Inactivation of porcine reproductive and respiratory syndrome virus and Seneca virus A in cell culture using chemical feed additives

by

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Abstract

Seneca virus A (SVA) and porcine reproductive and respiratory syndrome virus (PRRSV) have caused significant economic losses to swine production in the United States. Recently, contaminated feed and feed ingredients have been considered risk factors for swine virus transmission. The use of chemical feed additives has been discussed as one potential management strategy to mitigate this risk. The objective of this study was to evaluate the efficacy of medium chain fatty acid-based (MCFA) and formaldehyde-based (FORMALD) liquid antimicrobials against SVA and PRRSV in a cell culture model. Viral stocks of SVA and PRRSV (10^6 50% tissue culture infectious dose per ml, $TCID_{50}$/ml) were mixed with different concentrations of MCFA or FORMALD in minimum essential media. Ten-fold serial dilutions of each virus-mitigant mixture were performed in triplicate for inoculation onto confluent monolayers of porcine kidney (PK-15) and African green monkey kidney (MARC-145) cells for SVA and PRRSV, respectively. Viral titers after exposure to each mitigant were determined by the serial dilution endpoint method and calculated using the method of Reed and Muench. The differences between the titer of the control virus (no mitigant) and the mitigant-treated samples were used to measure antiviral activity. FORMALD reduced the titers of both SVA and PRRSV at concentrations above 0.6% and 0.15%, respectively. MCFA reduced the PRRSV titer at concentrations greater than 0.25%. No significant reduction of SVA titers were detected after exposure to MCFA up to 5%. This study provides evidence suggesting that both MCFA and FORMALD may be used as potential feed additives to mitigate the risk of SVA or PRRSV transmission through contaminated animal feed or ingredients.

Table of Contents

List of Figures

List of Tables

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Dedication

I dedicate this project to my family who have been supportive throughout my life.

Chapter 1 - Literature review

1.1 Transboundary animal diseases

Transboundary animal diseases (TADs) are among the greatest threats to animal health and livestock agriculture throughout the world. As defined by the United Nations Food and Agricultural Organization (FAO), TADs are of significant economic, trade and/or food security importance for numerous countries (Domenech et al. 2006). These diseases have the potential to spread to other countries and can reach epidemic proportions. Control, management, and exclusion requires cooperation between all affected countries. These diseases are categorized under List A diseases by the World Organization for Animal Health (OIE) as having had direct and indirect impacts on the economies of both developed and developing countries (Domenech et al. 2006). TADs are characterized by several factors, including 1) the severity of losses caused by the disease, 2) the distance over which these diseases can be transmitted by livestock or livestock by-products, 3) the speed of transmission, 4) the susceptibility of each disease to various control measures, 5) the availability of accurate diagnostics testing, and 6) the current scientific knowledge of the disease (Domenech et al. 2006; Rossiter and Hammadi 2009). Historically, the OIE has reported on the global cases of economic and livestock production losses caused by TADs worldwide. For example, in 1997, there was an outbreak of foot and mouth disease (FMD) in Taiwan. To contain and eliminate the disease, 3,850,746 pigs at 6,147 infected pig farms were culled within two months (Yang et al. 1999a). This outbreak cost Taiwan an estimated $378.6 million U.S. dollars, with economic losses associated with the cost of disease control and containment. Additionally, costs associated with eliminating pork exported to Japan from Taiwan was around $1.6 billion U.S. dollars (Yang et al. 1999). In that same year (1997), the Netherlands encountered significant economic losses from pig production

by culling of 429 infected herd and about 1300 at-risk herds were slaughtered due to an outbreak of classical swine fever (CSF) (Stegeman et al. 2000). As another example, the FMD outbreak of 2001 in the United Kingdom led to tremendous economic losses, including those directly and indirectly related to agriculture and related industries, up to 3.1 billion Pounds Sterling (Thompson et al. 2002). The U.S. has remained free of FMD for almost a century. An FMD outbreak in the U.S. would greatly affect the export market, as the U.S. is one of the largest exporters of pork and beef in the world (Hayes et al. 2011). Most of these costs will be associated with attempts to eliminate the virus by culling both infected and at-risk herds. Costs due to market losses have been estimated to be approximately $14 million in the U.S. due to an FMD outbreak (Paarlberg, Lee, & Seitzinger, 2002). TADs have affected the U.S. market by directly affecting production and the cost of managing the outbreak. This, in particular, is evidenced by an outbreak of porcine epidemic diarrhea virus (PEDV) in the U.S. in 2013 (Stevenson et al. 2013) and the current cost of porcine reproductive and respiratory syndrome virus (PRRSV), a disease which has emerged and re-emerged in U.S. swine over the past 3 decades (Charerntantanakul 2012; Nelsen, Murtaugh, and Faaberg 1999). First, the PEDV outbreak impacted the supply of pork products due to the significant loss of pigs to disease; within one year after the initial PEDV case had been detected, an estimated 7 million pigs had died due to PED (Schulz and Tonsor 2015). This resulted in a substantial animal welfare and economic impact to pork producers. Despite the substantial effect of PED on U.S. pork production over the last several years, PRRSV has been widely considered to be the costliest disease of swine production in the U.S. since its introduction. PRRSV is estimated to cost the U.S. more than $600 million annually, equivalent to $1.8 million/day (Holtkamp et al., 2012). Furthermore, Seneca virus A (SVA) is a third disease of importance to the U.S. swine industry

due to its emergence as a cause of vesicular disease since 2015 and its important clinical similarities to FMD (Baker et al. 2017; Raquel Arruda Leme et al. 2019; Segalés et al. 2017). Globally, there are many factors that can contribute to the spread of TADs to new areas, countries or regions. Specifically, human travel, live animal movement, animal by-product transport, fomites such as transportation vehicles, agricultural equipment, and the more recently recognized movement of contaminated animal feed and feed ingredients have all been described as potential risk factors for pathogen introduction and spread (Bowman et al. 2015; S. A. Dee et al. 2018).

1.2 Risk factor analysis for introduction of transboundary swine viruses

The U.S. swine industry first recognized the significant role that imported and distributed animal feed and ingredients may play in pathogen introduction and transmission after the 2013 outbreak of PEDV. The initial introduction and subsequent spread of PEDV was linked with contaminated swine feed (S. Dee et al. 2014). Consequently, most studies related to virus transmission and spread in feed have been performed on PEDV, which may serve as a model for other swine pathogens of transboundary significance such as PRRSV and SVA.

1.2.1 Porcine epidemic diarrhea virus (PEDV)

PEDV is an enveloped positive-sense, single-stranded RNA virus belonging to the family *Coronaviridae* and the genus Alphacoronavirus (Stevenson et al. 2013). It is a moderately sized virus with a genome of 28kb and is the causative agent of porcine epidemic diarrhea (PED) (Lee 2015). Since the introduction of PEDV in the U.S., it quickly became widespread in over 200 herds in thirteen states (Lee 2015). Within 1 year, PED caused major economic losses and the loss of approximately 10% of the swine population due to mortality (Schulz and Tonsor 2015; Stevenson et al. 2013).

PEDV was later reported in Canada and Mexico by 2014 (Lee 2015). Following PEDV's introduction and spread in the U.S., many sources of transmission were proposed including aerosol, contaminated transport vehicles, human movement, breaches in biosecurity, and other fomites (Lowe et al. 2014; Scott et al. 2016). Similarly, in Canada, studies involving animal feeds and animal by-products demonstrating PEDV positivity provided a potential clue on the role of feed in PEDV introduction to the country (Pasick et al., 2014). Following the recognition of contaminated animal feed as a potential risk for virus transmission, several studies have been performed to help define this risk. One such study included feeding naïve piglets contaminated porcine by-product (Opriessnig, Xiao, Gerber, Zhang, & Halbur, 2014), however another study done by (Pasick et al., 2014) indicated that excretion from piglets that were fed PEDV positive feed, tested positive for PEDV for over 9 days post-infection. Following these studies, the hypothesis was developed that contaminated porcine plasma (spray-dried plasma) included in feed had the potential to transmit the virus through natural feeding conditions. Dee et al.,2014 proved the concept of PEDV contaminated animal feed being capable of inducing PEDV infection in naïve piglets. These studies brought attention to whether contaminated feed and/or feed ingredients frequently imported to the U.S. from high-risk countries could have served as sources of PEDV introduction to the country. Whole-genome sequence analysis showed that the U.S. PEDV isolate had 96.6-99.5% identity with all known PEDV strains, and over 99% similarity with that of 2011-2012 Chinese strains, suggesting the possibility that the U.S. PEDV strain may have been introduced from China (Stevenson et al., 2013). Another important study showed that the minimum infectious dose of PEDV in feed was 5.6×10^1 $TCID_{50}$/g and that feed with a PCR cycle threshold value of 37 was sufficient to cause infection in piglets (Schumacher et al., 2016). Importantly, this information revealed the low dose of PEDV required for infection

and highlighted an important pathogenesis trait of the virus which may have contributed to its rapid spread. Overall, these findings supported the risk of contaminated feed as a risk factor for the introduction and transmission of PEDV and exposed a possible route for other TADs, such as African swine fever virus (ASFV) and PRRSV (Bowman, Krogwold, Price, Davis, & Moeller, 2015).

1.3 Virus survival

The concept of virus transmission through animal feeds and feed surfaces has been associated with emerging and endemic porcine pathogens in the U.S. and increased concerns associated with the risk of introduction or re-introduction and transmission of foreign animal diseases to the U.S. Mitigating the risk of pathogen introduction and transmission through contaminated animal feed and feed ingredients is a primary goal. To further define risk, studies have been conducted to demonstrate the ability of major transboundary swine viral pathogens to survive in feed when subjected to different environmental conditions such as temperature and humidity (S. Dee et al. 2015, 2016) and trans-oceanic shipping models such as trans-Pacific and trans-Atlantic shipping models have been used to demonstrate a survivability of important viruses in swine feed ingredients that are frequently imported to the U.S. (S. A. Dee et al. 2018; Stoian et al. 2020). These models simulated shipping environmental conditions and timelines. Specifically, the trans-Pacific model represents the transportation of swine feed ingredients from China to the U.S., including the simulated contamination of feed ingredients in Beijing, China (S. A. Dee et al. 2018). The trans-Atlantic transportation model represents feed ingredients imported from Europe, specifically from Poland to the U.S. (S. A. Dee et al. 2018).Overall, the model demonstrated that the survival was dependent on virus and ingredient combinations (i.e., certain ingredients promoted the stability of several diverse viruses). Specifically, SVA was shown to be

highly stable across feed ingredients, surviving in 11 of 12 feeds or feed ingredients across the 37-day trans-Pacific model. PRRSV was demonstrated to be less stable, surviving in only 2 ingredients after 37 days. ASFV was also shown to be highly stable with infectious virus being detected in 9 of 12 ingredients after 30 days (Niederwerder et al. 2019).

These viruses survived in specific feed ingredients such as conventional soybean meal, lysine hydrochloride, choline chloride, vitamin D and pork sausage casings. Survival of viruses depends on their structural properties and the specific feed matrices (S. A. Dee et al., 2018). Another study done by (Trudeau et al., 2017) showed that soybean meal preserved PEDV viral infectivity with titers of 0.83 log $TCID_{50}$/ml compared to other animal feed and animal by-products that that had titers of 0.50 log $TCID_{50}$/ml when incubated for 56-day. In a recent review, the impact of feed manufacturing, supply environment, and transport systems were highlighted as additional contributors to virus transmission (Jones et al. 2019). Contaminated surfaces and equipment at feed manufacturing facilities can play a role in the dissemination of viral pathogens following production of contaminated feed and cross-contamination of equipment (Huss et al., 2017).

Recent studies confirmed isolation of mammalian Ortho reovirus in porcine by-product, ring-dried swine blood meal (RDSB) and pig feces in the U.S. (Narayanappa et al., 2015). SVA was recently isolated in swine feed ingredients in Brazil and was associated with a vesicular disease outbreak in commercial swine (Leme, Miyabe, Dall Agnol, Alfieri, & Alfieri, 2019).

1.3.1 Porcine reproductive and respiratory syndrome virus (PRRSV)

Porcine reproductive and respiratory syndrome virus is a small, single-stranded, positive sense enveloped RNA virus, belonging to the family *Arteriviridae* and the order Nidovirales (Rossow 1998). The genome of the virus is about 15kb in length with 11 open reading frames and 2

untranslated regions at the 3’ and 5’ ends (Han, Xu, and Wang 2019). PRRSV is relatively unstable in the environment and generally susceptible to most disinfectants. This virus survived in 2/12 (17%) of the feed ingredients tested in a trans-Pacific shipping model study (S. A. Dee et al. 2018). PRRSV is the causative agent of porcine reproductive and respiratory syndrome, which can cause disease in all age groups of pigs, including disease of significant economic importance to the U.S. with costs estimated at approximately $1.8 million per day (Holtkamp et al. 2012).

1.3.2 Seneca virus A (SVA)

SVA is a small, single stranded RNA virus that is non-enveloped, belonging to the family *Picornaviridae* similar to other important virus causing vesicular diseases, such as FMD (Raquel A. Leme, Alfieri, and Alfieri 2017; Segalés et al. 2017). SVA is the only member of the genus Senecavirus. SVA was initially identified as a cell culture contaminant in 2002. In 2015, SVA was identified as a causative agent of vesicular disease in U.S. swine (Joshi, Fernandes, et al. 2016; Joshi, Mohr, et al. 2016). Although pigs are identified as the natural hosts, antibodies to the virus have also been isolated from cattle and mice (Joshi, Mohr, et al. 2016).

SVA is considered an emerging virus due to a recent increase in cases and distribution of the virus (Segalés et al. 2017). Importantly, clinical signs of SVA are indistinguishable from clinical signs of other important swine vesicular diseases. SVA is stable in the environment and recent studies highlighted the stability of the virus in animal feed, that may contribute to virus introduction and spread (S. A. Dee et al. 2018; Joshi, Mohr, et al. 2016; Raquel Arruda Leme et al. 2019)

1.4 Feed mitigation strategies

Contaminated animal feed and/or feed ingredients have been recognized as a potential source of virus introduction and spread. To mitigate this risk, various mechanisms to inactivate viruses or reduce virus titers in feed have been proposed. Point-in-time mitigation methods such as heat inactivation and irradiation reduce or eliminate the quantity of infectious virus, however, they are prone to cross-contamination (Gebhardt et al. 2018). Chemical feed additives such as medium-chain fatty acids (MCFA), formaldehyde-based additives, essential oils and dietary acidifiers have also been proposed as potential mitigation strategies (Cochrane et al. 2016; S. Dee et al. 2016; Gebhardt et al. 2018). Feed additives associated with antiviral properties may have the advantage of residual duration of activity that controls cross-contamination (Gebhardt et al. 2018).

1.4.1 Medium chain fatty acids (MCFA)

MCFA are comprised of carbon atoms that are 6 to 12 long. They have been used as antimicrobials and have also shown an ability to promote growth when included in animal feed (Hanczakowska 2017; Takeuchi, Sekine, and Aoyama 2008). A recent study has shown the effect of MCFA are concentration dependent with higher concentrations having more effect on viral membranes than lower concentrations (Cochrane, Dritz, et al. 2016; Thormar et al. 1987). MCFA appear to have increased efficacy at a lower pH around 4.2 than at a neutral or high pH (Thormar et al. 1987). Capric acid (10:0) and lauric acid (12:0) have been shown to have high viricidal activity against Herpes Simplex Virus (HSV) and Visna Virus (VV), when used at a low pH (Thormar et al. 1987; Welsh, Skurrie, and May' 1978). Increasing the contact time between MCFA and each virus increased efficacy with the higher the contact time translating to increased

virucidal effect (Hilmarsson et al.2006). The study by (Hilmarsson et al., 2006) on Visna Virus showed that virus titers were reduced to undetectable levels, a reduction of 4.5 $\log_{10}$ after exposure to lauric acid. This study also showed that lauric acid (12:0) had a greater effect on the virus compared to capric acid (10:0) which had no viricidal effects at a similar concentration. Lauric acid appears to inhibit the titer of Juvin Virus by reducing virus replication (Bartolotta et al., 2001). MCFA reduced the quantity of detectable PEDV RNA and render inactive PEDV in swine bioassay in feed trial (Cochrane, Saensukjaroenphon, et al. 2016; S. Dee et al. 2016; Gebhardt et al. 2018).

1.4.2. Formaldehyde

Formaldehyde based feed additives have been manufactured with the goal of pathogen reduction in feed. An example of one formaldehyde-based feed additive is Sal CURB® (Kemin Industries, Des Moines, IA, USA), which is 37% formaldehyde with a combination of organic acids and their salts (propionic acid and its salt, formic acid and its salts, sorbic acid, phosphoric acid). This product is approved by FDA to control *Salmonella* contamination in poultry and swine feed for up to 21days in feeds or feed ingredients (S. Dee et al. 2015, 2016).

A recent study showed that Sal CURB® rendered some enveloped viruses such as coronavirus inactive (S. Dee et al. 2015, 2016). Although there is no clear mechanism of its action, it may cause cross-linking of proteins or nucleic acid, denaturation of viral cell membrane or lipoprotein membrane by alkylation (Möller et al. 2015; Wilton et al. 2014). Sal CURB® was shown to reduce PEDV infection in naïve piglets (S. Dee et al. 2014). However, Sal CURB® is not approved by the FDA as a means of PEDV mitigation. Formaldehyde-based products have been used as antimicrobial compound in poultry feed (Ricke, Richardson, and Dittoe 2019; Wales, Allen, and Davies 2010).

MCFA AND FORMALDEHYDE BASED FEED ADDITIVES: PROPOSED MECHANISMS OF ACTION AND EFFECT ON VIRUSES		
CHEMICAL/PRODUCT	PROPOSED MECHANISM OF ACTION	EFFECTS ON VIRUSES
MCFA	Penetrates the viral envelope by hydrophobic effects and increase permeability to small molecules (Hilmarsson et al., 2006) that causes leakage through the viral envelope. At high concentrations, can cause disintegration of the viral envelope and viral particle (Thormar et al., 1987).	Effective against enveloped viruses such as vesicular stomatitis virus (VSV), Herpes simplex virus type 1 (HSV-1) and visna virus (VV) (Thormar et al.,1987). Lentivirus related to HIV (Hilmarsson et al., 2006), Herpes simplex virus type 1and 2 (Hilmarsson et al.,2006) Juvin virus (Bartolotta et al., 2001). PEDV (S. Dee et al. 2015, 2016)
FORMALDEHYDE	Interacts with virus proteins and nucleic acid, can cause denaturation of microbial protein and acts on nucleic acid by alkylation (Maris,1995).	Effective against enveloped viruses-such as PEDV and Turkey coronavirus (TCoV) (S. Dee et al. 2015, 2016) Poliovirus (Wilton et al.2014)

Table 1: The proposed mechanism of MCFAs and formaldehyde and their effects on example viruses that are rendered inactive by the mitigants.

1.4.3 Advantages and disadvantages of chemical feed additives

MCFA have been added to nursery diets to replace in-feed antimicrobials for reducing pathogens and improving pig performance with no significant effect on the gut microbiome (Gebhardt, Thomson, and Woodworth 2017). One study suggested that MCFA can be more easily digested than other sources of lipids and may reduce the accumulation of body fat (Takeuchi et al.2008).

Several studies have been performed on MCFA to demonstrate antiviral activity against many human enveloped viruses (Hilmarsson et al.,2006). MCFAs have been used as a blend of caproic, caprylic and capric acids, at a 1:1:1 ratio in feed studies (Cochrane, Saensukjaroenphon, et al. 2016; Jordan T. Gebhardt et al. 2018), where 2 inclusion levels have showed efficacy against PEDV. Medium-chain triglyceride (MCT) added in weanling pigs diet improved average daily gain and digestibility during the first 2 weeks post-weaning (Lai, Yen, Lin, & Chiang, 2014). Lauric acid is a medium-chain fatty acid with the additional benefit of being formed into mono-Laurin in the human or animal body (Hanczakowska, 2017). Mono-Laurin is the antiviral, antibacterial and antiprotozoal monoglyceride that has demonstrated effects on enveloped viruses such as HIV, herpesvirus, cytomegalovirus, and influenza virus (Hanczakowska, 2017). Formaldehyde based feed additives or 10% medium-chain fatty acid (MCFA) treatment of rice hulls reduced the quantity of detectable PEDV RNA compared to untreated rice hulls. Subsequently, the chemically treated feed was demonstrated to be non-infectious (Gebhardt et al., 2018). Formaldehyde has been used as a broad-spectrum antimicrobial in poultry and swine farms to reduce microbial contamination (Ricke, Richardson, and Dittoe 2019; Wales, Allen, and Davies 2010). (Campbell et al. 2018) suggested that formaldehyde added into pig feed did not reduce pig growth performance and pigs were fed with formaldehyde treated spray dried porcine plasma showed increase in growth rate compared to the pigs in control group. In human medicine, there have been reports that MCFA has an ability to increase satiety and reduce feed intake over time (Takeuchi, Sekine, and Aoyama 2008) Thus, MCFA have been used to prevent and/or treat obesity (Coleman et al., 2016).Formaldehyde can be highly irritating and is a toxic agent to both humans and animals when inhaled or via direct contact (Watt 1912). Formaldehyde has also been described as a carcinogenic agent to humans; however, 0.1% of formaldehyde

included in animal feed is considered to have minimal effect in both animals and humans (Swenberg et al. 2013), However, because of feed safety and biosecurity, proper handling of treated feed is highly recommended. It also suggested that treatment of feed with 37% formaldehyde (Sal CURB®) reduced pig growth rate (Campbell, 2018b). Formaldehyde based feed additives may hinder protein availability in animal feed due to the ability to denature and cause cross-linking of protein (Devi, Balasubramanian, Kim, & Kim, 2016).

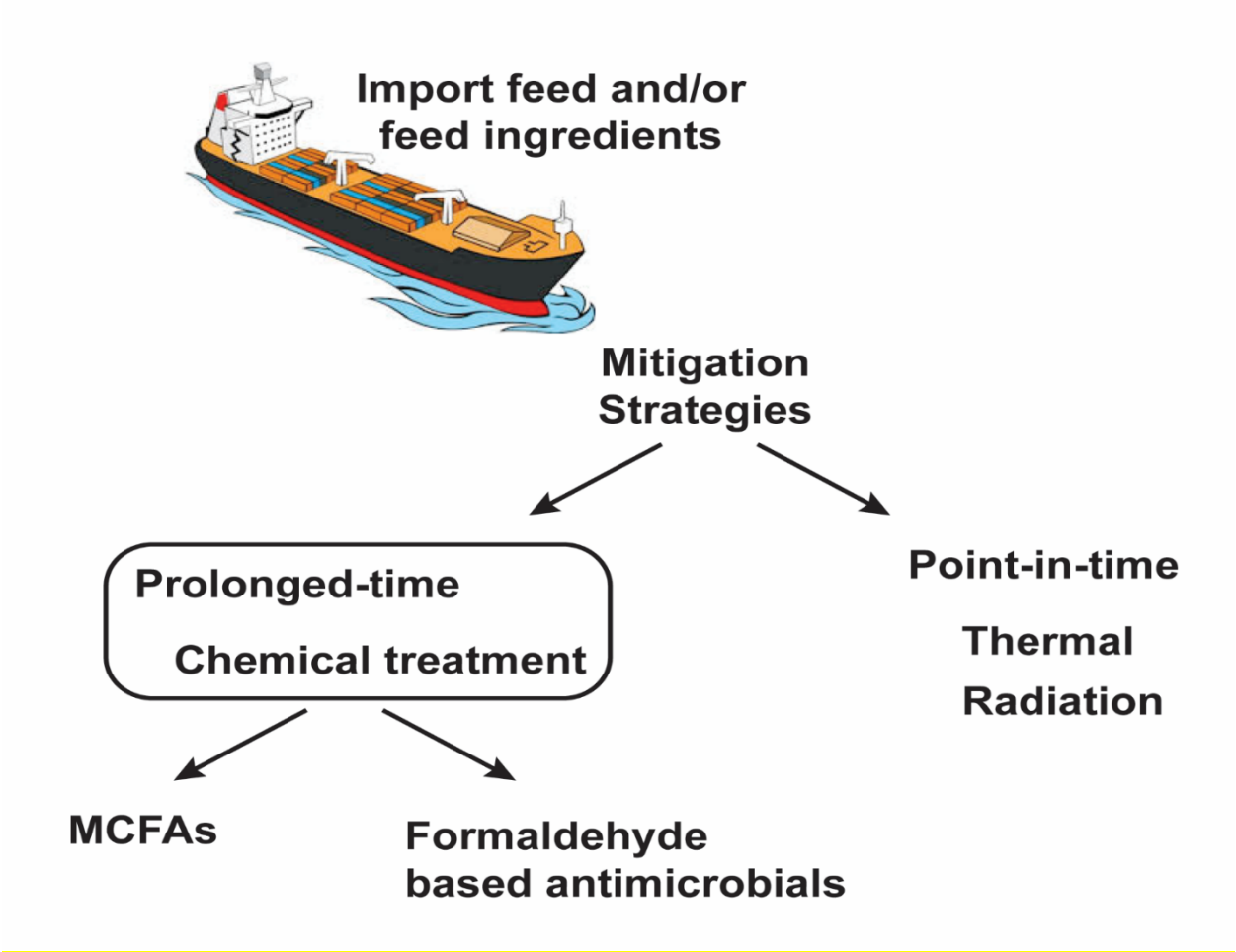

Figure 1: Schematic illustration of mitigation strategies for potentially contaminated imported feed and/or feed ingredients to the U.S.

Chapter 2 - Inactivation of Seneca virus A using medium-chain fatty acids and formaldehyde-based feed additives in cell culture

2.0 Introduction

U.S. swine endemic viruses

Endemic swine viruses for the purposes of this work are considered those viruses that are currently circulating in the U.S. Although endemic viruses are present within the country, there are substantial differences on the distribution and prevalence of each virus from herd to herd, county to county, and state to state. There are several factors that may contribute to the prevalence, introduction and re-introduction, transmission or spread of these viruses. Based on the recent finding that many viruses can survive in animal feed and or feed ingredients that are widely distributed throughout U.S. swine herds, this work has focused on investigating mitigation strategies for two important endemic diseases of swine to the U.S., including SVA and PRRSV. SVA and PRRSV are both currently circulating swine viruses that have potential to impact the health, welfare, and economy of the swine industry. Both viruses were shown to survive in animal feed and/or feed ingredients subjected to transoceanic conditions (S. A. Dee et al., 2018), with SVA surviving in 11 of the 12 ingredients tested and PRRSV surviving in 2 of the 12 ingredients tested. As both endemic diseases and foreign viral diseases of swine are of great importance to sustainable pork production and markets within and outside the country, this calls for significant investigation and control strategies to mitigate the risk (Jones, Woodworth, Dritz, & Paulk, 2019).

2.1 Seneca virus A

SVA is a small, single-stranded, positive sense, non-enveloped RNA virus belonging to the *Picornaviridae* family and the only member of the genus Senecavirus (Joshi, Mohr, et al.

2016).The virus is also known as Seneca Valley virus (SVV). The genome of SVA is approximately 7.2 kb and the virus has an internal ribosomal entry site that allows translation in the cap-independent manor (Segalés et al. 2017).

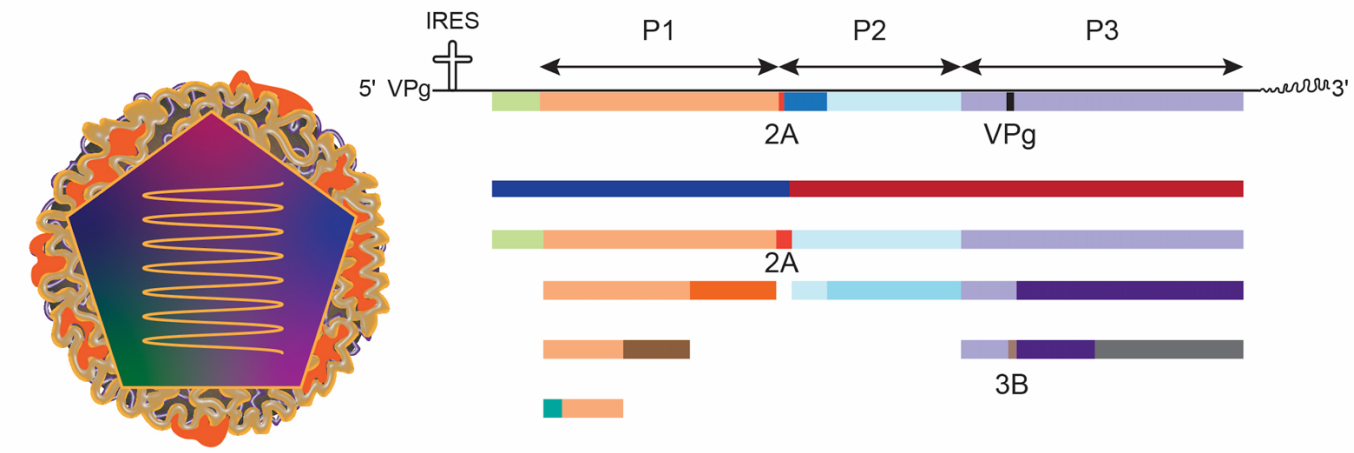

Figure 2: Genome and structure of Seneca virus A.

SVA was incidentally discovered as a cell culture contaminant during cultivation of PER.C6 cells (fetal retinoblastoma cell line) (Burke, 2016). The source of the virus was associated with the use of animal by-products such as serum or trypsin of porcine origin (Burke, 2016). Following sequence and serological analysis, retrospective studies between 1988 and 2005 identified 6 closely related picorna-like viruses isolated in different regions of the U.S. that seemed to cause various clinical signs. SVA was one of these viruses and this showed that the virus has been circulating in U.S. pigs for a long time before it was officially identified (Zhang et al., 2018).

Figure 3: World distribution of SVA. Countries marked in blue have detected SVA cases in pigs.

2.2 Why is SVA important

SVA is a causative agent of porcine vesicular disease in pigs, initially identified as an idiopathic vesicular disease (IDV), which has a wide geographic distribution (Segalés et al. 2017). The disease is characterized by infrequent cases of vesicles and erosions on the skin, snout, oral cavity, and coronary bands (Raquel A. Leme, Alfieri, and Alfieri 2017b). Clinical signs of the disease are indistinguishable from that of other (foreign) animal vesicular disease causative agents. The virus is of less economic significance, but due to clinical signs resembling that of devastating vesicular foreign animal diseases, such as foot and mouth disease, swine vesicular disease, vesicular stomatitis, and vesicular exanthema of swine, diagnostic testing is always required to rule out important foreign animal vesicular diseases. SVA has been reported in seven other countries (Leme, Alfieri, and Alfieri 2017).

There is no commercially available vaccine for SVA. Various research has been done to describe the virus along with the establishment of rapid diagnostic methods (Zhang et al., 2018) . SVA is considered to be an emerging or re-emerging virus in different geographical locations due to the increased incidence of the disease (Segalés et al. 2017).This highlights the ability of the virus to emerge and re-emerge in new geographical locations and ability to cause novel conditions in pigs around the world. SVA has been used as an important surrogate for foot-and-mouth disease virus, a foreign animal disease pathogen in the U.S., which requires biosafety-level 4 (BSL-4) laboratory containment conditions. Both SVA and FMDV are in the *Picornaviridae* family and have similarity in genetic and physiochemical properties (S. A. Dee et al., 2018).

2.3 Materials and Methods

1. Mitigants and effect on cells

Mitigants tested in this experiment included MCFAs, which consisted of a blend of capra fatty acids (C6: Caproic acid, C8: Caprylic acid and C10: Capric acid at a ratio of 1:1:1) and formaldehyde-based liquid (FORMALD, SalCURB®, Kemin Industries, 37% formaldehyde with organic acids and their salts). Cells utilized in this experiment were PK -15 (porcine kidney cells, PK15, PK15; ATCC® CCL33™). To determine if the mitigants induced a cytopathic effect on PK-15 cells, 100 µl of a 5% concentration of MCFAs or FORMALD was added onto confluent monolayer of PK-15 cells. Cells with Minimum essential media (MEM) only was used as a negative control where 100 µl of MEM was added onto confluent monolayers of PK-15 cells followed by three days incubation at 37°C. Mitigants were discarded after three days and cells were washed three times using 100 µl of PBS and viewed under fluorescent microscope.

2. Cells and virus

PK-15 cells in DMEM media with 7% heat-inactivated fetal bovine serum (FBS), streptomycin, penicillin and antimycotics were seeded in a 96-well plate, incubated in a 5% CO_2/95% air atmosphere at 37°C. Cells were grown to 95-100% confluence. The Seneca virus A isolate (SVA; GenBank accession No. KX349734) (Chen et al. 2016) had a green fluorescent tag and was kindly obtained from Dr. Ying Fang at the University of Illinois.

3. Virus titration

Confluent PK-15 cells in a 96-well plate were utilized to titer the initial virus stock. Serial ten-fold dilutions were performed in MEM media and each dilution was inoculated in triplicate onto confluent cells. Cells were incubated at 37°C for 2-3 days. Plates were examined under a fluorescent microscope every 24 hours. The endpoint dilution method was used to determine the virus titers calculated using the method of Reed and Muench (REED and MUENCH 1938) Initial virus stocks had titers of 10^8 - 10^9 $TCID_{50}$/ml.

4. Mitigants

Concentrations of each mitigant were created between 10% and 0.1% in MEM media.

5. Assay of antiviral activity

A 20% solution of mitigant was prepared from the original stock solution and 100 µl of 20% mitigant solution was mixed with 100 µl of SVA (10^6 $TCID_{50}$/ml), resulting in a final concentration of mitigant of 10%. This was repeated for other mitigant concentrations tested between 0.1% and 5%. Positive control included 100 µl of virus and 100 µl of MEM maintenance media in place of the mitigant. Negative controls included media alone with cells (no virus or mitigants). The 96-well plate was then used to perform 1:10 serial dilutions of the mitigants/SVA mixtures of the virus described above. Plates were incubated for 30 minutes at room temperature prior to being plated onto a separate 96-well plate previously seeded with PK-

15 cells. The media on cells was discarded prior to transfer of 100 µl from each well of the dilution plate to the corresponding well of the plate containing PK-15 cells. Cells were 95-100% confluent at the time of infection. Plates were incubated for 48 hours at 37±2°C with 5% CO_2. After 24 hours and again at the end of the 48-hour incubation period, we inspected all wells under fluorescent microscopy. We categorized each well as either positive or negative based on the presence or absence of virally infected cells. Endpoint titers were calculated as described above and mitigant treated samples were compared to positive controls.

2.4 Results

Mitigants had no significant cytopathic effect when inoculated onto the PK-15 cells after three days incubation period.

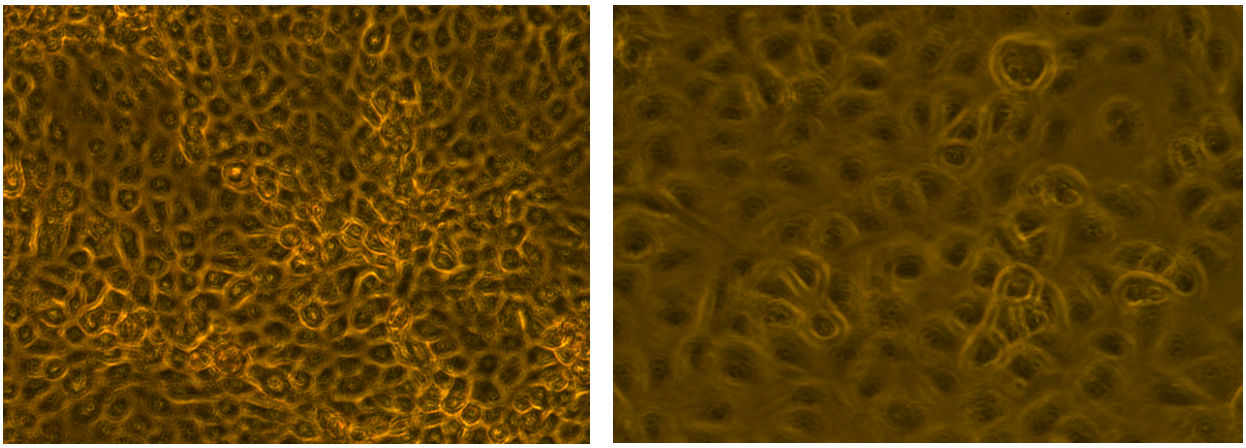

Figure 4: PK-15 cell line treated with MCFAs (left) and FORMALD (right) after three days incubation period. Magnification of 200x.

Assay of antiviral activity:

Exposure to FORMALD demonstrated efficacy for reducing SVA titers in the cell culture model. Specifically, SVA was undetectable under fluorescent microscope when a high concentration (5%) of FORMALD was used. No significant reduction of SVA titers were noted after exposure to up to 5% MCFA.

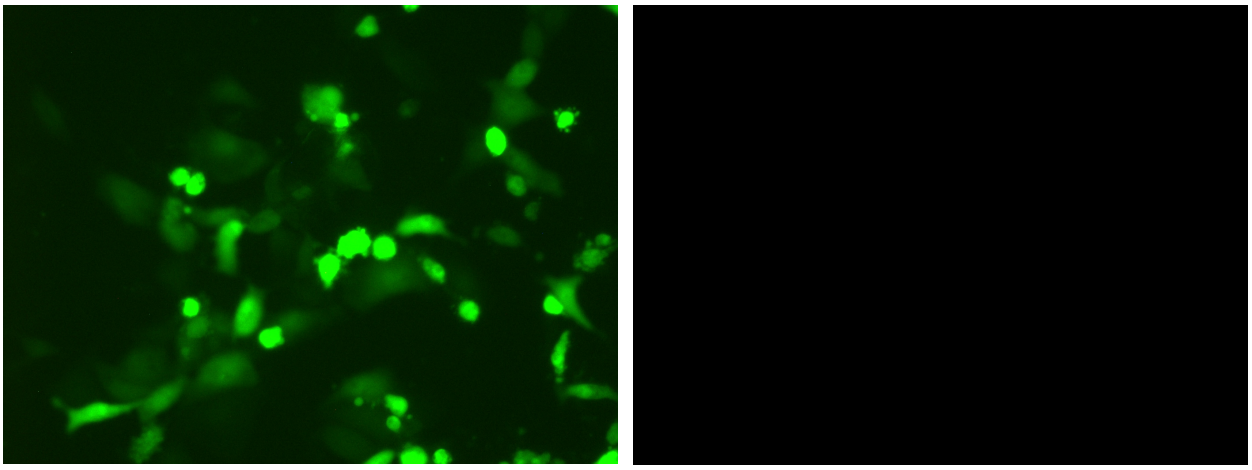

Figure 5: PK-15 cells after being challenged with virus-mitigant mixture, SVA-MCFA (left) and SVA-FORMALD (right). Magnification of 200x.

Dose response inactivation.

Inactivation of SVA using FORMALD:

FORMALD showed efficacy at reducing SVA titers. Concentrations higher than 0.6% caused inactivation of SVA titer to below the level of detection on cell culture while concentrations between 0.15%-0.3% reduced SVA titers by approximately 1 log ($3.16x10^6$ $TCID_{50}$/ml to $3.16x10^5$ $TCID_{50}$/ml). The lowest concentration of FORMALD tested was 0.01875% and no significant reduction of SVA titer was noted as the titer was similar to that of the positive control virus at $3.16x10^6$ $TCID_{50}$/ml.

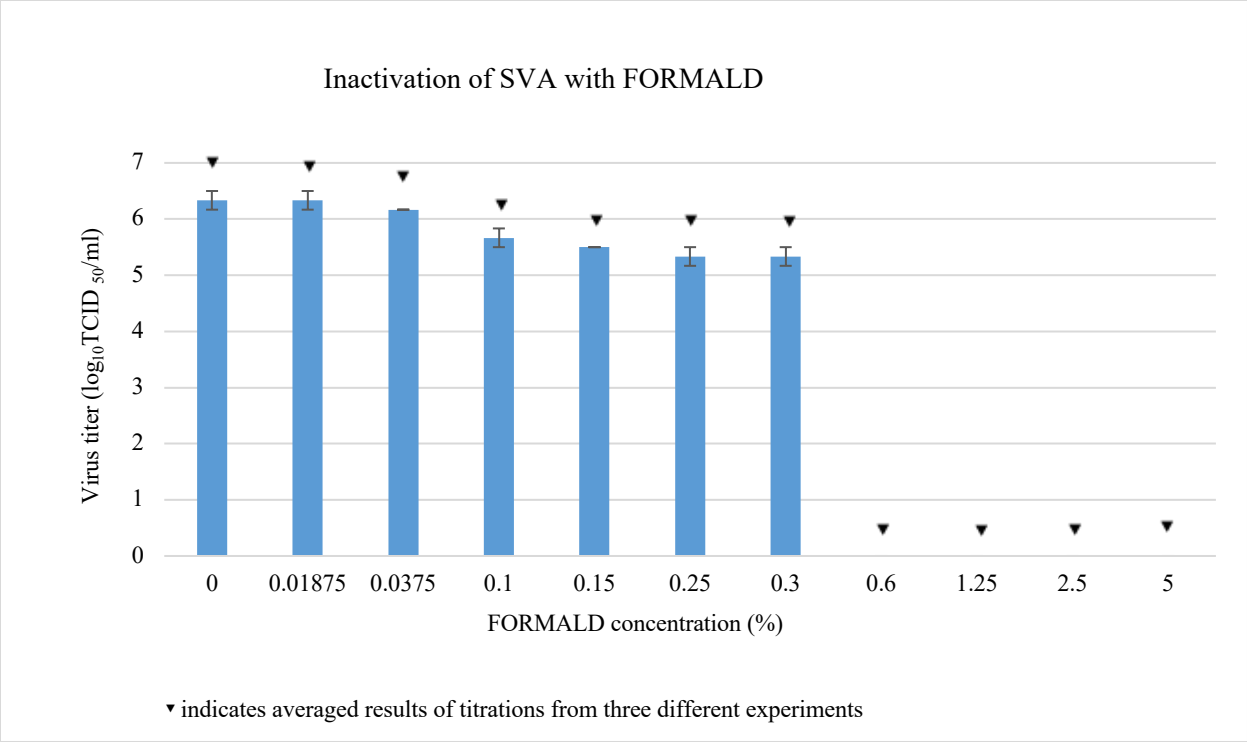

Figure 6: Dose response inactivation curve of FORMALD against SVA.

Inactivation of SVA with MCFA:

Exposure to MCFAs caused no significant reduction of SVA titer even at the highest concentration of 5% up to 30 minutes incubation period.

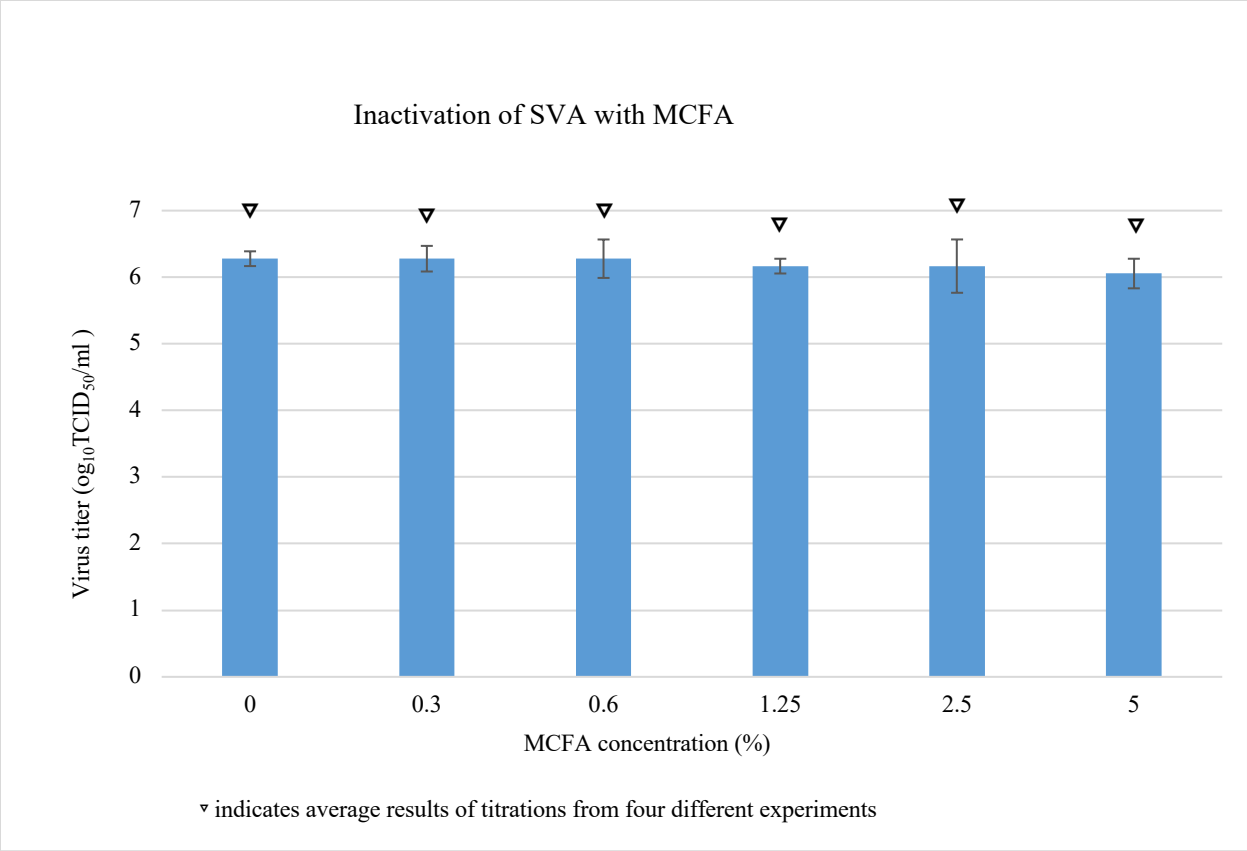

Figure 7: Dose response inactivation curve of MCFA against SVA. Data is shown for titers after no incubation period.

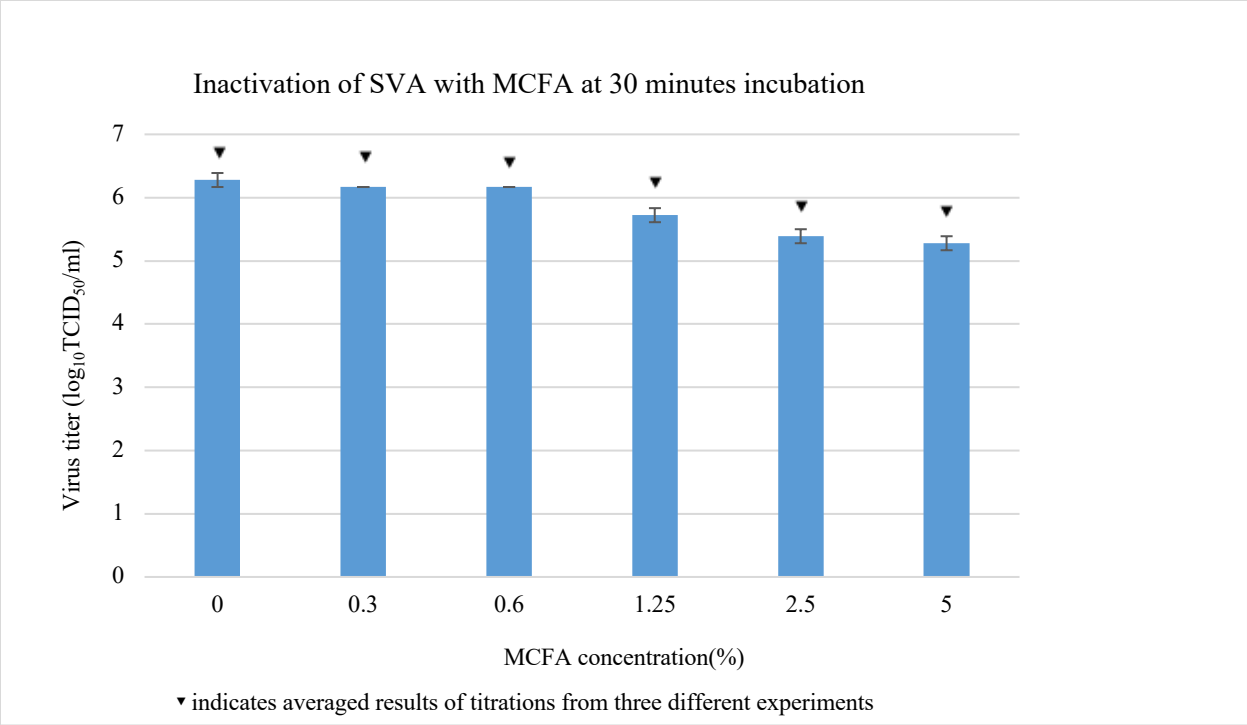

Figure 8: Dose response inactivation curve of MCFA against SVA. Data is shown for titers after 30 minutes of incubation.

The incubation period was later increased to 45 minutes where 5% concentration reduced the SVA titer by approximately 1log.

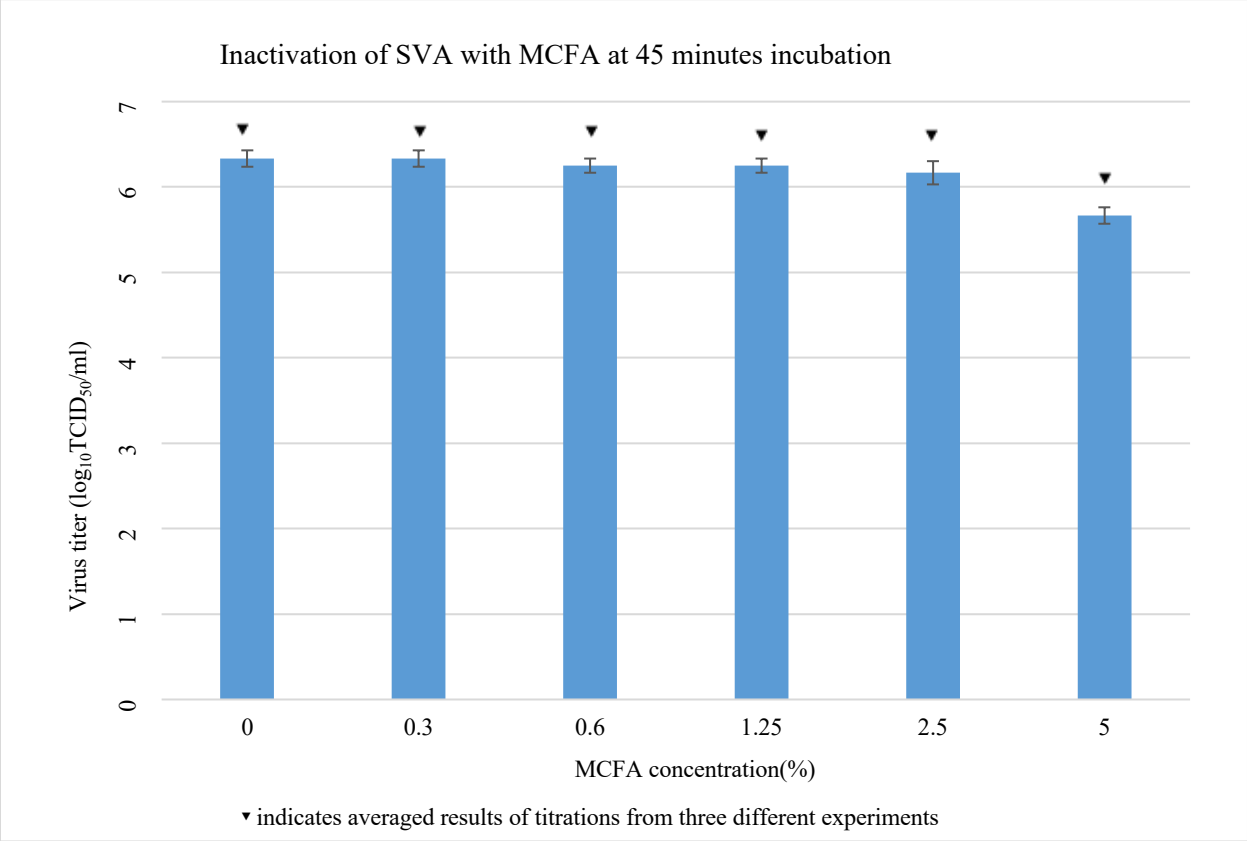

Figure 9: Dose response inactivation curve of MCFA against SVA. Data is shown for titers after 45 minutes of incubation.

2.5 Discussion

FORMALD was effective toward SVA with concentrations greater than 0.6% reducing the virus titer to below that which can be detected in cell culture. The efficacy may be due to the ability of formaldehyde to denature the virus particle or viral proteins by alkylation. However, no significant reduction of SVA titers were noted after exposure to MCFA up to 5% at 30-minutes incubation period. SVA titer was reduced by approximately 1log after the incubation period of 45-minutes at 5% MCFA concentration. Stability of SVA to MCFA may be due to lack of the

viral envelope as MCFAs is considered to have a proposed mechanism of action on viral envelopes.

Chapter 3 - Inactivation of porcine reproductive and respiratory syndrome virus (PRRSV) using medium chain fatty acids (MCFAs) and Formaldehyde based feed additives in cell culture

3.0 Introduction

3.1 Porcine reproductive and respiratory syndrome virus

PRRSV is a single stranded, positive sense enveloped RNA virus. The virus belongs to the family *Arteriviridae* similar to equine arteritis virus, mouse lactate dehydrogenase-elevating virus, and simian hemorrhagic fever virus within the order Nidovirales (Dokland 2010).

The viral nucleocapsid is surrounded by envelope proteins, glycoproteins and membrane proteins to form virion particle (Nelsen, Murtaugh, and Faaberg 1999).The genome has 11 open reading frames (ORF) that code for both structural and non-structural proteins (Charerntantanakul 2012). ORF 1a and 1b code for non-structural protein pp1a and pp1b; these are polyproteins that are later processed into 14 peptides (Rossow 1998). ORF 2-11 carries information for structural proteins such as glycoproteins (2-5) translated from ORF 2-5 (Rossow 1998).The rest of the ORFs encode for three un-glycosylated membrane proteins (E, ORF5a, and M) (Nelsen, Murtaugh, and Faaberg 1999; Snijder, Kikkert, and Fang 2013). A nucleocapsid protein (N) of GP 5 is most abundant and the primary contributor to host immune response (Rossow 1998).

PRRSV is characterized into two genotypes, including the European and North American genotype, also named as genotype one and type two, respectively (Gauger et al. 2012; Rossow 1998; Snijder, Kikkert, and Fang 2013). Both genotypes are found worldwide in countries where PRRSV is endemic. Both genotypes cause similar clinical signs in swine but have significant variability in their nucleotide sequence, with only 60-75% nucleotide similarity (Pei et al. 2009).

Pigs are the natural host of PRRSV. The virus particle has tropism to immune cells like those of the monocytic lineage (Loving et al. 2015). Porcine alveolar macrophages are known to be the primary replication site for the virus; however, replication also occurs in other immune cells like dendritic cells (Loving et al. 2015). Other cell lines such as MARC-145 cells and other related cell lines derived from African green monkey kidney cells are permissive in vitro (Lunney et al. 2016a). Different receptors for the virus have been described with CD163 being the primary receptor (Jay G Calvert et al. 2007). Viral glycoproteins GP2a and GP4 facilitate virus attachment (Dokland 2010). Virus enters the cells through clathrin-mediated endocytosis; endosome acidification after fusion facilitates genome release into the host cell cytosol followed by genome replication in the cytoplasm (Lunney et al. 2016a).

Viral infection can be acute, sub-acute or persistent (Lunney et al. 2016a). Replication of the virus in porcine alveolar macrophages and dendritic cells can lead to lung infection (Lunney et al. 2016b; Rossow 1998). Blood of infected pigs can be PRRSV positive 6-12 days after infection (Lunney et al. 2016b; Rossow 1998). Persistent infection occurs when virus is localized to the tonsil and lymph nodes (Charerntantanakul 2012). The last stage of infection occurs when virus replication ceases, following gradual declines (Charerntantanakul 2012). The severity of disease depends on herd immunity and virus strain (Mondaca-Fernández et al. 2007; Nelsen, Murtaugh, and Faaberg 1999).

PRRSV can be transmitted both directly and indirectly. Direct transmission, which is very efficient, can be horizontal or vertical within infected herds (Charerntantanakul 2012). Infected pregnant sows can be the source of virus to piglets as PRRSV is capable of crossing the placenta during the third trimester (Larochelle and Magar 1997; Lunney et al. 2016a).

Indirect transmission involves contaminated fomites, including transport vehicles, needles, and personnel (Charerntantanakul 2012). PRRSV infection stimulates antibody production by day 7 post infection; however, this antibody does not neutralize the virus and does not provide protection (Loving et al. 2015; Pei et al. 2009). Neutralizing antibodies may be produced by day 28 post infection, however, they still do not provide complete protection (Loving et al. 2015). The virus has been mentioned to suppress innate immune responses (Ansari et al. 2006). Multiple vaccinations or repeated infection may produce neutralizing antibodies that are protective. Serum from infected animals when transferred to naïve pigs has been shown to passively protect the naïve animal (Larochelle and Magar 1997). Over a time, various virus strains have evolved and caused considerable loses as well as increased severity of disease. The virus is prone to mutation and recombination, leading to the development of different strains (J. G. Calvert et al. 2007). In Asia, the highly pathogenic PRRSV strains cause over 20% mortality, associated with high fever and respiratory syndrome (Charerntantanakul 2012). Both the Lena strain and the highly virulent 184 strain have caused severe clinical signs to pigs in Eastern Europe and north-central America, respectively (Charerntantanakul 2012). PRRSV has been isolated as a cofactor in porcine respiratory disease complex (PRDC) and is the main cause of respiratory infection in most cases (Gauger et al. 2012).

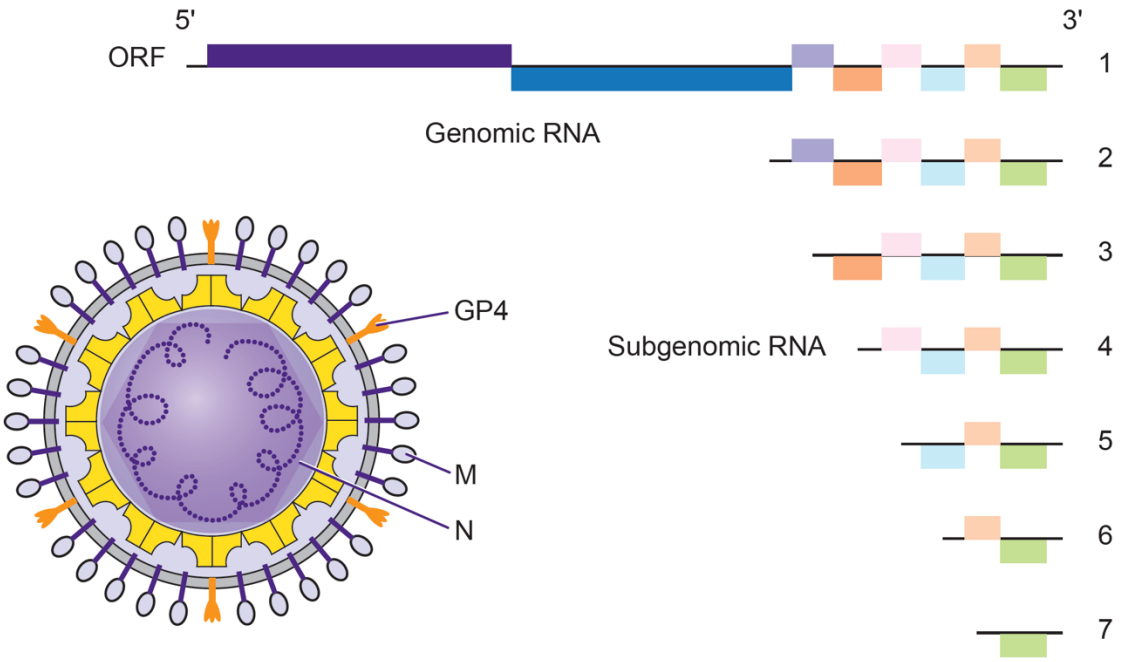

Figure 10: The genome and structure of PRRSV.

Porcine reproductive and respiratory syndrome virus (PRRSV) affects all age groups (Rossow1998). Reproductive impairment or failure is typically noted by increases in the number of stillborn piglets, mummified fetuses, premature farrowing, and weak-born pigs (Larochelle and Magar 1997). Abortions typically occur in less than 10% in the endemic herd (Rossow, 1998). The respiratory syndrome is seen more often in young growing pigs but can also occur in naïve finishing pigs and breeding stock; infection in these age groups can last for up to four months (Mondaca-Fernández et al. 2007; Rossow 1998). PRRSV can reduce weight gain by 85% and cause mortality up to 10%-25% in the infected farm (Rossow 1998). Although reported initially in only a few countries in the late 1980s, PRRS now occurs worldwide in most major swine-raising countries (Charerntantanakul 2012). PRRS is prevalent in the United States and exists both in epidemic and endemic forms. Various control strategies have been developed and established; however, there is no clearly successful control strategy to date (Lunney et al. 2016a).

This is likely due to viral mutation and recombination that leads to highly pathogenic, highly virulent, or new viruses that may reduce the efficacy of available vaccines. (Gauger, Faaberg, Guo, Kappes, & Opriessnig, 2012).

Figure 11: World distribution of PRRSV. Countries marked in blue have detected PRRSV cases in pigs.

3.2 Why is PRRSV important

PRRS causes a tremendous economic loss in the swine industry worldwide. Losses are primarily associated with reproductive failure and mortality of young piglets due to respiratory problems as well as reduced growth (Nelsen, Murtaugh, & Faaberg, 1999). In the United States, PRRSV results in losses of $1.8 million per day, equivalent to $664 million per year (Holtkamp et al., 2012).Veterinary and biosecurity expenses can cost up to $140.11 million and $191.86 annually, respectively, while other related costs can reach $145 million (Holtkamp et al., 2012).

	Individual cost	**Total cost**
Breeding herds	**$302.06 million**	**$664 million annually** ($1.8 million per day). (Holtkamp *et al*., 2012)
Growing pig herd	**$361.8 million**	
Veterinary cost	$140.11 million	**$477.79 million** (Holtkamp *et al*., 2012)
Biosecurity	$191.86 million	
Other outbreak-related costs	$145.82 million	

Table 2: Estimated Annual Cost of PRRS in the U.S.

There is a continual threat from the potential emergence of increasingly divergent and often virulent PRRSV strains (Han et al. 2019) One example is the disease outbreak in China in 2006, where infected pigs developed various clinical signs that led to an increase in the death of pigs of all age groups (Han et al. 2019). PRRSV was identified to be the dominant virus by 40% in the outbreak and the virus was rapidly transmitted between herds in various provinces (Tong et al. 2007). The virus was identified as highly pathogenic and associated with high mortality between 20%-100% and a morbidity rate between 50%-100% (Tong et al. 2007). Glycoprotein 5 (GP5) was identified to have mutations in the highly pathogenic PRRSV virus and other strains (Nelsen, Murtaugh, and Faaberg 1999). Mutation and recombination of PRRSV contributed to emergence of two novel stains recently isolated (ZJnb16-2, SDbz16-2) in China that seemed to not be protected by available vaccines (Han, Xu, and Wang., 2019). The tendency for viral

mutation makes the development of accurate diagnostic tools and a universal vaccine against PRRSV challenging.

The possibility of developing endemic herd infections after an outbreak and the ability of the virus to persist for up to 200 days in carrier pigs contributes to the introduction and maintenance of the virus to the pig population (Charerntantanakul 2012). The virus can be introduced into the herd through direct contact of infected pigs to healthy pigs, as well as various fomites and potentially aerosol transmission. Infected pregnant sows can transmit viruses vertically to piglets and lead to the development of congenital PRRS infection, while shedding of viruses from piglets and sows can lead to infection of other pigs (Charerntantanakul 2012; Dokland 2010b). Infected boars contribute to virus transmission by shedding the virus in semen for up to three-months post-infection and infect sows through natural breeding or artificial insemination (Dokland 2010a; Rossow 1998; Song, Moon, and Kang 2015).

3.3 Materials and Methods

1. Determining effects of mitigants on cells

Mitigants tested in this experiment included MCFAs, which consisted of a blend of capra fatty acids (C6: Caproic acid, C8: Caprylic acid and C10: Capric acid at a ratio of 1:1:1) and formaldehyde-based liquid (FORMALD, SalCURB®, Kemin Industries, 37% formaldehyde with organic acids and their salts). Cells utilized in this experiment were MARC-145 (African green monkey kidney cells). To determine if the mitigants induced a cytopathic effect on MARC-145 cells, 100 µl of a 5% concentration of MCFAs or FORMALD was added onto confluent monolayers of MARC-145 cells. Cells with minimum essential media (MEM) only was used as a negative control where 100 µl of MEM was added onto confluent monolayers of MARC-145

cells followed by three days incubation at 37°C. Mitigants were discarded after three days and cells were washed three times using 100 µl of PBS and viewed under a fluorescent microscope.

2. Cells and virus

MARC-145 cells in DMEM media with 7% heat-inactivated fetal bovine serum (FBS), streptomycin and penicillin as well as antimycotics were seeded in a 96-well plate, incubated in a 5% CO_2/95 % air atmosphere at 37°C. Cells were grown to 95-100% confluence. The PRRSV isolate used was a type 2 was derived from an infectious clone of P129 strain (Pei et al. 2009) and had a green fluorescent tag.

3. Virus titration

Confluent MARC-145 cells in a 96-well plate were utilized to titer the initial virus stock. Serial ten-fold dilutions were performed in MEM media and each dilution was inoculated in triplicate onto confluent cells. Cells were incubated at 37°C for 2-3 days. Plates were examined under a fluorescent microscope every 24 hours. The endpoint dilution method was used to determine the virus titers calculated using the method of Reed and Muench (REED and MUENCH 1938). The initial virus stocks had titers of 10^6 $TCID_{50}$/ml.

4. **Mitigants**

Concentrations of each mitigant were created between 10% and 0.1% in MEM media.

5. Assay of antiviral activity

A 20% solution of mitigant was prepared from the original stock solution and 100 µl of 20% mitigant solution was mixed with 100 µl of PRRSV (10^6 $TCID_{50}$/ml), resulting in a final concentration of 10% mitigant. This was repeated for all other mitigant concentrations tested between 0.1% and 5%. Positive control included 100 µl of virus and 100 µl of MEM maintenance media in place of the mitigant. Negative controls included media alone with cells

(no virus or mitigants). The 96-well plate was then used to perform 1:10 serial dilutions of the mitigants/PRRSV mixtures described above. Plates were incubated for 30 minutes at room temperature prior to being plated onto a separate 96-well plate previously seeded with MARC-145 cells. The media on cells was discarded prior to transfer of 100 µl from each well of the dilution plate to the corresponding well of the plate containing MARC -145 cells. Cells were 95-100% confluent at the time of infection. Plates were incubated for 48 hours at 37±2°C with 5% CO_2. After 24 hours and again at the end of the 48-hour incubation period, we inspected all wells under fluorescent microscopy. We categorized each well as either positive or negative based on the presence or absence of virally infected cells. Endpoint titers were calculated as described above and mitigant treated samples were compared to positive controls.

3.4 Results

Mitigants had no cytopathic effect when inoculated onto the MARC-145 cells, there were no significant changes on the cells compare to the normal monolayer of MARC-145 cells.

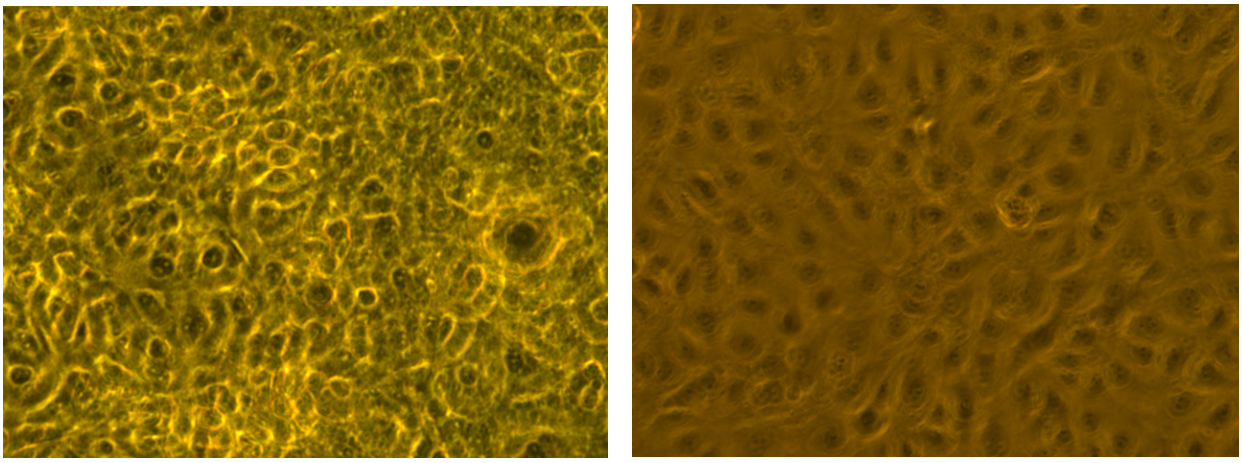

Figure 12: MARC-145 cell line treated with MCFAs (left) and FORMALD (right) after three days incubation period. Magnification of 200x.

Assay of antiviral activity:

Exposure to FORMALD and MCFAs demonstrated efficacy for reducing PRRSV titers in the cell culture model. Specifically, PRRSV was undetectable under fluorescent microscope when a high concentration (5%) of FORMALD and MCFA was used.

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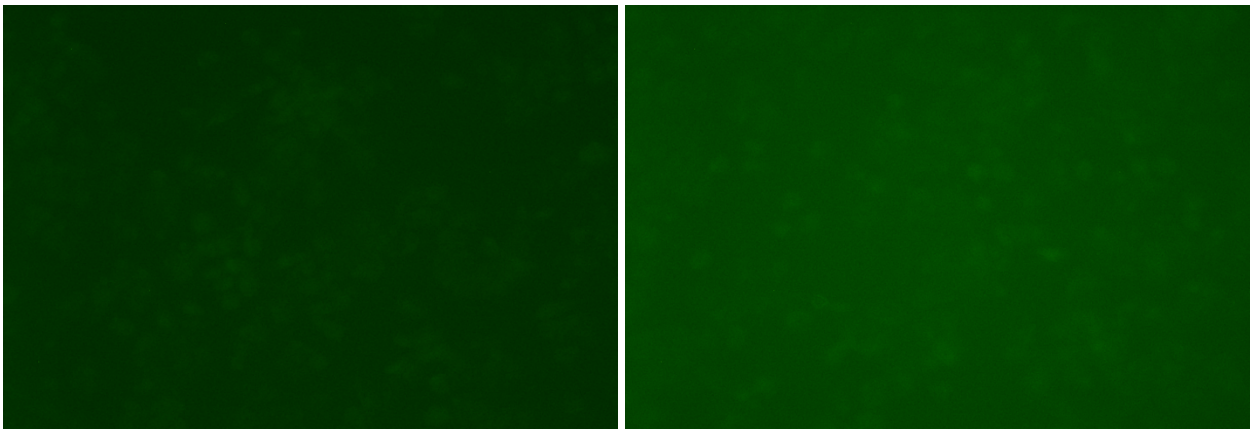


Figure 13: MARC 145 cells after being challenged with virus-mitigant mixture, PRRSV-MCFA (left) and PRRSV-FORMALD (right). Magnification of 200x.

Dose response inactivation.

Inactivation of PRRSV with FORMALD

FORMALD showed efficacy at reducing PRRSV titers. Concentrations higher than 0.15% caused inactivation of PRRSV titer to below the level of detection on cell culture while the concentration of 0.1% reduced PRRSV titers by approximately 2 logs ($3.16x10^6$ $TCID_{50}$/ml to $3.16x10^4$ $TCID_{50}$/ml). The lowest concentration of FORMALD tested was 0.0375% and no significant reduction of PRRSV titer was noted as the titer was similar to that of the positive control virus at $3.16x10^6$ $TCID_{50}$/ml.

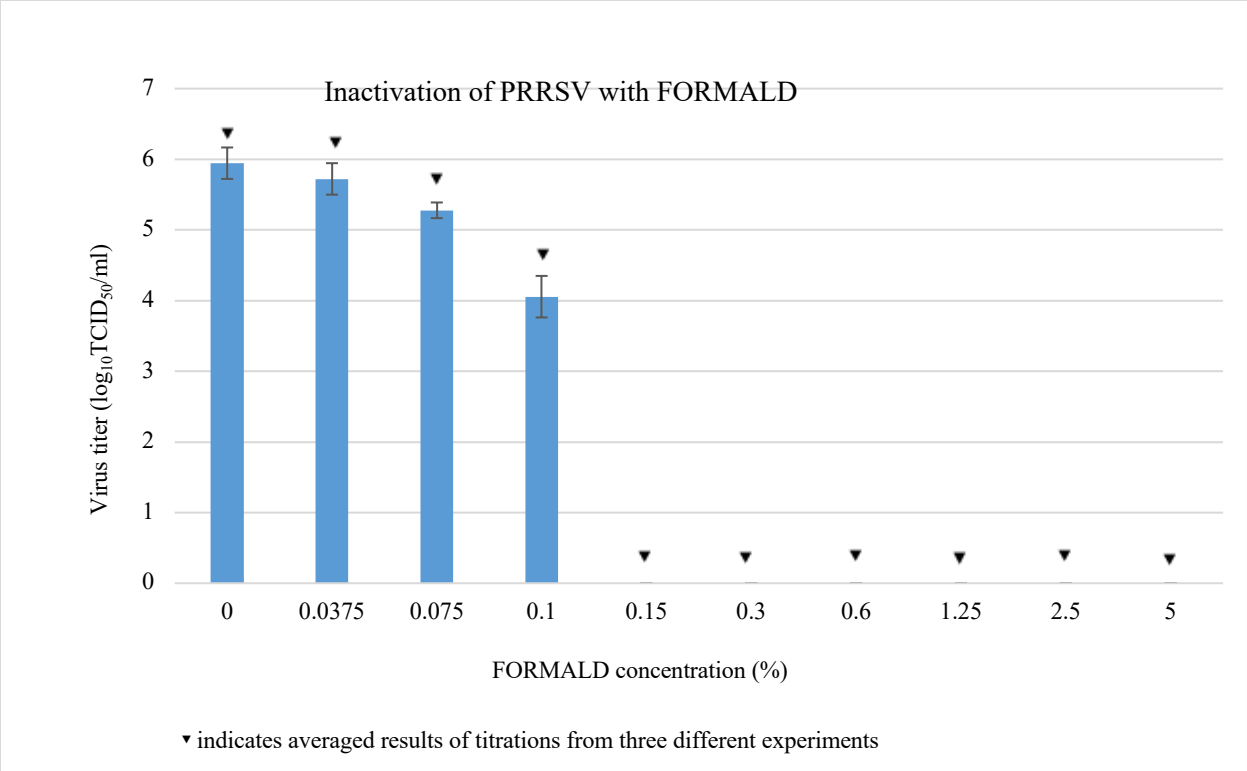


Figure 14: Dose response inactivation curve of FORMALD against PRRSV

Inactivation of PRRSV with MCFA

MCFA showed efficacy at reducing PRRSV titers. Concentrations higher than 0.25% caused inactivation of PRRSV titer to below the level of detection on cell culture while concentration of 0.2% reduced PRRSV titers by approximately 3 logs ($3.16x10^6$ $TCID_{50}$/ml to $3.16x10^3$ $TCID_{50}$/ml). The lowest concentration of MCFA tested was 0.1% and no significant reduction of PRRSV titer was noted.

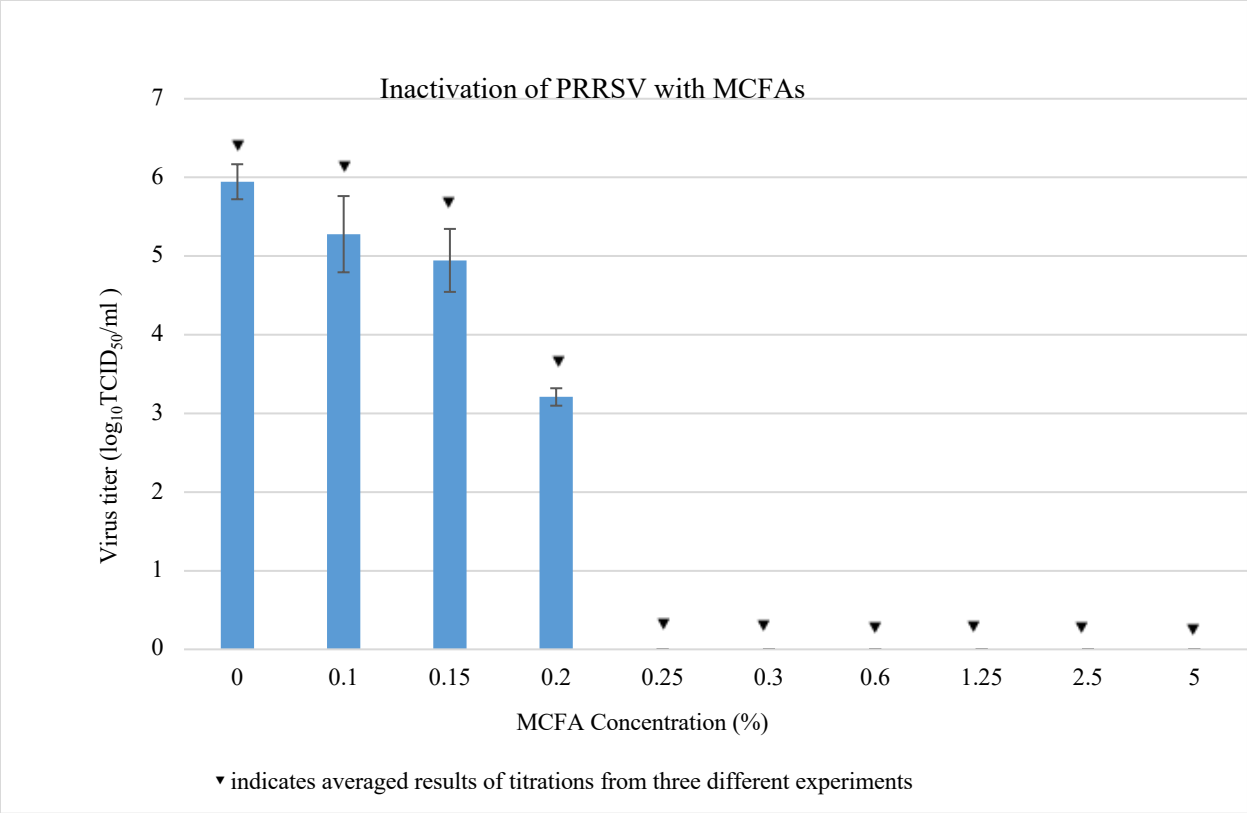


Figure 15: Dose response inactivation curve of MCFAs against PRRSV.

3.5 Discussion

The objectives of this study were to evaluate the efficacy of MCFAs and FORMALD in reducing the titer or inactivating PRRSV in a cell culture model and determining the dose response inactivation curve. To achieve these goals, various dilutions of FORMALD and MCFAs were prepared and used to assess efficacy in reducing or inactivating PRRSV. FORMALD showed efficacy by inactivating PRRSV at concentrations as low as 0.15%. The efficacy of FORMALD toward PRRSV may be due to denaturation of viral proteins caused by formaldehyde as the primary active ingredient, which is mentioned to have an antiviral ability to mitigate the risk of PEDV in animal feed and feed ingredients (Maris,1995).

On the other hand, MCFAs reduced PRRSV titer to below detectable levels after exposure to MCFA between 5% to 0.25% concentration. We hypothesize this may be due to the proposed mechanism of MCFAs to increase the permeability of the viral envelope and at high concentrations, it may cause disintegration of the viral envelope and viral particle (Thormar et al., 1987).

Chapter 4 - Animal feed trial

4.0 Introduction

Contaminated complete feed has been implicated as one of the potential sources for animal disease spread within a country. Following an outbreak of PEDV in the U.S. in 2013, this risk was recognized as a potential contributor to virus transmission and rapid spread across pork producing states (S. Dee et al., 2014). Various studies have been done to show the possibility of other U.S. endemic viruses being spread through contaminated animal feed and/or feed ingredients, where certain viruses survived in specific feed ingredients (S. A. Dee et al., 2018). Among those, SVA was mentioned to be stable in most of the feed ingredients and PRRSV was less stable but still showed some degree of stability (S. A. Dee et al., 2018; Joshi et al., 2016; Stoian et al., 2020a). Having this background, it is important to consider risk mitigation strategies that will inactivate the viruses and hence reduce the chance of virus introduction/re-introduction, transmission and spread from one place to another through contaminated animal feed (S. Dee et al., 2015; Gebhardt et al., 2019; Pasick et al., 2014).

The objective of the current study was to investigate the stability of SVA in complete swine feed when subjected to MCFAs and FORMALD in a cell culture model.

4.1 Materials and Methods

1. Cells and virus

PK-15 cells in DMEM media with 7% heat-inactivated fetal bovine serum (FBS), streptomycin and penicillin and antimycotic were seeded onto 96-well plates and incubated in a 5% CO_2/95% air atmosphere at 37°C. Cells were grown to 95-100% confluent. PK -15 cells were used for experiments with Seneca virus A (SVA; GenBank accession No. KX349734) (Chen et al. 2016) as described above.

6. Virus titration

Confluent PK-15 cells in 96-well plates with 180µl MEM media was inoculated with 20 µl of virus. Inoculation was done in triplicate, followed by 1:10 serial dilutions, and incubated at 37°C for two to three days. Plates were examined under a fluorescent microscope after 24 hours. The virus titers were determined by end-point dilution method using Reed and Muench (REED and MUENCH 1938).

7. Feed preparation and inoculation

Five grams of complete swine feed was added to 50ml conical tubes in duplicate. Two tubes were prepared per batch, including mitigant treated, positive controls and negative controls. All procedures were completed in a BSC to avoid cross-contamination. To prepare negative controls, 100µl of sterile PBS was added into 5g of feed in 50ml tubes and vortexed at the highest speed for 30 sec. For positive control, 100µl of virus (10^6TCID_{50}/ml SVA) was added into 5g of feed in 50ml tubes and vortexed at the highest speed for 30 sec. For mitigant treated samples, 50µl (1% MCFAs) or 16.5µl (0.33% FORMALD) was added into 5g of feed in 50ml tubes, vortexed at the highest speed for 30 seconds, followed by inoculation of 100µl of virus (10^6TCID_{50}/ml SVA) and vortexed for 5 seconds. Samples were vortexed for 10 seconds at the

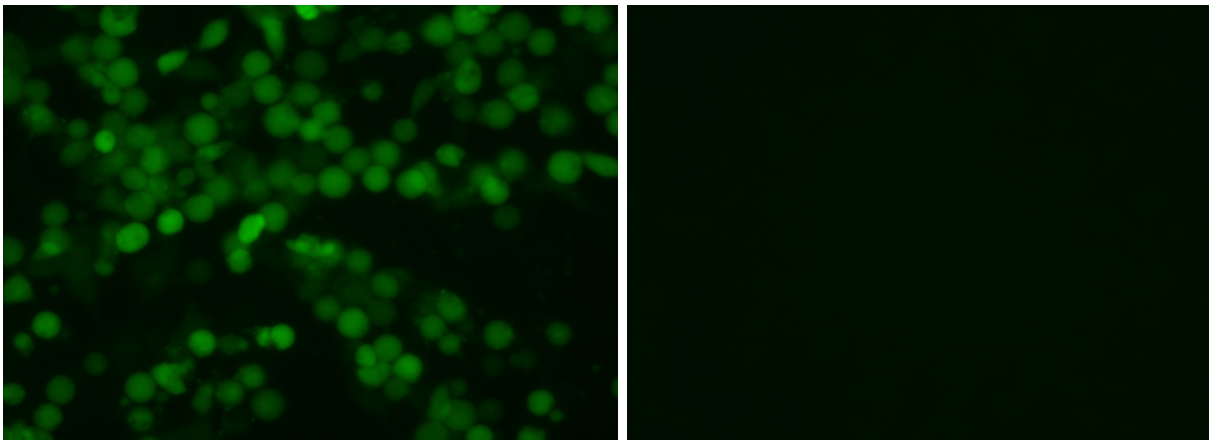
highest speed after mock inoculation or inoculation. After vortexing, 15 ml of sterile PBS with antibiotics and anti-mycotic were added to each sample for processing, vortexed for 10 seconds and centrifuged at 10,000 *g* for 5 min at 4°C. The supernatants were then aliquoted into cryovials and stored at −80°C.

8. Virus isolation and titration

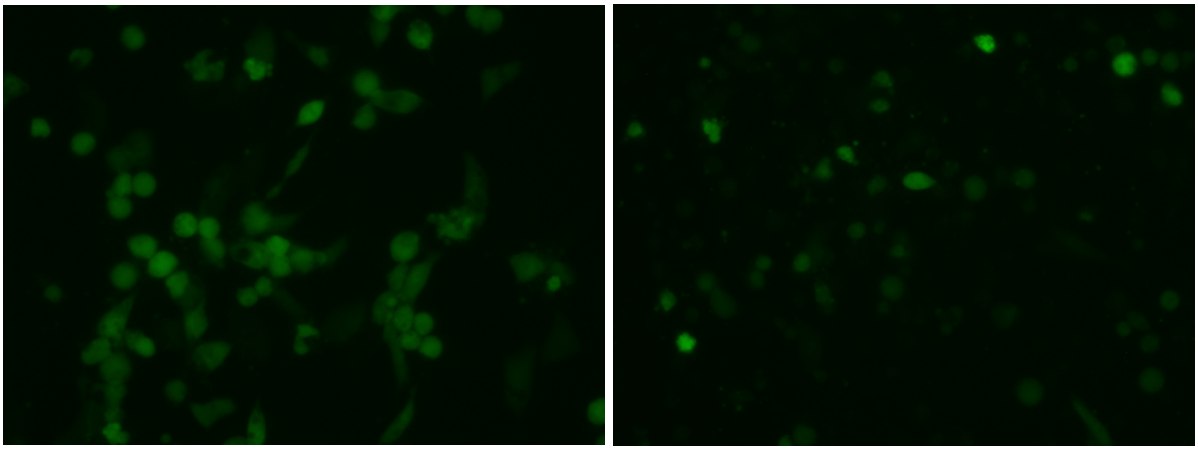
For titration of feed supernatant samples, 2-fold serial dilutions of each sample were prepared in MEM in triplicate and plated onto confluent monolayers of PK-15 cells. After incubation at 37°C for 2 to 3 days, cells were examined under fluorescent microscope for evidence of infection and titers were calculated as described above.

4.2 Results

SVA remained viable in complete feed after treatment with MCFAs and FORMALD as there was no significant reduction of titer of mitigant treated samples. The titers were not significantly different from that of complete feed positive control after three days incubation period, as shown in table 9. No significant reduction in SVA titer was noted after exposure to mitigants in a complete feed model with no incubation time.



a) Complete feed-SVA: positive control. b) Complete feed: negative control.



c) MCFA-complete feed treated PK-15 cells d) FORMALD-complete feed treated PK-15 cells

Figure 16: Complete feed trial, the presence of green cells indicates infected cells by SVA. Images representative of A) the positive control is the result of complete feed spiked with SVA b) negative control, the mock inoculation of complete feed with sterile normal saline c) MCFA-SVA treated feed with SVA d) FORMALD treated feed with SVA. Magnification of 200x.

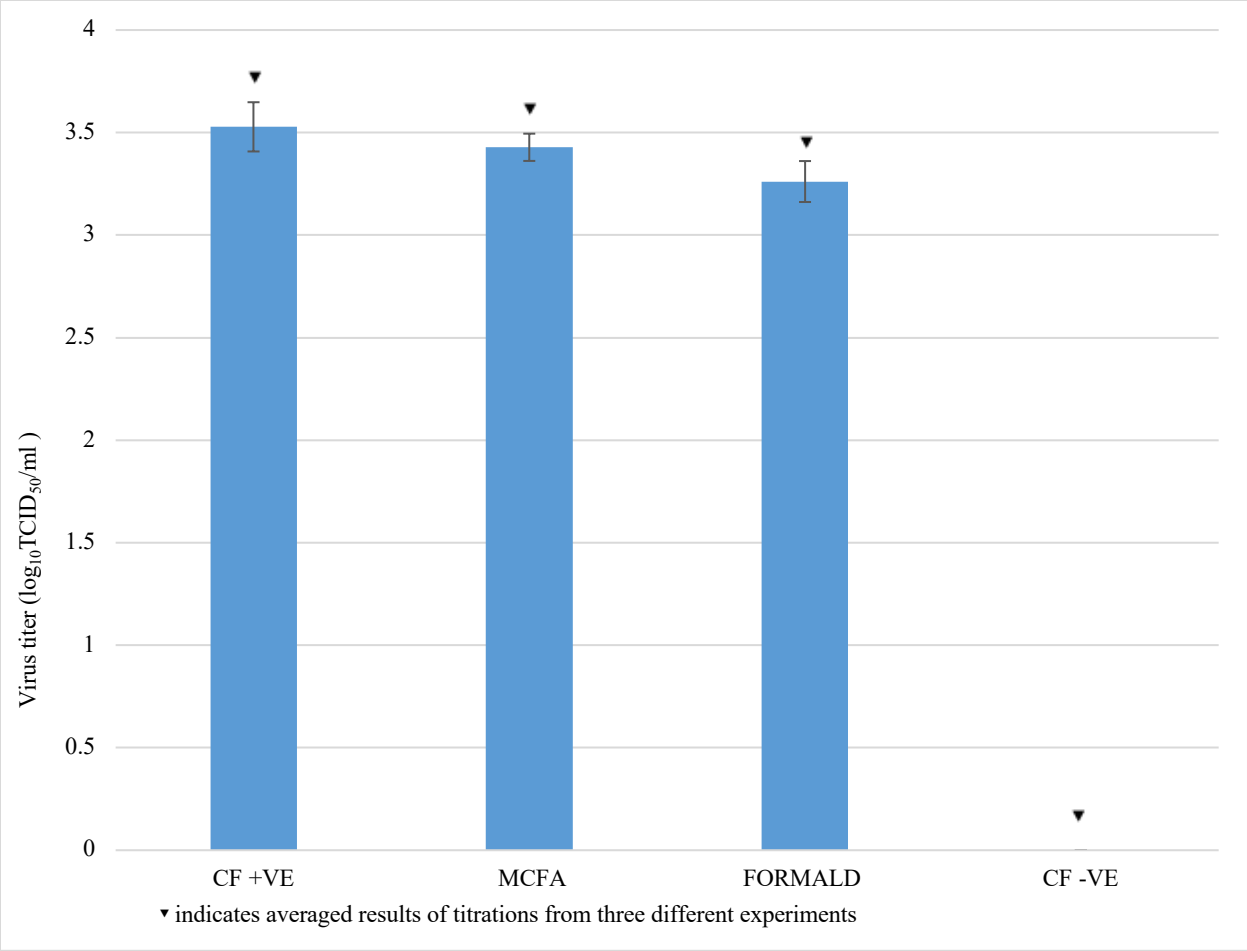


Figure 17: Complete feed trial, dose response inactivation curve of SVA-complete feed-mitigants and controls (positive control and negative control).

4.3 Conclusion

Overall, these studies provide evidence suggesting that FORMALD and MCFAs may be used as potential feed additives to reduce the risk SVA and PRRSV transmission and dissemination in animal feed and feed ingredients. This data is supported by studies performed on a cell culture model which indicated the concentration necessary to reduce the virus titers and mitigate the risk of virus transmission through feed. The use of feed additives is a potential strategy to reduce the risk of endemic swine disease spread through feed and plays a role in the concept of feed biosecurity. Further studies should be performed in complete feed at various incubation times,

other feed ingredients, and *in vivo* experiments to confirm the efficacy of feed additives in reducing the risk of infection to pigs. This will further assess risk mitigation in control of emerging and re-emerging transboundary animal diseases in feed and/or feed ingredients.

Chapter 5 - References

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