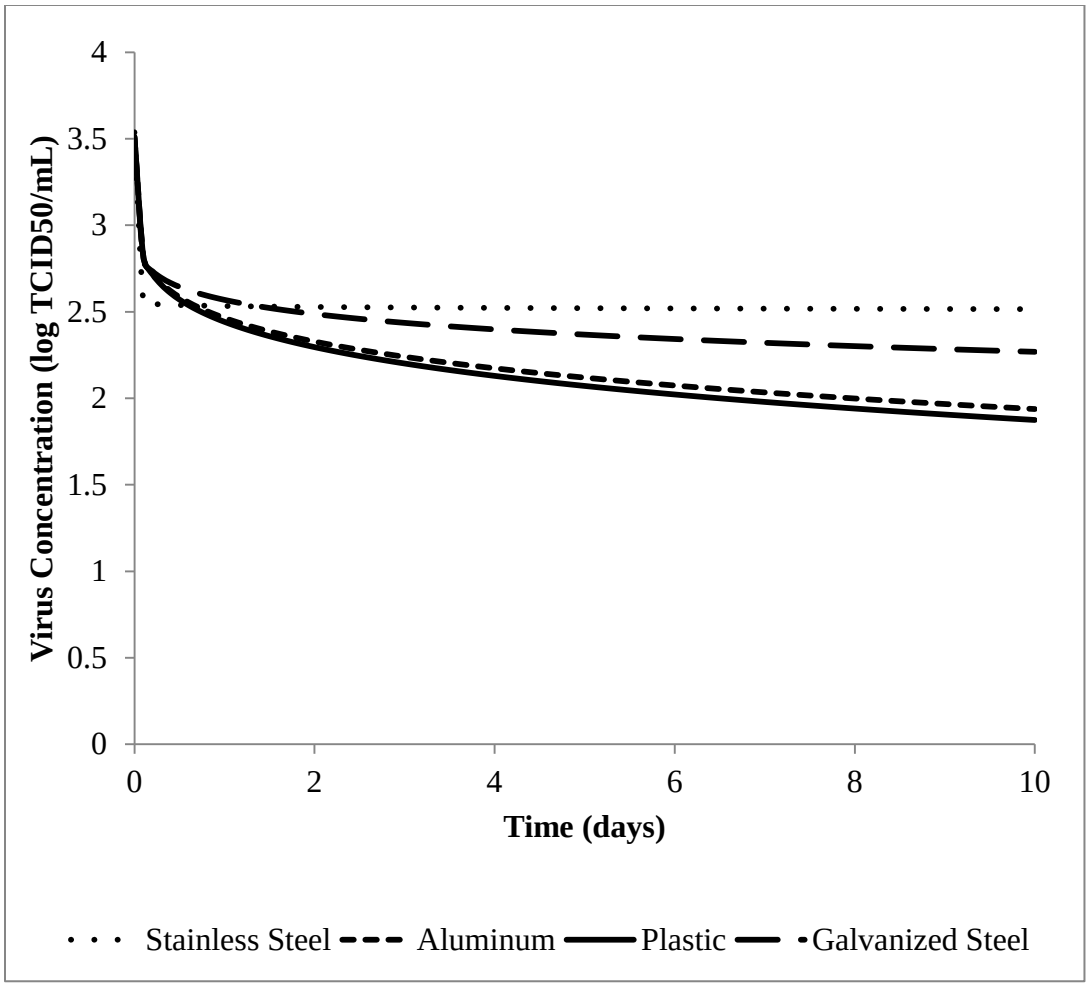

Figure 4 Inactivation curves developed by the Weibull model for Porcine Epidemic Diarrhea Virus on different surfaces

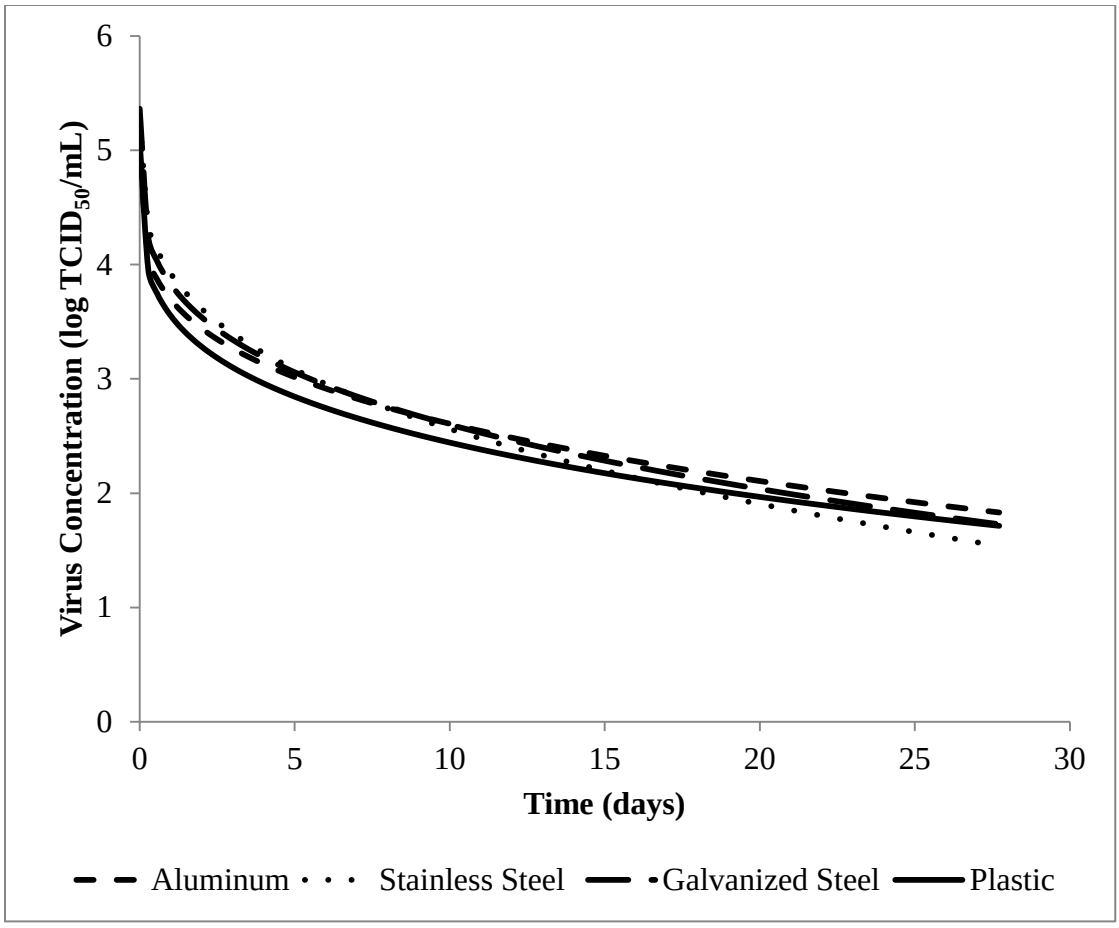

Figure 5 Inactivation curves developed by the Weibull model for Porcine Delta Corona Virus on different surfaces

Overall Summary

The overall goal of this research was to evaluate the risk of porcine coronavirus transmission through feed ingredients. This included the characterization of virus survival in feed ingredients through storage, thermal processing, eBeam irradiation, when exposed to feed additives, and when in contact with surfaces in a feed mill. By identifying virus survival through these processes, the evaluated conditions can be compared to the conditions used in feed mills and during feed ingredient processing. This comparison can then be used to identify critical control points as in a HACCP plan in the feed manufacturing chain where virus may contaminate the feed. It can also be used to make recommendations on how ingredients should be handled and stored to reduce the risk of virus contamination and transmission. When all the information is combined, the overall findings can then be used to create strategies to improve feed safety by keeping feed free from coronavirus contamination.

Results in Chapter 2 suggest that coronavirus survival is higher in soybean meal compared to other feed ingredients. This indicates that the handling of soybean meal may need to be adjusted to take extra precautions to avoid contamination. When reviewing the processing conditions in soybean meal, drying (55-60 ºC for 30 min or less for drying) and solvent extraction processes (65-75 ºC for conditioning) should be sufficient to reduce the concentration of PEDV in the ingredient. This is based off of our findings that heating at 60 ºC for 30 min was able to cause a 3 log reduction in PEDV concentration. After the toasting process, soybean meal should follow the most direct route to the feed mill and extra biosecurity procedures should be taken during its transportation. This

could include truck washing, and additional requirements for truck driver contact with animals. Since storage has minimal impact on the survival of coronaviruses in soybean meal, these extra steps should be developed towards prevention of the initial contamination of the ingredient. In addition to the findings on the behavior of coronavirus in soybean meal, we observed an increase in virus survival with an increase in ingredient moisture content for TGEV and PDCoV survival and moisture content. It is important to note that this correlation does not indicate causation, but it does indicate an association that would be an interesting topic for future research. This is especially true considering the multiple drying steps in processing the different feed ingredients. A better understanding of coronavirus survival comparing humidity, moisture content, and water activity would help to fulfill some of these research gaps.

Results from Chapter 3 identified that thermal treatment was effective at reducing PEDV concentration in feed ingredients. There were no statistical differences in the virus survival for the nine feed ingredients tested, indicating that same processing conditions (time and temperature) can be effective to inactivate PEDV in any ingredient. The results from this experiment showed a maximum reduction of 4 logs after the combination of temperatures of 90°C for 30 min. The method of drying corn uses 94 °C for 2 hours and based on our findings, this would be capable of significantly reducing the risk of PEDV survival. Other findings suggest that the conditions used for drying soybeans (55-60°C for 30 min) are capable of reducing virus concentration by 3 logs. Overall, none of the thermal treatments between 60-90°C were able to cause complete inactivation of PEDV, but the time and temperature treatments can be compared with current feed ingredient processed to determine an approximate log reduction at that step. Since each feed

ingredient has multiple processing steps, the total log reduction at the end of the process can be estimated by adding the partial virus reduction after each step.

Results from chapter 4 identified multiple additional processing steps that can be combined with thermal processing to maximize the overall reduction in virus concentration. These methods included additional temperature treatments, irradiation, and the use of additives. This could mean adding a 50 kGy irradiation treatment to the complete feed or using a feed additive to add a 4-5 log reduction in addition to the log reduction experienced in the previous ingredient processing.

Results from chapter 5 focused on the survival of PEDV and PDCoV on surfaces that may be encountered in a feed mill. This information is useful if a feed mill is contaminated and it needs to be determined how long the virus can survive in the environment. In our experiment, complete inactivation was not achieved in 28 days for PDCoV or 10 days for PEDV. This suggests that if a feed mill is contaminated, coronaviruses can survive an extended period of time and alternative measures such as feed sequencing will need to be used to clean the mill.

The presented experiments had a few limitations that were experienced across all 4 experiments. The first of these was the initial virus titer for the PEDV experiments. Our maximum titer for PEDV was 10^4 $TCID_{50}$/mL. These virus titers are much lower than those observed in the field (10^{10} $TCID_{50}$/mL) and so even if we were able to achieve complete inactivation, this may not indicate complete inactivation in the wild type strain of PEDV in an industry setting. In addition to this, because of our low virus titers, 1mL of liquid media containing virus was added to each feed and ingredient sample. This addition of liquid impacted the moisture content of the ingredient (about 18%), which

could also impact the overall survival of the viruses. This is especially true considering the correlation between moisture content and virus survival in chapter 2. Because the virus titer was already low, the amount of inoculum could not be reduced without once again limiting our potential for log reduction any more. The final limitation in our experiments was the variability between replicates. Though measuring the $TCID_{50}$/mL was the most efficient way to measure viable virus without completing a bioassay using piglets, which would take into account all the aspects of the virus infecting a live pig, it still relied on an individual reading and interpreting plates to determine the extent of cytopathic effects. Though our experiments used the same, trained individual to complete this task, there is still a level of variability introduced when relying on human interpretation of the data. Increasing the number of replicates for each experiment can reduce the error induced using human measurements.

Even with these limitations, our results were still able to provide a comparison between survival in different feed ingredients and the effectiveness of different processing treatments to reduce virus survival. These results can then be used as stepping-stones for future research on virus survival in feed ingredients. In addition to this, it provides information that can be used to improve and develop feed mill biosecurity plans to ensure feed that arrives on the farm is safe and free of pathogens.

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