Senecavirus A in pigs: Epidemiology, Transmission, and Diagnosis

A DISSERTATION

SUBMITTED TO THE FACULTY OF THE

UNIVERSITY OF MINNESOTA

BY

Guilherme Milanez Preis

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

Dr. Fabio Augusto Vannucci, Dr. Cesar Agustin Corzo

November 2022

Acknowledgments

I do not think I can fully express my gratitude to all the fantastic people that were part of this wonderful journey in my graduate studies. I feel so honored, blessed, and happy to have learned from the best mentors, colleagues, and friends I have met in Minnesota. Thank you for your support, advice, and friendship during my program.

I am very grateful for having two amazing individuals advising me these past four years. Drs. Fabio Vannucci and Cesar Corzo, I cannot thank you enough for all the doors you have opened for me. I will never forget your constant mentorship, advice, feedback, patience, and friendship during my graduate program. Thank you so much for all the trust you have put in me. I will be forever grateful to you.

I also want to thank the members of my committee, Drs. John Deen and James Lowe. Your support, feedback, and constructive criticism were essential for completing this thesis.

I also would like to thank all faculty members and post-doctoral associates from the University of Minnesota's swine group. You were an essential part of this journey, and the knowledge you shared with me was priceless. A special thanks to Drs. Montserrat Torremorell, Marie Culhane, Peter Davies, Maria Pieters, Albert Rovira, Matthew Sturos, Igor Paploski, and Mariana Kikuti.

I am lucky to have met many amazing people in the UMN VMED graduate program. My friends, I will never forget any of you and the amazing times we shared. Thank you so much for your friendship, all the moments, and all the help during my projects. Thank you so much Mariana, Gustavo, Albert, Berta, Igor, Mariana, Jorge,

Erika, Chong, Lan, Cipriano, Alyssa, My, Beatriz, Eliana, Talita, Juan, Carles, Marcello, Joaquin, Miranda, and Emily.

None of my studies would have been possible without funding from the National Pork Board, the American Association of Swine Veterinarians, and the Minnesota Rapid Agriculture Response Fund. A very big thank you to all the swine veterinarians and companies that helped with several projects during my graduate program. I would also like to thank the Carlos Pijoan and Bob Morrison Graduate Student Fellowships and the MnDrive Global Food Ventures Program for their financial support.

I am so grateful for being born into such an amazing family. I want to thank my parents, Wanderley and Luciane, my brother, Christian, and Sophia, for all the support you have given me, the encouragement, and the amazing little time I was able to spend with you during my graduate program.

Dedication

Dedico este trabalho à minha mãe e ao meu pai. Muito obrigado por todo o suporte, carinho, conselhos, e por sempre encorajar a minha busca pelos meus sonhos.

Abstract

Senecavirus A (SVA) has been linked to several swine vesicular disease outbreaks worldwide. Several countries have already reported the presence of the virus, and the risk of new cases is constant due to the movement of animals, people, and materials, among other potential SVA-carrying agents, between pig herds. Despite the disruptions and confusion that SVA can cause due to its clinical similarities to high-consequence foreign animal diseases, such as foot-and-mouth disease, little is known about its epidemiology and control. The main objective of this thesis was to provide new information on the epidemiology, transmission, and detection of this virus.

Understanding the extent of SVA spread within a country and the risk factors associated with its exposure are the first steps toward building control strategies. Therefore, a study was designed to estimate the seroprevalence of the virus in the United States (U.S.). The seroprevalence of SVA in breeding and growing pig farms was relatively low, with 17.3% of breeding and 7.4% of growing pig farms being classified as positive. Among sow farms, the disposal of dead animal carcasses through rendering was associated with SVA seropositivity.

The information about how SVA transmits between farms is scarce. Therefore, another study was conducted to evaluate the role of fomites in the indirect transmission of SVA between pig populations. Study personnel carrying fomites had direct contact with SVA-infected pigs and moved to different rooms housing SVA-naïve animals under low, medium, and high biosecurity standards. The virus was successfully transmitted to the

rooms under low biosecurity standards, evidencing the role of fomites in the indirect transmission of SVA between pig populations.

A third study focused on understanding the methods to detect the virus at different stages of infection, which is crucial for developing control and surveillance efforts. It is known that SVA-infected pigs shed the virus for approximately 21 to 28 days after infection. The study was designed to compare the results from oral swabs, rectal swabs, tonsil swabs, and tonsil scrapings in diagnosing SVA in experimentally-inoculated animals up to 48 days after infection. Tonsil scrapings were the only sample type where SVA was found at the late stages of the infection. At the same time, other sample types, such as rectal swabs, were easier to collect and had higher chances of SVA detection at earlier stages of infection.

Developing an alternative aggregate sampling method would facilitate the detection of SVA at the population level. The fourth study in this chapter aimed to estimate the average number of SVA-positive weeks in processing fluids (PF) after an SVA outbreak. After longitudinally sampling 10 breeding farms, PF samples were estimated to remain positive on average for 11.8 weeks after an outbreak. Testing of PF may be a cost-effective method to detect SVA presence in breeding farms.

In summary, this thesis has uncovered and provided novel information on the epidemiology of SVA and the knowledge gaps that remain. The information generated will serve as the foundation work for the development of further studies as well as monitoring, surveillance, and control strategies.

Table of Contents

List of Tables

List of Figures

General Introduction

Senecavirus A (SVA) is a non-enveloped, single-stranded, positive sense RNA virus belonging to the *Picornavidae* family (1). This pathogen shares the same virus family as the foot-and-mouth disease virus (FMDV), a highly contagious viral disease affecting cloven-hoofed animals such as pigs, cows, sheep, goats, and deer (2). Both viruses (SVA and FMDV) cause vesicular diseases in pigs that are clinically indistinguishable from each other. Due to the clinical similarities, foreign animal disease investigations (FADI) must be conducted in every swine case where vesicles are seen to rule out FMDV and other swine vesicular diseases. It is known that SVA outbreaks seem to be self-limiting and last for approximately 1-2 weeks. Vesicles and skin lesions in animals of all ages and an increase of 30-70% in neonatal mortality may be seen (3–5). The mechanisms responsible for clinical signs development, mortality, and the economic impact of production losses are yet to be characterized. Still, SVA has been challenging the United States (U.S.) swine industry and local state and federal animal health authorities by causing a rampant increase in the yearly number of FADI conducted in the past years (6).

Limited information regarding how SVA is transmitted within and between pig farms is available. Initial investigations considered that farm size, carcass disposal procedures, entry of replacement animals, biosecurity gaps, and other factors might be associated with the introduction of the virus into susceptible farms (7). Feed ingredients (8), rodents, and insects (9) were also suggested to be potential vectors of SVA in pig farms. The transmission of SVA between pigs is believed to result from direct contact with oro-nasal secretions, feces, and vesicular fluids originating from infectious pigs

(4,10,11). However, there are currently no published studies designed to evaluate the transmission of this virus between pigs or what farm-level factors may be associated with exposure to this virus.

Infected pigs are known to shed early after infection, but previously-infected animals may also serve as sources of SVA transmission long after disease resolution. Vesicular lesions usually appear 4 days after infection (11,12), and virus shedding can be detected for up to 28 days in oral and nasal secretions and feces (11). The virus can also be found in different tissues after infection, including the lungs, heart, liver, spleen, intestines, kidneys, tonsils, and lymphnodes (11). The tonsils of the soft palate appear to have an essential role as a primary site of SVA replication due to replicating virus being found in this tissue during the early and late stages of infection (13–15). Under experimental settings, SVA was isolated from the tonsils of pigs 60 days after infection, and intermittent shedding in oral and nasal secretions and feces several weeks following disease resolution was detected after the animals were challenged with different stressful conditions such as transportation stress and farrowing (16). A persistently infected state characterized by animals harboring the virus for prolonged periods has been documented for other picornaviruses, including FMDV (17). The persistently-infected animal is defined as being virus-positive for at least 28 days post-infection, which may serve as a potential source of infection to susceptible animals (18). The similar nature of these viruses and the apparent persistence of SVA in the tonsillar tissue warrant further investigation to better understand the epidemiology of this virus and help swine veterinarians and caretakers control this disease.

This virus seems to have complex infection dynamics, and practical strategies for its detection have not been developed. To further advance the knowledge on the epidemiology of SVA and aid in its control efforts, the following specific objectives were proposed for this thesis: 1) estimate the seroprevalence of SVA at the national U.S. level and determine the risk factors associated with seropositivity, 2) assess whether SVA can be transmitted indirectly between pig populations through fomites, 3) compare different sample types to detect SVA in animals at different stages of infection, and 4) better understand the use of processing fluids to monitor SVA at the population level on sow farms after an outbreak.

Even though a very high number of false alarms for foreign animal diseases led to an expressive increase in the yearly number of FADI conducted in the U.S., the epidemiology of this disease is poorly understood. No studies had been designed to estimate the extent to which U.S. pig farms were exposed to this virus. Chapter 2 estimated the seroprevalence of SVA antibodies in U.S. breeding and growing pigs farms. Additionally, the farm-level risk factors associated with seropositivity were also determined.

SVA infection dynamics were previously described, and SVA transmission events between pigs on a farm were suggested to be likely the result of pigs in direct contact with secretions, feces, and vesicular fluid containing SVA (4,10,11). However, direct or indirect transmission of SVA between pigs had never been described before. In Chapter 3, SVA transmission between pigs and the role of fomites in this virus's indirect

transmission was described. The results reported help to understand how this disease moves within and between pig populations.

Developing well-designed monitoring and surveillance protocols is necessary for disease control. However, understanding what kinds of samples can be used to detect SVA in positive animals is warranted, especially in the case of persistently-infected animals. Currently, SVA is known to be found in oral and nasal secretions and feces, but it is unknown which sample type is more likely to yield a positive test result. Also, extracting and testing dead animals' whole tonsils is the only described method to detect persistently-infected pigs. Chapter 4 describes the success of different sample types in detecting SVA-positive animals at various stages of infection.

Using aggregate samples such as PF has made disease monitoring more cost-effective (19) and has aided the swine industry's efforts to control pathogens such as PRRSV (20–23). SVA was shown to be present in PF for over 50 days after an outbreak in a preliminary study (24), potentially causing long-term disease transmission in the farrowing room. Characterizing SVA detection over time in PF from multiple breeding farms will significantly advance the epidemiological knowledge of this disease—which was accomplished in Chapter 5 of this thesis.

Novel information in this thesis will enable the swine industry's stakeholders to understand SVA's epidemiology better and make science-based decisions to monitor and control this virus.

Chapter 1: Literature Review

1.1. Senecavirus A

Senecavirus A (SVA) is a non-enveloped, single-stranded, positive sense RNA virus belonging to the *Picornavidae* family and the only member of the genus Senecavirus. It has an icosahedral capsid of approximately 27 nm, similar to other picornaviruses (1). The capsid comprises 60 protomers, and four structural proteins form each protomer: VP1, VP2, VP3, and VP4 (25). The genomic RNA consists of approximately 7,200 nucleotides (nt), with 666 and 71 extra nt in the 5' untranslated region (UTR) and 3'UTR portions, respectively, and a poly(A) tail. The viral genome has a single open reading frame encoding a polyprotein of approximately 2180 amino acids (1).

1.2. History of SVA

This virus was once named *Seneca Valley virus* but had its name changed to Senecavirus A in 2015 (26). The virus was accidentally discovered in 2002 at Genetic Therapy Inc., a laboratory close to the Seneca Valley in Gaithersburg, MD, USA. During a PER.C6 cell cultivation process (1) and after observing an unexpected cytopathic effect, the virus was discovered, and the investigators concluded that the virus was presumably introduced via porcine trypsin or bovine serum (27). Upon electron microscopy analysis, a purified virus sample revealed the presence of icosahedral particles of approximately 27 nm in diameter, consistent with viruses from the *Picornaviridae* family. After complete genome sequence analysis, SVA was recognized as a novel picornavirus and proposed to be classified on its novel genus named Senecavirus (1).

The virus also receives attention for its potential in oncolytic virus therapy in humans due to its ability to penetrate tumors, inability to integrate into human genomic DNA, and showing a selective tropism for cancer cells (28). In addition, the virus has demonstrated efficacy against several types of cancers, such as certain types of blastomas and lung small cell carcinoma (29), and has reached phase I (30) and phase II (31) clinical trial stages. However, even severely immunocompromised patients seem to be able to build a robust antiviral response and develop SVA-specific antibodies after SVA exposure (28)—an issue that needs to be addressed for its therapeutic success.

1.3. First reports of SVA-associated vesicular disease in pigs

SVA has been present in swine populations since at least 1988 (27), but it was only sporadically detected in pigs with clinical vesicular signs until the last eight years. The term *porcine idiopathic vesicular disease* (PIVD) is used to identify the sporadic cases in which pigs develop skin lesions and vesicles without a known causative agent (32). Due to the clinical similarities between vesicular diseases, foreign animal disease investigations (FADI) need to be conducted in all PIVD cases to rule out the introduction of foot-and-mouth disease (FMD), vesicular stomatitis, vesicular exanthema of swine, and swine vesicular disease into the swine herd. One of the most concerning diseases is FMD, a highly contagious viral disease affecting cows, pigs, sheep, goats, deer, and other animals with cloven hooves (2), given that North America is free of this virus. This disease is a worldwide concern due to its potential to cause socio-economic disruptions and billions of dollars in economic losses if the virus is eventually introduced and its spread goes undetected (33).

The first known case of PIVD associated with SVA was reported in a group of 187 Canadian hogs that arrived in a harvest facility in Minnesota in 2007 and originated from Manitoba (34). In this report, the authors described that 80% of the pigs were lame pigs and approximately 25-30% of pigs had ruptured vesicles. All other known vesicular diseases were ruled out after laboratory investigations; however, the authors briefly described the identification of SVA by polymerase chain reaction (PCR), even though no association with any disease was known in pigs at the time. The second vesicular disease report where SVA was detected was related to a male Chester White boar at the Indiana State Fair in 2010 (35). The pig showed signs of anorexia, lethargy, and lameness and had intact and ruptured vesicles in its oral cavity and coronary bands. Attempts to isolate SVA failed, but SVA RNA was detected by PCR in the collected samples.

1.4. Large-scale SVA vesicular disease outbreaks throughout the world

In late 2014 several commercial pig farms from different parts of Brazil started reporting acute outbreaks of vesicular disease (36). Vesicles and coalescing erosions on the snouts and coronary bands of sows were commonly seen as well as increased neonatal mortality ranging from 30 to 70%. The outbreaks were described as self-limiting, with clinical signs lasting approximately 1-2 weeks. Claudication and locomotion limitations were also reported in some of the first Brazilian outbreaks, together with decreased feed intake, feed efficiency, and weight gain (3). All other known infectious agents that cause vesicular disease in pigs were ruled out after the local authorities conducted foreign animal disease (FAD) investigations (3,36).

In March and May of 2015, the first cases of SVA were also reported in China (37). Two farms from the province of Guangdong noticed lameness and the presence of vesicles in sows and piglets. The clinical presentation was similar to what was reported in the Brazilian outbreaks. Phylogenetic analysis placed the Chinese strains in the same clade as previously reported US strains, but the pathway of SVA introduction to China is unknown. During the summer of 2015, the first North-American SVA-associated vesicular disease outbreaks were reported in the US. Similar to Brazil, there was a rise in vesicular disease reports associated with increased neonatal mortality affecting piglets less than seven days of age (4,5). The United States Department of Agriculture (USDA) has registered a dramatic increase in yearly FAD investigations driven by SVA cases in pigs since 2015 (6) (Figure 1.1). The rampant increase in false FMD alarms after SVA outbreaks became endemic in the US is causing a significant economic impact due to the reallocation of resources for differential diagnosis and the need for round-the-clock availability of trained veterinary clinicians and laboratory technicians personnel for the timely closure of all investigations.

In February 2016, a 300-sow farrow-to-wean commercial pig farm in Colombia reported the country's first SVA-related vesicular disease outbreak (38). The vesicles first appeared in sows in the farrowing barn, but it was soon also detected in the gilt development unit and gestation barn. However, no clinical signs were seen in suckling piglets, and no differences were seen in pre-weaning mortality. Sequence alignment analysis indicated a 98.50%-98.98% nucleotide (nt) identity between the Colombian strains and the most recent US strains, whereas sequences were 97.66%-97.71% and

96.50%-97.80% identical when comparing it to Brazilian and Chinese sequences, respectively.

In October 2016, the first Asian case of SVA outside China was detected in Thailand (39). A 6,000-head grow-to-finish pig farm detected clinical signs of lameness, decreased feed intake, and lesions on the coronary bands of pigs. The outbreak lasted for two weeks, and no mortality was recorded. Interestingly, the Thai SVA isolates from this outbreak showed higher nt similarities with a Canadian strain (98.2%) than with the Brazilian, Chinese, and US strains. In January 2018, Vietnam reported its first SVA-related vesicular disease outbreak in the province of Kon Tum (40). There was no information about the farms or clinical signs in pigs besides the vesicular lesions. The complete sequence from the Vietnamese SVA isolate had a homology ranging between 98.5% to 99% with Chinese SVA isolates from 2015 and 2016. More recently, the first SVA reports were made in Chile (41) and Mexico (42) in 2022.

Several potential vectors of SVA introduction into farms have been suggested, such as mice, houseflies (9), and feed products (43). The movement of SVA-infected pigs can potentially contribute to the introduction of SVA to pig populations. Despite the temporal distribution of these first reports of SVA-related vesicular disease in different parts of the world, there is not enough evidence to indicate its place or region of origin.

1.5. Pathogenesis and shedding

Characteristic lesions include vesicles on the snout, oral mucosa, and coronary bands, with lameness and lethargy being reported occasionally. The role of SVA as the causative agent of a vesicular disease was first described by Montiel *et al.* in 2016 (12)

after the experimental intranasal inoculation of SVA in 9-week-old pigs. The clinical signs appeared 4 days post-inoculation (dpi) in 7 of 16 inoculated pigs with intact or ruptured vesicles developing on the coronary bands and minimal-to-mild lameness. This was further demonstrated in a different study (11), where lameness, lethargy, and vesicular lesions were first detected at 4 dpi. First, erythema in the skin may be seen, which progresses into the appearance of vesicles of up to 3cm in diameter that may rupture around 5 to 6 dpi. The affected areas of the skin develop crusts at 8 to 9 dpi, and the lesions completely resolve by dpi 12 to 16 (11).

The mechanisms for cell entry and development of vesicular lesions in pigs are uncertain. The anthrax toxin receptor 1 has been identified as the cellular receptor for infection of human cancer cell lines (44), but the receptors associated with infection in pigs are still unknown. The mouth and feet's epithelia continuously undergo mechanical stress as the pig interacts with other animals and the environment. This causes continuous trauma and results in an increased cell-membrane activity which, in the case of FMD, has been shown to increase the spread and infection of local epithelial cells, contributing to the development of vesicles (45).

Viremia is short-lived when the presence of SVA RNA in serum is assessed by quantitative reverse transcriptase PCR (qRT-PCR). Peak SVA RNA copies were found at 3 dpi and constantly decreased afterward, with the last serum viral RNA positive sample detected at 10 dpi (11). The length of viremia seems to coincide with the acute clinical phase of the vesicular disease.

Virus shedding has been detected for up to 28 dpi by qRT-PCR testing of oral and nasal secretions and feces. All inoculated animals tested qRT-PCR positive at 3, 5, 7, 10, and 14 dpi, with fewer animals testing positive at 21 and 28 dpi and all animals testing negative at 35 dpi (11). Viral shedding appeared to peak between 1 and 5 dpi, especially in oral secretions. The virus has been isolated from nasal, fecal, and oral samples for up to 7, 10, and 21 dpi, respectively. However, it is unknown whether the virus contained within the oral-nasal secretions and feces is infectious to naïve animals during these stages of infection.

Different viral loads have been found by qRT-PCR across different tissues, including lungs, heart, liver, spleen, small and large intestines, kidneys, tonsils of the soft palate, mediastinal and mesenteric lymph nodes until 7 dpi (11). Most of the tissues mentioned above were also SVA-positive by virus isolation at 3 and 4 dpi, but the tonsil was the only tissue where SVA was isolated at 7 dpi. It is possible that the pigs' viremic state during the first 10 dpi interfered with the qRT-PCR and virus isolation results. Therefore, the detection of SVA may have been due to the virus circulating in the blood rather than replicating in the collected tissues. However, *in-situ* hybridization (ISH) testing of tissues collected from SVA-affected farms revealed the presence of replicating SVA in the snout epithelium, heart, lymph nodes, spleen, lungs, liver, colon, small intestine, tongue, and tonsils of pigs (13).

SVA RNA is constantly found in the tonsils of infected pigs in all stages of SVA infection (9,11,14,46) at higher concentrations than other replication sites (14,15). Due to

the early and late detection of SVA in the tonsils of exposed animals, the tonsil has been suggested as a potential primary site of SVA replication (14,15).

1.6. Immune response and antibody detection

Virus-specific neutralizing antibodies (NA) have been detected as early as 5 dpi, with peak titers observed at 10 dpi and still detected at 38 dpi by a virus neutralization assay (VN) (11). Humoral antibodies (e.g., IgG) have been detected by indirect immunofluorescence (IFA) at 10 dpi and were still detected at high levels by dpi 38. Interestingly, the levels of viremia seen in this study started to decrease after NA detection, and animals were less likely to be viremic at 10 dpi when NA titers were highest and IgG antibodies were present (11).

In another study, SVA-specific NA antibodies were detected as early as 5 dpi, and titers peaked at 7 dpi (47). IgM and IgG antibodies were first detected at dpi 5 and 7, respectively, and both antibody isotypes titers peaked at 10 dpi. Early detection of NA seems to be due to IgM presence in serum. SVA IgG antibodies were detected by IFA for up to 13 months in a longitudinally sampled cohort of sows after an outbreak was detected in a commercial sow farm (24). This finding suggests that testing animals for the presence of SVA-IgG is a suitable method to assess previous exposure to the virus in pig farms.

Specific T-cells are induced as early as 3 dpi, with $CD4^+$ T-cells detected earlier than $CD8^+$ T-cells (47). $CD4^+$ T-cell responses coincided with decreasing levels of viremia and increased levels of NA, IgM, and IgG antibodies. $CD8^+$ T-cells peak levels coincided with disease resolution and SVA clearance from different tissues after

infection, except lymph nodes and tonsils at 14 dpi (47). Increasing T-cell responses were also correlated with decreasing levels of viremia and virus shedding in another study (48).

1.7. SVA epidemiology and persistent infection

Serological surveys identified the presence of SVA-specific antibodies in pigs, cattle, and mice. This knowledge, together with the isolation of SVA from pigs in different regions of the United States (US) between 1988 and 2005, suggested that pigs are natural hosts for SVA (27).

It is currently known that SVA has been present in pig populations since at least 1988 (27). However, the large-scale SVA-related vesicular disease outbreaks only started after 2014. This recent increase in disease occurrence may be due to evolutionary changes in the SVA genome. A genetic analysis study split SVA whole-genome sequences into historical and contemporary SVA strains identified before and after 2010, respectively (49). The authors reported a 6.32% genetic diversity between the historical and contemporary strains at the nucleotide level, with several amino acid substitutions observed in the VP1, VP2, and VP3 structural proteins. Interestingly, only a 2.8% genetic diversity was found between all whole-genome sequences from contemporary strains found in Brazil, Canada, China, Colombia, Thailand, U.S., and Vietnam. This finding suggests that SVA might be transmitted between countries.

Even though SVA is present in several countries, little is currently known about its transmission and risk factors associated with infection at the farm level. An initial epidemiological investigation considered that farm size, number of employees, carcass

disposal procedures, removal of cull sows, entry of replacement animals, and biosecurity gaps, among other factors, may be associated with the introduction of the virus into naïve farms (7). The virus has been shown to survive in different feed matrices (43), and SVA-contaminated feed can lead to the infection of susceptible pigs (50). In a recent investigation of SVA outbreaks in a historically negative country, the virus' genetic material was found in soybean meal imported from an SVA-positive country (8). However, it is unknown whether the genetic material found in the imported soybean meal was infectious, and no genetic sequence analysis between the viruses found in the soybean meal and animals was performed. The causal pathway between the consumption of naturally-contaminated feed ingredients and infection needs to be further studied to understand their role in introducing the virus into a farm.

On-farm transmission events between pigs are likely the result of pigs being in contact with oral and nasal secretions, feces, and vesicular fluids originating from infectious pigs (4,10,11). However, rodents and insects may also contribute to spreading the disease within and between pig farms (9) as SVA RNA has been detected in mice feces, their small intestines, mice bait boxes, and houseflies collected at a farm undergoing an SVA outbreak. The virus was also isolated from mice feces, intestinal samples, and bait boxes, suggesting their potential role as vectors of infectious virus to naïve pigs. The virus was also isolated from several surfaces and equipment across the farm, such as dust collected from exhaust fans, a tractor bucket used to transport dead animals, ground samples from outside the farm, and internal hallway swabs.

Persistent infection, defined by the presence of the virus for long periods after disease resolution, may be another mechanism by which SVA persists in pig populations. As previously discussed, the tonsil has been suggested as one of the primary sites for virus replication (14,15). This anatomical site may be of further importance since SVA has been detected in the tonsils for long periods after an SVA outbreak detection (51). In experimental settings, SVA has been shown to persist in the tonsils of convalescent animals for up to 38 dpi, approximately 3.5 weeks after the resolution of clinical disease. All experimentally-infected pigs showed high levels of SVA RNA in the tonsils detected by qRT-PCR and in situ hybridization, specifically targeting a reverse complementary sequence of the VP1 mRNA, detecting replicating SVA virus mainly in tonsillar crypt epithelium and subepithelial lymphocytes (11). These findings raise important questions about potential strategies the virus may have developed for subclinical maintenance and persistence in pig populations.

A carrier state characterized by persistently-infected animals has been documented for other picornaviruses, including FMDV (17) and encephalomyocarditis virus (EMCV) (52). These asymptomatic carriers play an essential role in the epidemiology of these diseases, especially as potential sources for new outbreaks in the case of FMDV (17). Feces and oral/nasal secretions of carrier animals can test SVA positive after stressful conditions (16); therefore, regular stressful events in the life of a pig on the farm, such as farrowing, social stress due to comingling of different animals, and even heat stress may trigger transmission events in asymptomatic herds. In FMDV, a persistently-infected animal is defined as being virus positive for at least 28 days post-

infection (18). In order to test live animals and identify carriers, oesophageal-pharyngeal fluid from cattle is collected by the probang cup method (17), enabling better disease and transmission control. Given the similar nature of SVA and apparent persistence in tonsillar tissue, further investigations are needed to understand this epidemiological feature to better help veterinarians and caretakers control this disease.

1.8. SVA diagnosis and surveillance

The clinical diagnosis of SVA is not possible due to its lesions being macroscopically identical to FMD and other swine vesicular diseases. Therefore, as previously discussed, ruling out FMD through laboratory testing is necessary to prevent the economic losses it can cause (33).

Several serological and molecular diagnostic methods are available to diagnose SVA infection or exposure. The detection of SVA RNA provides a powerful diagnostic tool for assessing an active infection through the detection of the virus in vesicles, viremia, oro-nasal secretions, feces, tissues, and environmental contamination (9,11). The RT-PCR test targeting SVA's polymerase gene (53), a conserved genomic fragment of the VP1 protein (54), and a nested RT-PCR targeting a segment of the VP1 gene (55) have been developed. An RNAscope ISH technique was developed to detect specific areas of the VP1 gene in tissues (13). The authors reported positive ISH results even when animals showed no clinical signs of disease or lesions. Immunohistochemistry was also designed with monoclonal antibodies to detect SVA in fixed tissue (56).

Serological tests can identify previously infected animals as early as 5 dpi. Indirect ELISA tests have been developed to target multiple structural proteins such as

VP1, VP2, and VP3. However, VP1 and VP2 ELISAs had higher sensitivities and specificities (4,57). Competitive ELISA (58), VN (58,59), and IFA (57,58) tests are also available for the serological assessment of SVA antibodies.

As previously discussed, SVA has limited detection in oral/nasal fluids and feces for approximately 28 days, limited availability of vesicles and vesicular fluids after infection, and short-termed viremia for up to around ten days. Therefore, both serological and molecular diagnostic methods should be concomitantly used for the correct assessment of the SVA status of pigs at both the individual and population levels.

1.9. SVA prevention and control

A robust cleaning and disinfection protocol is essential to help control and prevent pathogens' transmission and environmental spread. Sodium hypochlorite was shown to inactivate more than 95% of SVA at 4°C and 25°C on various surfaces (e.g., aluminum, stainless steel, rubber, cured cement, and plastic) after a 5-minute contact time. A quaternary ammonium compound with glutaraldehyde only achieved similar results after 60 minutes of contact (60).

Field reports state that the transmission of SVA within a breeding farm can be stopped, and continuous weaning of SVA-negative pigs can be achieved (61). In two different farms, herd closure and mass exposure of the resident population through SVA-positive feces were conducted. One farm introduced the first SVA-naïve sentinel pigs 9 weeks later, while the second farm waited 5 months before opening the herd. In both cases, the sentinel pigs remained negative after the introduction, and the veterinarians concluded that the disease elimination process was successful (61). In a third farm,

vesicular fluid from infected animals was collected and diluted in PBS to prepare a solution to mass inoculate the herd using the intranasal route. Some animals developed clinical signs, and 96% of the inoculated pigs seroconverted after this procedure (61).

However, a comprehensive study still needs to be done to understand better SVA's infection and transmission dynamics in a sow farm undergoing a disease elimination program. Additionally, persistently infected animals' role in transmitting this disease still needs to be assessed. It is currently unknown whether mass SVA exposure can create persistently infected pigs that will remain on the farm and potentially transmit SVA to naïve replacement gilts.

1.10. SVA production impact

Information about SVA's production and economic impact on a pig farm is scarce. SVA-affected litters have had up to 75% of piglets dying with signs of diarrhea, anorexia, and lethargy (5). Mortality rates of 30% among all piglets born in the same farrowing group have been reported (62), with clinical signs present in up to 70% of all piglets. Another study reported up to 75% morbidity and 50% mortality in piglets housed in SVA-affected farrowing rooms (9). The increased neonatal mortality seen in SVA-affected farms is perhaps the most critical impact on production, but it is not seen in all SVA-exposed farms. Morbidity rates in adult animals can be as high as 90%, but mortality is negligible in this category of animals (62).

SVA-affected animals have difficulties moving and decreased feed intake and efficiency (3). However, the impact of an SVA outbreak on feed efficiency or other parameters has not been measured and/or published.

Chapter 2: Senecavirus A seroprevalence and risk factors in United States pig farms

This chapter was published in:

Guilherme Preis, Juan Manuel Sanhueza, Carles Vilalta, Fabio Augusto Vannucci, Marie Rene Culhane, Cesar Corzo. Senecavirus A seroprevalence and risk factors in United States pig farms. **Front. Vet. Sci**. Sec. Veterinary Epidemiology and Economics

doi: 10.3389/fvets.2022.1011975

2.1. Summary

Senecavirus A (SVA) is a non-enveloped, single-stranded, positive-sense RNA virus belonging to the Picornaviridae family. SVA is constantly associated with outbreaks of vesicular disease in pigs and has been reported in several countries since its first large-scale outbreak in 2014. SVA's clinical disease and lesions are indistinguishable from other vesicular foreign animal diseases (FAD). Therefore, an FAD investigation needs to be conducted for every SVA case. For this reason, SVA has been attributed as the cause of an alarming increase in the number of yearly FAD investigations performed by the United States Department of Agriculture. The objectives of this study were to estimate the seroprevalence of SVA antibodies in breeding and growing pig farms in the United States and to determine the farm-level risk factors associated with seropositivity. A total of 5,794 blood samples were collected from 98 and 95 breeding and growing pig farms in 17 states. A farm characteristics questionnaire was sent to all farms, to which 80% responded. The responses were used to conduct logistic regression analyses to assess the risk factors associated with SVA seropositivity. The estimated farm-level seroprevalences were 17.3% and 7.4% in breeding and growing pig farms, respectively. Breeding farms had 2.64 times higher odds of SVA seropositivity than growing pig farms. One key risk factor identified in breeding farms was the practice of rendering dead animal carcasses. However, the adoption of a higher number of farm biosecurity measures was associated with a protective effect against SVA seropositivity in breeding farms.

2.2. Introduction

Senecavirus A (SVA), previously known as Seneca Valley Virus (26), is a non-enveloped, single-stranded, positive-sense RNA virus belonging to the Picornaviridae family and the only member of the genus Senecavirus (1). The virus was first isolated in 2002 as a contaminant from PER.C6 cell lines, presumably introduced via fetal bovine serum or porcine trypsin during the cell cultivation process (1). Current data suggest that SVA has existed in the United States (U.S.) swine population for at least three decades (27). However, the presence of SVA in pigs with vesicular lesions had not been reported until 2007 after a trailer transporting 187 market hogs from the Canadian province of Manitoba arrived at a harvest facility in Minnesota, U.S. (34). Larger multi-state SVA outbreaks of this vesicular disease in pigs were reported in Brazil in 2014 (36), in the U.S. (5,9) and China (37) in 2015, followed by Colombia (38), Thailand (39) in 2016, Vietnam (40) in 2018, and Mexico (42) and Chile (41) in 2022. Characteristic vesicular lesions usually start developing approximately four days post-infection, consisting of multiple-sized vesicles on the snout, oral cavity, and feet (i.e., coronary band and interdigital space), which may lead to lameness and lethargy. Vesicles tend to rupture five days post-infection (11,47) and are clinically indistinguishable from high-consequence foreign animal diseases (FAD) such as foot-and-mouth disease (FMD). In neonatal pigs, diarrhea and a sudden increase in pre-weaning piglet mortality have also been reported (5–7), contributing to production losses.

The clinical disease and lesions caused by SVA are indistinguishable from other vesicular animal diseases: swine vesicular disease, vesicular exanthema of swine,

vesicular stomatitis, and FMD. Therefore, an FAD investigation needs to be conducted by local government authorities for every SVA case. This practice, while prudent, has resulted in an increase in false alarms for FADs in the United States. The average yearly number of FAD investigations conducted by the United States Department of Agriculture (USDA) in all animal species in the U.S. between 2008 to 2014 was 487. This number increased almost four times between 2015 and 2020, with an average of 1808 FAD investigations per year. Around 75% of FAD investigations were attributed to swine vesicular disease in pigs in the last four years of this period (6).

Despite the considerable number of SVA outbreak reports in swine farms across the past few years and the associated problems, the epidemiology of this disease is poorly understood. Basic information, such as prevalence and risk factors associated with the disease, is scarce. Therefore, the objectives of this study were to 1) estimate the seroprevalence of SVA antibodies in breeding and growing pig farms in the U.S. and 2) determine the farm-level risk factors associated with seropositivity.

2.3. Materials and Methods

The University of Minnesota (UMN) Institutional Animal Care and Use Committee (IACUC) approved this study (protocol 1804-35818A).

2.3.1. Experimental design

A cross-sectional study was designed and conducted to estimate the seroprevalence of SVA in U.S. pig farms. Participation in the study was voluntary. Major veterinary clinics and production systems throughout the country were invited to participate. After agreeing to participate, each production system or veterinary clinic was

asked to select breeding and growing pig farms for sample collection randomly. Both participating veterinarians and, in some cases, investigators collected the study samples.

2.3.2. Sample size calculation

Number of farms: To calculate the number of breeding and growing pig farms to be included in the study, the following formula was used (63):

$$N = \frac{Z^2 pq}{L^2}$$

where N = number of farms to be sampled, Z = 1.96 (Z-score value for 95% confidence), p = expected true farm-level prevalence (50% was used as the default as data was not available at the time of the study), q = 0.5 (1-p), L = precision of the estimate was set at 0.1. A total of 97 breeding and 97 growing pig farms were needed for this study, which brings to a total of 194 farms.

Number of pigs sampled per farm: The number of samples needed within each farm to classify the farm as either seropositive or seronegative was calculated using the following formula (64):

$$N = \frac{\log(1 - C)}{\log(1 - TP)}$$

where N = number of animals to be sampled in each farm, C = 0.95 (confidence of 95%), TP = 0.1 (assuming that the expected true within-farm prevalence was 10%). Therefore, 29 blood samples were necessary to reach a 95% confidence level that at least one positive sample would be detected when the within-farm prevalence was at least 10%, assuming perfect sensitivity and specificity.

2.3.3. Sample collection, handling, and testing

In breeding farms, sampling was performed randomly across sow parities. In the case of growing pig farms, samples were collected from 20-weeks-old or older pigs to avoid the possible detection of maternal antibodies (4). Blood samples were collected, refrigerated, and shipped to the UMN Food Centric Corridor Laboratory. Blood samples were sorted, organized, de-identified upon reception, and submitted to the UMN Veterinary Diagnostic Laboratory for testing. SVA IgG presence was tested through an immunofluorescent antibody test (IFA), which was reported to have 90% and 100% diagnostic sensitivity (Se) and specificity (Sp), respectively (58). Briefly, NCI-H1299 (ATCC® CRL-5803™) cells were inoculated with SVA and fixed with cold acetone. Sera samples were screened for SVA-specific IgG in two dilutions (1:40 and 1:80) using PBS (Gibco). After incubating for 60-75 minutes, plates had the sera removed and washed with PBS. DyLight® 650 anti-pig IgG (Abcam, Cambridge, MA) was added to the wells, and plates were incubated for 60-75 minutes. Plates were washed with PBS and observed under fluorescence microscopy (58) by the same laboratory technician. Fluorescence observed at a sample dilution of 1:40 or 1:80 indicated that the serum sample was positive for SVA IgG antibodies.

2.3.4. Farm characteristics questionnaire

Two questionnaires—one for breeding and another for growing pig farms—were designed to capture general information such as farm type, farm size, personnel flow, animal sourcing, and other details on biosecurity measures. The survey was electronically sent to all participating veterinarians to answer on a per-farm basis. Data from the

questionnaires were then transcribed to an electronic spreadsheet (Microsoft Excel 2016, Microsoft Corporation) for analysis.

2.3.5. Data analysis

Estimating farm-level SVA seroprevalence: The proportions of seropositive breeding and growing pig farms were estimated after calculating the cut-point number of positive samples needed to classify a farm as being positive. The cut-point number of positive samples was determined by maximizing herd sensitivity (HSe) and herd specificity (HSp) values (65), based on the SVA IgG IFA antibody test's Se and Sp (90% and 100%, respectively) (58). Clopper-Pearson 95% confidence intervals for the proportions of seropositive farms were also calculated.

Association between farm type and SVA seropositivity: A chi-square test of independence was used to determine if there was a significant association between farm type (breeding or grow-finishing pig farms) and SVA seropositivity. The odds ratio and 95% CI for SVA seropositivity between farm types were calculated using the unconditional maximum likelihood estimation method (Wald).

Assessment of farm-level factors associated with seropositivity: The risk factor analyses for breeding and growing-pig farms were conducted separately. Univariable logistic regressions were fit to determine the unconditional associations between all risk factors recorded in the questionnaires and the outcome (SVA farm seropositivity).

Linearity between continuous variables and the outcome in the logit scale was assessed visually using scatterplots and statistically. If the relationship between the continuous variable and the outcome was not linear, continuous variables were

categorized based on their median values (less than/equal to the median vs. greater than the median).

Variables with multiple categories where all positive cases were within the same category or had categories with few observations and no cases (indicating a lack of substantial variability for the analysis) were either excluded from the analysis or had their observations regrouped in a new two-factor categorical variable.

A new variable was created to evaluate the association between biosecurity measures and SVA-seropositivity. The list of biosecurity measures included in the survey was 1) Visitor check-in is required to enter the farm, 2) Shower in/out procedures, 3) Danish bench-entry system is installed, 4) Use of farm-specific boots are required, 5) Use of farm-specific clothing is required, and 6) A downtime is required before entering the farm. Since all farms responded to either having or not having these six different biosecurity measures in place, they were categorized as having "four or less" or "five or six" biosecurity measures in place if they responded to having any combination of <=4 or >=5 biosecurity measures, respectively.

Unconditional associations between each predictor variable and the outcome were tabulated. Only variables with associated p-values below 0.2 were selected for inclusion in the multivariable analysis. A backward elimination process was used to build the final multivariable logistic model. First, a maximum model was fit using all the previously screened variables. Variables were then removed one at a time, and the likelihood ratio test was used to compare the nested models until a model with a maximum likelihood was found.

All statistical analyses were performed using R statistical software (66).

2.4. Results

This study involved pig farms from nine production systems, eight veterinary clinics, and two private practitioners. Thirty-six swine veterinarians contributed to this study by collecting 5,794 blood samples from 193 farms. The overall survey response rate was 80% and included data from 155 (77 breeding and 78 growing pig farms) out of 193 tested farms, including all positive breeding farms and six out of the seven positive growing-pig farms.

Blood samples were collected from 193 participating farms: 98 and 95 breeding and growing pig farms located in 17 different states (Table 2.1). Recruitment and sample collection at all farms occurred between October 2018 and October 2019.

2.4.1. Classification of farm status

The number of positive samples needed to classify a farm as seropositive was 1. This cut-off value maximized the HSp and HSe values, which reached 100% and 94%, respectively. Changing the cut-off value to 2 or 3 did not alter HSp, but HSe decreased to 77% and 51%, respectively.

2.4.2. Seroprevalence results

The overall proportion of IFA-positive sera samples from breeding and growing pig sites was 4.6% (268/5,794). Of the 268 IFA-positive sera samples, 95.1% (255/268) were positive at the 1:80 dilution, and 4.9% (13/268) were positive at the 1:40 dilution. Twenty-four out of 193 (12.4%) sampled farms had at least one seropositive serum

sample. The median, mean, and standard deviation for the number of positive samples within positive farms were 6.5, 11.2, and 10.1, respectively.

Overall, the proportion of IFA-positive sera samples from breeding farms was 5.9% (174/2,943). Seventeen out of 98 (17.3%, 95%CI – 10.4, 26.3%) breeding farms had at least one positive sample and were located in Illinois, Indiana, Kansas, Minnesota, North Carolina, and Texas. The overall estimates of SVA farm level seroprevalence among breeding farms in different states or regions are shown in Figure 2.1. Among seropositive breeding farms, the median, mean and standard deviation of the number of positive samples were 4, 10.2, and 9.8, respectively (Figure 2.2). The average within-farm apparent prevalence among seropositive breeding farms was 34% (95% CI – 17.8, 53.5%).

Seven out of 95 (7.4%, 95% CI – 3, 14.6%) growing pig farms had at least one positive sample, and these were detected in the states of Illinois, Indiana, Minnesota, North Carolina, and Oklahoma. The proportion of IFA-positive sera samples from growing-pig farms was 3.3% (94/2,851). The overall estimates of SVA farm level seroprevalence among growing pig farms in different states or regions are shown in Figure 2.3. The median, mean, and standard deviation of the number of positive samples within positive growing pig farms were 8, 13.4, and 11.3, respectively (Figure 2.2). The average within-farm apparent prevalence among seropositive growing pig farms was 44.7% (95% CI – 26.6, 63.8%).

2.4.3. Association between farm type and SVA seropositivity

A significant association between farm type and SVA seropositivity was detected ($X2 = 4.411$, $df = 1$, $p=0.035$). Breeding farms had 2.64 (95% CI - 1.04, 6.69) times higher odds of SVA seropositivity when compared to growing pig farms.

2.4.4. Risk factors associated with SVA seropositivity

After tabulation of unconditional associations between the surveyed predictors and the outcome of SVA seropositivity, six breeding, and six grow-finishing pig farm predictors were selected to enter the multivariable model selection process.

The results from the univariable and multivariable logistic regression analysis for the breeding farms' characteristics are shown in Table 2.2. The final multivariable logistic regression model showed that breeding farms that reported rendering dead animal carcasses were more likely to be SVA-seropositive (OR = 9.2, CI – 2.5, 33.7), while farms that reported practicing five or six different biosecurity measures were less likely to be SVA-seropositive (OR = 0.2, CI – 0.1, 0.99). A summary of the biosecurity measures and associations is shown in Table 2.2.

It was not possible to fit a multivariable logistic regression model for the growing-pig farms due to the low number of seropositive farms. The risk factors in growing-pig farms that appeared to be positively associated with SVA-seropositivity ($p<0.2$) in the univariable analysis were 1) pigs are loaded into trucks by an external pig-loading crew, and 2) More than one external crew is hired to perform jobs at the farm. Alternatively, the risk factors that appeared to be negatively associated with SVA-seropositivity ($p<0.2$) were 1) people that load pigs into trucks have direct access to pigs

in the barn after loading a truck, 2) all pigs in the farm are sourced by a single breeding farm and 3) all trucks that arrive in the farm are cleaned and disinfected (Table 2.3).

2.5. Discussion

The present study demonstrates that SVA antibodies exist in the U.S. swine population. To the authors' knowledge, this is the first national study designed and sampled to estimate the seroprevalence of SVA. Despite the high incidence of swine-vesicular FAD investigations (16), the estimated farm level apparent seroprevalences of 17.3% (95%CI – 10.4, 26.3%) and 7.4% (95% CI – 3, 14.6%) among U.S. breeding and growing pig farms, respectively, were relatively low. These proportions change slightly when accounting for the imperfect HSe estimate of the applied methodology. Considering the calculated HSe (94%) and HSp (100%), breeding and growing pig farms had estimated true seroprevalences of 18.5% (95%CI – 11.1, 28%) and 7.8% (95%CI – 3.2, 15.5%). Although slight numerical increases are seen when comparing the apparent and true prevalence estimates, there are no significant changes due to the overlapping confidence intervals.

Currently, there is scarce information on the serological response to SVA at a population level. In a recent study, SVA IgG was detected in a cohort of 60 sows from a 6,000-sow farrow-to-wean farm that underwent an SVA outbreak for up to 13 months after the outbreak, using the same IFA procedure (24). This suggests that antibodies can be detected for an extended period after exposure. Therefore, the IgG detection in this study is likely the result of naturally-infected breeding and growing pig farms, even if

exposure happened a long time before sampling, as maternally derived antibodies may be undetectable after six weeks of age (4).

It is currently unknown what may have caused the onset of large-scale SVA outbreaks after 2014-2015. The virus is likely to have been circulating within and between U.S. pig farms since at least 1988, as was shown by the sequence analysis of picorna-like viruses isolated from pigs in the U.S. (27). Conversely, another retrospective study attempted to assess the presence of SVA in Brazil through the serological testing of samples collected between 2007 and 2016 (59). The authors concluded that SVA was likely absent in the major Brazilian pig-producing states before 2014. However, the reported results must be interpreted carefully since a low number of samples were tested and collected from asymptomatic farms for other research purposes not related to vesicular diseases. Therefore, the study design likely introduced a selection bias that significantly reduced the probability of detecting SVA-exposed animals. A more comprehensive study design is needed to rule out the presence of SVA among Brazilian pig farms before 2014. It may be possible that SVA can remain present and undetected in pig populations until a formal vesicular disease investigation is conducted and SVA is ruled out.

The results from this study differ significantly from the results of another seroprevalence study conducted in U.S. pig farms using samples collected in 2016 (67). The estimated farm-level seroprevalences were 75.8% in breeding farms and 42.7% in growing pig farms versus 17.3% and 7.4% in this study. The discrepancies in the proportions reported in both studies may be explained by fundamental differences in the

study designs, time periods when samples were collected, and interpretation of the serological assays. This study's source population was U.S. pig farms from major swine-producing companies and veterinary clinics, regardless of their SVA or other infectious diseases status. However, in the study by Houston et al. (67), the source population was pig farms conducting porcine reproductive and respiratory syndrome virus (PRRSv) monitoring at one Veterinary Diagnostic Laboratory with no known history of SVA. It is currently unknown if the presence of other infectious diseases (e.g., PRRS) could be associated with the presence of SVA, which may have introduced potential biases. It is possible that biosecurity failures in PRRSv-positive farms are also responsible for the introduction of SVA; thus, assessing the prevalence of SVA exposure exclusively in farms monitoring for PRRSv is not appropriate. The parallel interpretation of two different serological tests with fair-to-moderate results agreement by Houston et al. (67) may have overestimated the proportion of positive farms, partially explaining the significant differences between both studies.

Very little is known about how SVA transmits between farms. SVA-infected animals appear to develop a short-term viremia for up to 10 days post-infection and shed the virus for up to 28 days post-infection in oral/nasal secretions and feces (11). While this information can help us mitigate transmission between animals on a farm, more information is needed to prevent the infection of pig farms in the first place. To shed some light on this matter, we performed a risk-factor analysis to identify what farm characteristics might be associated with SVA exposure.

Implementation of biosecurity measures in breeding farms yielded a sparing effect in this study. While not surprising, it does remind the industry of the importance and needs that most modern pig farms have when adopting such preventive measures to avoid the introduction of new pathogens carried by people themselves or the boots and clothes they are wearing (68). However, rendering was another predictor in the model that was found to have a significant association with SVA seropositivity. Breeding farms that reported disposing of the carcasses of dead animals via rendering had 9.2 higher odds of being seropositive compared to farms that either compost, bury or incinerate the dead animals. One possible causal pathway for this association is the indirect transmission of the pathogen between farms through the trucks transporting the carcasses since the truck may need to visit several farms before filling and returning to the rendering plant. Similar associations involving the disposal of dead animals via rendering have been reported in other studies, such as with the increased risk of respiratory disease outbreaks in pig farms (69), PRRSv positivity (70), porcine epidemic diarrhea positivity (Morrison Swine Health Monitoring Project science page, personal communication), H5N2 highly pathogenic avian influenza virus (71), and H7N2 avian influenza virus in commercial poultry farms in the United States (72). More studies are needed to understand whether other carcass disposal methods should be considered to reduce the probability of introducing SVA or any other pathogen to the farm.

Due to the low number of positive observations, it was not possible to build a multivariable logistic regression model for the growing pig farms. The positive univariable associations between "external pig loading crew" and "hires more than one

external crew" with the outcome of seropositivity (Table 2.3) highlight the potential role of people in the introduction of pathogens. As for the protective associations, it is not surprising that single-sourcing of weaned pigs and disinfecting all incoming trucks would decrease the odds of seropositivity since such measures prevent the comingling of pigs from negative and positive populations and the cross-contamination between different batches of animals, respectively. However, the statistically significant association of being at lesser odds of positivity when people had direct contact with pigs in the barn after loading pig trucks is unexpected and challenging to explain. Upon further investigating this artifact of the analysis, it was seen that the only farms where people went back into the barns and had contact with the remaining pigs were the ones that did not hire an external pig-loading crew. All farms that hired an external pig-loading crew reported that people left the farms after loading the trucks without contacting the remaining pigs. Therefore, it is likely that this association is measuring a similar effect as the association with the farms that hire external pig-loading crews.

Although the current study design is not optimal for estimating within-farm prevalences, a broad range of SVA-positive sera samples was detected. The range of positive samples per positive farm was somewhat similar between the breeding and growing pig farms (Figure 2.2), with an average of 10.2 and 13.4 out of 30 tested samples in breeding and growing pig farms, respectively (Figure 2.2). As reported in the results section, the estimated within-farm prevalence yielded wide confidence intervals due to the reduced sample size per farm. However, these results may still provide helpful information for further investigations. Nevertheless, interpreting these results is rather

difficult in cross-sectional studies since there is no information about the previous SVA history on tested farms.

2.6. Conclusion

This is the first study specifically designed to estimate the seroprevalence of SVA at a national level, with a broad selection of farms from producing companies and veterinary clinics as the source population. After sampling and testing 5,794 sera samples from 98 breeding and 95 growing pig farms, it was shown that SVA antibodies are present among U.S. pig farms. Seroprevalence was higher in breeding farms than in growing pig sites.

Key risk factors identified were the rendering of dead animals and access of external working crews to the farms. At the same time, the implementation of biosecurity measures seemed to have a protective effect against SVA seropositivity. These findings may be applied in pig farms to help reduce the risk of SVA exposure. Other carcass disposal methods could be considered, such as composting or incineration, or the dead-animal disposal areas should be located away from the farms, and the trucks used for carcass collection should be prohibited from coming close to the barns. Furthermore, attention should be given to biosecurity measures to reduce the risk of pathogen introduction through any incoming personnel or fomites. Although this is the first assessment of farm-level risk factors associated with SVA seropositivity, more studies need to be specifically designed to understand these associations.

Chapter 3: Evaluation of biosecurity procedures to prevent the indirect transmission of Senecavirus A

This chapter is in preparation to be submitted to a peer-reviewed journal

3.1. Summary

Despite several countries reporting Senecavirus A (SVA) outbreaks, little is known about how SVA transmits between pig populations. The objective of this study was to evaluate the role of fomites in the indirect transmission of SVA between pig populations. Forty-eight pigs were allocated into five groups housed in eleven separate biocontainment rooms. Eight pigs were assigned to the infected group (INF), 12 pigs (3 replicates of 4 pigs) to the low biosecurity (L.B.1, L.B.2, and L.B.3) sentinel group, 12 pigs (3 replicates of 4 pigs) to the medium biosecurity (M.B.1, M.B.2, and M.B.3) sentinel group, 12 pigs (3 replicates of 4 pigs) to the high biosecurity (H.B.1, H.B.2, and H.B.3) sentinel group; and 4 pigs to the negative control (NC) group. The INF group was experimentally infected at day 0, and nine movement events occurred from days 2 to 10 post-inoculation between the INF and L.B., M.B., and H.B. groups. Before each movement event, personnel spent 20 minutes of direct contact with the pigs in the INF group and moved to one of the biosecurity groups' replicates, and direct contact with the pigs occurred for 20 minutes. The virus was successfully transmitted to pigs in L.B.1, L.B.2, and L.B.3 rooms, while all M.B. and H.B. replicates remained negative during the entire study. SVA can be indirectly transmitted between pig populations through fomites. This study provides information necessary to mitigate the spread of SVA between pig populations within and across farms and production systems.

3.2. Introduction

Senecavirus A (SVA) causes a vesicular disease in pigs clinically indistinguishable from what is observed in vesicular stomatitis, swine vesicular disease,

vesicular exanthema of swine, and foot-and-mouth disease (FMD) (73). This clinical similarity, especially to FMD, prompts foreign animal disease investigations that must be performed every time any vesicular disease sign is detected in pigs. SVA is a member of the *Picornaviridae* virus family, of which the FMD virus is also a member. This virus is a non-enveloped, single-stranded, positive sense RNA virus and the only member of the genus Senecavirus (1).

The onset of large-scale swine vesicular disease outbreaks due to SVA started in 2014 in Brazil (36) and 2015 in both China (37) and the United States (4,5) (U.S.). Even though several countries reported swine vesicular disease outbreaks due to SVA, very little is known about how SVA transmits between pigs and farms. The vesicular fluid contains large amounts of SVA (5,36), which may play an essential role in the direct transmission of the virus between pigs in the same environment. The virus is usually detected in feces and oral secretions for up to 21-28 days after infection (11,47), and pigs seem to shed in larger amounts during the initial 10 days post-infection before the onset of robust cellular and humoral responses (47). The rupture of these vesicles and shedding may also contribute to the indirect transmission through fomites, as has been reported for other infectious diseases (74,75). Pigs may also become persistently infected with SVA for long periods after disease resolution when clinical signs and shedding are no longer evident. The tonsils of the soft palate are known to harbor SVA and are an important site for viral replication (11). In a previous study, infectious SVA was isolated from the tonsils of two pigs 60 days post-infection (16). Additionally, SVA RNA has been found

in large quantities in the tonsils and testes of a boar 156 days after an SVA outbreak in a sow farm (76).

The main objective of this study was to evaluate the role of fomites in the indirect transmission of SVA between pigs in separated environments. As a secondary objective, we also assessed the infectivity of SVA found in the tonsils of persistently-infected animals.

3.3. Materials and Methods

This study was approved by the University of Minnesota (UMN) Institutional Animal Care and Use Committee (Protocol 1903-36847A). The UMN's Institutional Review Board (IRB) determined that this study does not involve human subjects (IRB ID: STUDY00010190).

3.3.1. Experimental design

This experiment was designed to be a proof-of-concept to determine the role of indirect transmission on the epidemiology of SVA. The sample size was selected for testing the hypothesis that indirect transmission of SVA to a sentinel group would occur, assuming an overall 90% probability of at least one transmission event happening if the probability of transmission to any biocontainment room is at least 53%.

Forty-eight nine-week-old pigs acquired from an SVA-negative farm were tested for SVA by serum indirect immunofluorescence (IFA) and rectal swab qRT-PCR. The animals were then assigned to one of five experimental groups housed in the UMN BSL-2 Veterinary Isolation Facility (VIF) and fed ad libitum commercial feed and water. Each experimental group was housed in separate biocontainment rooms. Eight pigs were

assigned to the infected group (INF), 12 pigs (3 replicates of 4 pigs) to the low biosecurity group (L.B.1, L.B.2, and L.B.3), 12 pigs (3 replicates of 4 pigs) to the medium biosecurity (M.B.1, M.B.2, and M.B.3) group and 12 pigs (3 replicates of 4 pigs) to the high biosecurity (H.B.1, H.B.2, and H.B.3) group. The negative control group had 4 pigs.

3.3.2. Entering the VIF and gaining access to the biocontainment rooms

Showering in was mandatory for all personnel before entering the VIF. Upon exiting the shower, personnel wore VIF-specific scrub pants, shirts, and disposable plastic boots before entering the VIF's clean hallway area. Before entering each biocontainment room, study personnel picked up the following PPEs: cloth coveralls, nitrile gloves, a bouffant cap, and a pair of clean and disinfected rubber boots. Upon entering the anteroom, personnel stepped into a hydrogen peroxide (Rescue™) footbath and put on all PPEs that were picked up in the hallway. All personnel stepped into two additional hydrogen peroxide footbaths to access the biocontainment room: one footbath before going through the door between the anteroom and biocontainment room and another footbath after walking past the door. The biocontainment room layout is shown in Figure 3.1.

3.3.3. SVA inoculation and exposure

The virus used for the inoculum was grown on NCI-H1299 cells (ATCC® CRL-5803™) in RPMI cell culture medium (ATCC®30-2001™). A total of six out of eight INF pigs were challenged at day 0 post-inoculation (pi), while the remaining two SVA-naïve pigs were housed in a different biocontainment room. Each pig was intranasally

inoculated with 4mL of RPMI-SVA solution with a titer of 1.2 X 10^7 $TCID_{50}$/mL upon laboratory preparation and 3.2 x 10^6 $TCID_{50}$/mL back titrated after inoculation of the animals. The two additional SVA-naïve pigs were moved to the INF biocontainment room on day 2 pi to act as sentinel pigs as these had direct exposure to the SVA-challenged pigs. The four animals from the negative control group were intranasally inoculated with 4mL of sterile RPMI medium. Personnel caring for the N.C. group did not have contact with the INF, L.B., M.B., and H.B. groups. All animals were monitored daily for clinical signs of vesicular disease or other diseases.

3.3.4. Fomite exposure to SVA and movement between experimental groups

All six personnel entered the INF room simultaneously and spent 20 minutes directly contacting INF animals by sampling, holding, or interacting with the pigs. This period allowed fomites (e.g., gloves, coveralls, boots) to become contaminated with infectious particles in feces and secretions. All study personnel interacting with pigs on any given day were assigned into three pairs, and each pair was assigned to either the L.B., M.B., or H.B. group. The two persons in each pair were then responsible for performing movement events to all three replicates of their specific biosecurity group. A movement event was defined as the movement of two persons from the INF to any of the biosecurity treatment rooms. One movement event happened daily in each biosecurity room between days 2 and 10 pi of the INF group (9 movement events) when SVA shedding was highest.

3.3.4.1. Moving from the INF room to the low biosecurity (L.B.) rooms

Following the 20-minute exposure period to the INF pigs, personnel walked directly from the INF room into one of the L.B. rooms using the VIF's dirty hallway area (Figure 3.1) without stepping into hydrogen peroxide footbaths, changing clothing, or PPEs. After spending 20 minutes of direct contact with the L.B. animals (sampling, handling, and interacting with pigs), L.B. personnel washed their rubber boots and entered the anteroom after stepping into two footbaths on each side of the door. Personnel then hung their boots in the anteroom, removed their cloth coveralls, placed them in a bag for washing and disinfection, discarded their gloves and bouffant caps, and washed their hands. Afterward, the two persons gained access to the VIF's clean hallway, where they picked up new PPEs and moved to the INF room again to repeat all the procedures until all three L.B. replicates were done.

3.3.4.2. Moving from the infected room to medium biosecurity (M.B.) rooms

Following the 20-minute exposure to the INF pigs and before entering the M.B. room, personnel washed their rubber boots and entered the INF's anteroom after stepping into two footbaths on each side of the door. M.B. personnel hung their boots in the anteroom, removed their cloth coveralls, placed them in a bag for washing and disinfection, and discarded their gloves and bouffant caps. The two persons picked up new sets of PPEs in the clean hallway (Figure 3.1) and entered the M.B. anteroom, following the same procedures until entering the M.B. biocontainment room. M.B. personnel spent 20 minutes directly interacting with the animals (sampling, handling, and

interacting with pigs). Afterward, personnel followed the same procedures described for the L.B. group to exit the M.B. biocontainment room. New PPEs were picked up again in the clean hallway, and the M.B. pair moved back into the INF room to repeat all procedures until all M.B. replicates were done.

3.3.4.3. Moving from the infected room to high biosecurity (H.B.) rooms

Following the 20-minute exposure to the INF pigs and before moving into one of the H.B. rooms, personnel followed the same procedures performed in the M.B. group; however, in this case, personnel showered and put on new scrub pants and shirts and a pair of disposable plastic boots, picked up new PPEs in the clean hallway, and entered the biocontainment H.B. room following the same procedures described for the other groups. H.B. personnel also spent 20 minutes directly contacting the H.B. animals (sampling, handling, and interacting with pigs). Afterward, H.B. personnel followed the same procedures described for other groups to exit the H.B. biocontainment room. New PPEs were picked up again in the clean hallway, and the H.B. pair moved back into the INF room to repeat all procedures until all H.B. replicates were done.

3.3.5. Sample collection

3.3.5.1. Sampling of animals

Feces and blood were collected from animals of all groups to evaluate SVA shedding, viremia, and IgG antibodies. The pigs' rectums were digitally evacuated, and feces were placed in individual tubes. Blood samples were collected from the external jugular vein into red-top BD Vacutainer® tubes. An animal sampling scheme with the

dates of feces and blood collections is shown in Table 3.1. Pigs from the L.B., M.B., and H.B. groups were euthanized and necropsied on day 24 pi (14 days after the movement period ended), except animals that were euthanized earlier due to non-SVA-related issues or participated in the bioassay portion of the study (as per section 3.3.7); INF pigs were necropsied on day 57 pi, and NC pigs were necropsied on day 67 pi (as per section 3.3.7). At necropsy, the tonsils of the soft palate were collected to assess the presence of SVA RNA.

3.3.5.2. Sampling of PPE fomites and personnel

Following the 20-minute interaction period with the INF group pigs, each person collected four separate rayon-tipped swabs (BBL CultureSwab™ liquid, Stuart single plastic applicator; Becton, Dickinson and Com. Sparks, MD, USA) from their 1) gloves, 2) coveralls, 3) boots, and 4) their nasal cavities. Personnel in the M.B. and H.B. groups repeated this procedure after wearing new PPEs inside the M.B. and H.B. anterooms and before contacting the M.B. and H.B. animals. The entire surface of the gloves was swabbed, beginning with all sides of the fingers and expanding to the palmar and dorsal sides of the hand. Coveralls were swabbed, starting with all sides of both legs and arms and then the chest and right/left sides of the body. Boots were swabbed, beginning with the bottom and then all sides covering the foot and lower legs. Lastly, each person inserted the tip of the swab approximately ¾ of an inch into one of the nasal cavities and slowly rotated the swab at least 5 times in each nostril.

3.3.6. Sample testing

The serum samples were tested for SVA-specific IgG antibodies by IFA (13) in the UMN Veterinary Diagnostic Laboratory (VDL). Feces, serum, and tonsils collected from the animals, swabs from PPE fomites, and personnel nasal cavities were tested for the presence of SVA RNA by qRT-PCR (4,13). All animal samples (feces and tonsils) were qRT-PCR tested at the UMN VDL (13) and were considered positive when the cycle threshold (Ct) value was below 36. All samples from INF pigs were tested individually, while L.B., M.B., and H.B. samples were initially tested in pools of 4 samples of the same kind within each biosecurity replicate. Whenever a feces pool tested positive, all samples from the positive pool were re-tested individually. Swab samples originating from PPEs and personnel nasal cavities were qRT-PCR tested at the Iowa State University VDL (4) and were considered positive when the Ct value was below 35. Swabs from the same type of PPE (boots, gloves, or coveralls) were pooled together for qRT-PCR testing. The fomite samples taken immediately after contact with INF pigs were tested in pools of two samples of the same kind from the same two persons moving into one of the biosecurity group replicates. Fomite swabs collected upon entering the M.B. and H.B. rooms were taken from washed/disinfected PPE and were therefore tested in pools of six samples of the same kind per treatment (e.g., two boot swabs taken upon entering M.B.1 + two boot swabs from M.B.2 + two boot swabs from M.B.3) per day to confirm negativity. All personnel nasal swab samples were tested in pools of two samples taken immediately after direct contact with INF pigs or upon entering the M.B. and H.B. rooms.

SVA virus isolation (VI) (9) was attempted from all INF pigs' tonsils and fomite samples with Ct. values <30.

3.3.7. Bioassay study - assessment of SVA infectivity from persistently-infected pigs

This study assessed the infectivity of SVA RNA found in the tonsils of persistently-infected animals as a secondary objective. Fourteen pigs were needed for this objective, eight INF pigs, four N.C. pigs, one H.B.1, and one H.B.2. Pigs from the INF, NC, and H.B. groups were housed in three separate biocontainment rooms.

All eight animals from the INF group were euthanized after SVA RNA was no longer detected in their feces by qRT-PCR on day 57 pi. At necropsy, the tonsils of the soft palate of all INF pigs were collected into separate Whirl-Pak® sterile sampling bags and refrigerated on ice for immediate transportation to the UMN VDL. Tonsil samples were suspended in 40mL of Hanks media and mechanically homogenized (Stomacher 80 microBiomaster; Seward) for 30 seconds. The entire content from the bags was poured into individual tubes with two 5/32-inch steel grinding balls and mechanically homogenized once more (2010 Geno/Grinder®; SPEX SamplePrep) for 6 minutes at 1,200 rpm. Two aliquots from each tonsil homogenate were tested through qRT-PCR and for VI. The 4 tonsil homogenates with the lowest Ct values were selected to inoculate all 4 NC pigs.

On the following day (58 pi), NC and H.B. pigs' SVA-naïve status was assessed by serum IFA testing, and each NC pig was inoculated with one of the selected tonsil homogenates. Each inoculum consisted of 30mL of tonsil homogenate solution. Due to

the large volume, 15mL of inoculum was intranasally administered (7.5mL in each nostril) at one time, and the remaining 15mL was administered 10 minutes after. Two days after the inoculation, the 2 SVA-naïve H.B. animals were moved into the N.C. biocontainment room to act as sentinels.

Feces and serum samples were collected on days 2, 5, and 7 after inoculating the N.C. pigs with the tonsillar material for qRT-PCR testing. All six animals were necropsied on day 9 after inoculation with the tonsillar material and had their tonsils and serum collected for qRT-PCR and IFA testing, respectively.

3.4. Results

3.4.1. Health monitoring

No clinical signs were visualized during the entire study. One L.B.2 pig had to be euthanized on day 11 pi due to a locomotion-related problem (not caused by SVA). Another L.B.2 pig was euthanized on day 13 pi due to rectal prolapse. One M.B.3 pig and one H.B.3 pig were euthanized on days 7 and 10 pi, respectively, due to hematomas related to the multiple blood sampling events.

3.4.2. Feces and serum qRT-PCR/IFA results

Animals from the INF group shed SVA in feces every day during the personnel movement period (Figure 3.2). The 2 sentinel pigs in direct contact with the inoculated animals on day 2 pi had SVA RNA detected in their feces starting on day 3 pi (1 day after commingling). The feces from the INF group had qRT-PCR-positive results (Ct <36) up until day 45 pi (Figure 3.3). The INF group had viremic pigs during the entire movement

period, as shown by the serum qRT-PCR results in Figure 3.4, and all pigs had seroconverted by 28 dpi (Table 3.2).

All pigs from the replicates in the L.B. group became infected with SVA as feces from the L.B.2 and L.B.3 groups had qRT-PCR-positive results at movement day 9 (Mv 9) (Figure 3.2) and L.B.1 and L.B.3 were qRT-PCR-positive by 11 days post-movement period (11 PMv) (Figure 3.2). One animal from the L.B.2 replicate was viremic on MV 8, and both L.B.2 animals were viremic at day PMv 4 (Figure 3.4). The serum samples from L.B.1 and L.B.3 replicates were qRT-PCR-positive when pool-tested at day 4 PMv (Table 3.3) and remained positive by 11 and 14 PMv (Figure 3.4).

Pigs from the M.B. and H.B. replicates had qRT-PCR-negative feces four days after the movement period and remained SVA-free at necropsy.

3.4.3. PPE fomites and human nasal swabs

Fomite qRT-PCR-positive results were only seen when samples were collected from PPEs after direct contact with the inoculated animals (Table 3.4). All PPEs were qRT-PCR-negative before entering the M.B. and H.B. rooms (Table 3.4). All human nasal swabs from Mv 1, Mv 4, and Mv 7 had qRT-PCR-negative results. The virus was successfully isolated from the swab samples taken from the boots of personnel leaving the INF room and heading to the L.B.3 room on Mv 5, and from the swab samples taken from the coveralls of personnel leaving the INF room and heading to the L.B.1 room on Mv 8.

3.4.4. Necropsy results

All INF pigs had qRT-PCR-positive tonsils at necropsy on day 57 pi (Table 3.2), but VI failed in all tonsil samples. All L.B. pigs' tonsils tested qRT-PCR positive for SVA at necropsy, while M.B. and H.B. pigs had qRT-PCR-negative tonsils (Table 3.2).

3.4.5. Bioassay results

All NC and H.B. pigs participating in the bioassay portion of the study were IgG-negative at the beginning of the trial. The tonsil homogenates from the four INF pigs (ID 141, 147, 186, and 191) had Ct values ranging from 24 to 26 (Table 3.2). All feces and serum tested negative on days 2, 5, and 7 post-tonsil homogenate inoculation. At necropsy, SVA RNA or anti-SVA IgG antibodies were not detected in all animals' tonsils or serum (Table 3.2).

3.5. Discussion

Although SVA has been present in U.S. pig populations since at least 1988 (27) and was recently reported in several countries, minimal information about its transmission and mitigating biosecurity practices is currently available. In this study, we provide evidence that SVA present in the secretions of infected pigs can contaminate fomites and be later indirectly transmitted to SVA naïve pig populations.

Oronasal secretions and feces from infected pigs have been previously suggested as a source of SVA to susceptible pigs (11), and virus shedding is detectable for at least 9 weeks after outbreak detection under field conditions (51). Direct pig-to-pig transmission has been reported previously (51) and is further evidenced in this study. The two sentinel pigs in direct contact with the six inoculated INF animals were successfully infected—

confirmed by the qRT-PCR-positive results in feces, serum (Figures 3.2 and 3.3), and tonsils at necropsy together with IFA-positive results (Table 3.2). Considering that no vesicles or skin lesions were present in the infected animals, there is strong evidence that SVA shed through the infected animals' secretions was the source of infection in the two sentinel pigs. However, it is unknown whether SVA can be aerosolized and transmitted to other pigs through the air, which may have also contributed to the sentinel pigs' infection. The aerial spread of SVA still needs to be studied.

SVA-naïve pig populations are at risk of infection if exposed to contaminated fomites, as evidenced by the successful transmission of SVA to all L.B. replicates (Figures 3.2 and 3.3, and Table 3.2). All L.B. replicates had at least one pig that shed SVA in feces, developed viremia, and seroconverted. On movement day 8 (Mv 8), the first L.B. pig (L.B.2) developed viremia, as shown by the serum qRT-PCR results (Figure 3.4). L.B.2 pigs only had qRT-PCR-positive feces on Mv 9 after a viremic pig was detected on Mv 8. Infection and viremia may occur a few days after exposure to SVA-contaminated environments, which could explain the first viremic L.B. animal detected on Mv 8. This finding contrasts with similar studies performed with PEDV (75) and IAV (74), where shedding was detected 1 and 5 days after pigs had contact with contaminated PPEs, respectively. The L.B. group was designed having two scenarios in mind: 1) some pig farms do not require changing of clothing and boots for incoming personnel, and 2) it is not uncommon in the swine industry for personnel to walk through all barns on a farm without changing clothes, boots, and other PPE. Therefore, contaminated PPEs may help disseminate SVA between pens, barns, and farms.

The M.B. and H.B. pigs remained SVA-negative throughout the experimental study (Figures 3.2 and 3.3 and Table 3.2). No SVA was found in fomites when entering the M.B. and H.B. rooms, suggesting that changing clothes, boots, and other PPEs prevents people from carrying SVA from infected to naïve populations. However, when visiting a pig farm, it is advisable to always shower in and out of the facilities to have higher confidence in the containment of this pathogen.

SVA positivity rate was higher in boots than in coveralls or gloves used by the study personnel (Table 3.4). Boots from 1/3 L.B. replicates, 1/3 M.B. replicates, and 2/3 H.B. replicates were positive at Mv 1, while all other fomites were negative. On Mv 4 and Mv 7, the boots from all L.B., M.B., and H.B. replicates were SVA-positive. Gloves were only positive in 1/3 L.B., 1/3 M.B., and 1/3 H.B. replicates on Mv 4, and 1/3 L.B. replicates on Mv 7. Similarly, coveralls were SVA-positive only in 2/3 M.B., and 1/3 H.B. replicates on Mv 4, and 1/3 L.B. and 1/3 H.B. replicates on MV 7. The boots may be at higher risk of exposure to higher amounts of SVA due to direct contact with feces and contaminated floors. Boots also seem to carry larger amounts of manure, where SVA can be found. Pigs tend to rub and chew on the boots and coveralls of farm personnel, potentially contaminating those surfaces. However, the SVA contamination of coveralls and gloves seemed to happen in lower amounts than in the boots under this study's experimental settings. Washing/disinfecting and changing boots may be critical points for mitigating SVA spread. The virus was successfully isolated from swab samples taken from boots and coveralls of personnel leaving the INF room and heading into the L.B. rooms, further evidencing the role of fomites in carrying and transmitting infectious virus

to susceptible populations. The amount of time that SVA may survive on fomites or in the environment is unknown. Still, infectious SVA has been detected in different SVA-spiked feed ingredients for up to 91, 35, and 14 days of incubation at 4°C, 15°C, and 25°C, respectively (50).

Even though SVA RNA was detected in the INF pigs' tonsil homogenates, all N.C. pigs remained SVA-negative after being inoculated under the conditions of the bioassay portion of this study. In a previous study, the infectious dose of SVA in finishing pigs was 1,260 $TCID_{50}$/ml, for a total of 6,300 $TCID_{50}$ when administering 5ml of inoculum (77). Using the UMN VDL's SVA qRT-PCR standard curve (data not shown), we can estimate the tonsil homogenates used for the inoculation had from 438 to 1,671 $TCID_{50}$/ml, which brings to a total of 13,140 to 50,130 $TCID_{50}$ per pig after administering 30ml of inoculum. Although it seems that the total dose should have been enough to infect a pig, a lower volume of inoculum with the same total $TCID_{50}$ could have increased the chances of infection. However, despite being qRT-PCR positive, the tonsils were VI-negative. Therefore, the true tonsil homogenates' $TCID_{50}$/mL and infectivity are unknown. Further studies are needed to evaluate the infectivity of SVA found in the tonsils of persistently infected pigs.

All human nasal swabs taken right after direct contact with the INF group and upon entering the M.B. and H.B. rooms were SVA-negative. The recovery of FMD virus from the nose, throat, saliva and air expelled from humans who had direct contact with infected animals has been shown up to 28 hours after the contact (78). The aerosolization of SVA by infected pigs has not been studied. However, this study's small number of SVA-

infected animals may not have been enough to produce detectable amounts of viruses in the personnels' nasal cavities.

The possibility of SVA transmission from persistently infected to naïve pigs should not yet be discarded. The tonsils of the soft palate are a known location for SVA replication (11), and different studies have reported the persistence of SVA RNA for long periods after disease resolution (11,16,47,51). In one study, infectious SVA was isolated from the tonsils of two pigs 60 days post-infection (16). Intermittent viremia and shedding were also reported up to 60 days post-infection after challenging the pigs with different stressors (16). A large-scale study may be needed to evaluate the risk of infectious SVA being transmitted from persistently infected to naïve animals. The risk of transmission in the presence of several carriers and naïve animals exposed to different stressful scenarios under farm conditions may still exist and thus should be assessed.

The small sample size of pigs used in this study is an important limitation since a higher number of pigs in the infected and biosecurity rooms could have changed the results. Transmission to the L.B. rooms could have happened earlier, or perhaps even the M.B. rooms could have been infected due to higher contamination of study personnel. The VI-negative tonsil homogenates from the infected pigs raise questions about the infectivity of SVA in these tissues, even though they were qRT-PCR-positive.

3.6. Conclusions

SVA can be indirectly transmitted between pig populations through contaminated fomites and under low biosecurity standards. Therefore, basic biosecurity procedures can

prevent the indirect transmission of SVA between pig populations, as demonstrated in this study.

Chapter 4: Comparison of sample types to diagnose Senecavirus A throughout different stages of infection and persistently infected pigs

This chapter is in preparation to be submitted to a peer-reviewed journal

4.1. Summary

Senecavirus A (SVA) is the causative agent of an important vesicular disease in pigs and is clinically indistinguishable from high-consequence foreign animal diseases such as foot-and-mouth disease. SVA is known to shed for up to 28 days in oral and nasal secretions and feces, and the existence of persistently infected pigs has also been reported. However, it is currently unknown what kind of sample matrix is better suited to detect SVA throughout different stages of infection or in persistently-infected animals. The main objective of this study was to compare different sample types to diagnose SVA throughout various stages of infection. Oral swabs, rectal swabs, tonsil swabs, tonsil scrapings, sera samples, and oral fluids were collected at 1, 3, 7, 10, 14, 21, 28, 35, 42, and 48 days post-inoculation of two different SVA strains. All samples were tested for the presence of SVA RNA by qRT-PCR, and sera samples were also tested for the presence of SVA-specific IgG antibodies. Tonsil scrapings appear to be the sample matrix of choice to detect SVA in persistently-infected animals, as it was the only sample type where SVA RNA was found at the later stages of infection. However, other samples, such as rectal swabs, may be the best suited sample type during the earlier stages of infection. Oral fluid samples were also successful at diagnosing SVA at the group levels.

4.2. Introduction

Senecavirus A (SVA; *Picornaviridae* family) has emerged as an important pathogen affecting swine populations worldwide, as it has been reported in multiple countries in the Americas (5,9,36,38,41,42) and Asia (37,39,40). Clinically, the virus causes a vesicular disease that is indistinguishable from high-consequence foreign animal

diseases such as foot-and-mouth disease (FMD). Vesicular lesions seem to develop approximately within four days after pigs become infected and resolve within ten days thereafter (11,47). Sudden increases in neonatal mortality have also been associated with SVA outbreaks (32).

Once a pig is infected, SVA RNA can be detected in serum for up to 10 days, but shedding through oro-nasal secretions and feces can last up to 28 days (11,47). The serological response is also detected early after infection, with neutralizing and IgG antibodies detected five and ten days after infection, respectively (47). Intriguingly, SVA RNA is consistently detected in different tissues of previously infected animals for long periods after clinical disease resolution. In a recent study by Joshi et al. (2016) (11), the virus was detected by reverse transcriptase quantitative (qRT-PCR) and *in situ* hybridization (ISH) in the tonsils of experimentally-infected animals 38 days post-inoculation (pi). Sturos et al. (2022) reported the detection of SVA RNA in the testicles and tonsils of adult boars for up to 156 days after an SVA outbreak was detected in a breeding farm, although the time of the boar's exposure is unknown (76). In another study by Maggioli (2019) (16), SVA was isolated from the tonsils collected from experimentally-infected pigs on day 60 pi. These findings raise important questions about the potential mechanisms for the virus to persistently infect pigs and remain asymptomatically present in pig populations. This asymptomatic carrier state plays an essential role in the epidemiology of other picornaviruses, such as FMD virus (FMDV)(17) and encephalomyocarditis virus (52), especially as sources for new

outbreaks in the case of FMDV (17). It has been found that carrier animals' feces and oral/nasal secretions can test SVA positive after stressful conditions (16).

Currently, there is no established method to identify SVA persistently infected pigs. However, oesophageal-pharyngeal fluids from cattle are collected through the probang cup method for FMDV (17). Identifying a sampling method for SVA could provide veterinarians and caretakers with a tool to control and eliminate this virus. In addition, there is no current parallel comparison of sample types to detect SVA throughout different stages of infection, which can help improve the monitoring and control of this disease in pig populations. Therefore, this study aims to compare different sample types to detect two different SVA strains in live pigs throughout the infection and their persistent-infection period. As secondary objectives, we aimed to characterize the infection dynamics of two different SVA strains, describing virus shedding, length of viremia, and serological IgG responses. We hypothesize that sampling the tonsillar tissue of pigs yields higher probabilities of SVA detection during later stages of infection compared to the collection of oro-nasal secretions and feces.

4.3. Materials and methods

The University of Minnesota (UMN) Institutional Animal Care and Use Committee (IACUC) approved this study (protocol 1810-36438A).

4.3.1. Animals and facilities

A total of 28 crossbred three-week-old gilts from a breeding herd with no prior history of SVA infections were enrolled in the study. Sera and oral swabs from all pigs were collected to confirm naïve status using indirect immunofluorescence (IFA) and

quantitative reverse-transcription real-time PCR (qRT-PCR). Animals were housed in the University of Minnesota's Veterinary Isolation Unit (BSL-2) and were fed ad-libitum commercial feed and water.

4.3.2. Virus isolates and cell culture

This study used two SVA isolates: a historical (99-14900\MN) and a contemporary (19-19343\MN) isolate. Both strains were isolated from clinically affected pigs in 1999 and 2017, respectively, and have 87.5% nucleotide similarity. The NCI-H1299 (ATCC® CRL-5803™) human lung carcinoma cell line was used with RPMI-1640 cell medium (ATCC®30-2001™), as previously described (76). The observation of cytopathic effects signaled virus growth. Two freeze-thaw cycles were performed on each flask of SVA-infected cells, and the supernatant was collected to be used as the inoculum. The virus titers were determined by Spearman and Karber's method (79), and titers were expressed as tissue culture infective dose ($TCID_{50}$) per milliliter.

4.3.3. Experimental design, animal health assessment, and sampling

Pigs were housed at the University of Minnesota BSL-2 Veterinary Isolation Facility. Each experimental group was housed in a separate room, and pigs were observed daily for any clinical signs of vesicular disease.

Animals were divided into three groups: negative control group (NC) (n=4), contemporary group (C) (n=12), and historical group (H) (n=12). All groups were intranasally inoculated with their respective treatment: Group C with a 5mL inoculum containing a total titer of 9.6×10^6 $TCID_{50}$ and group H with a 5mL inoculum with a total

titer of 1.7×10^7 $TCID_{50}$. The NC group was sham-inoculated with sterile RPMI culture media.

Sampling methods included oral, rectal, and tonsil swabs, as well as tonsil scrapings and blood (i.e., serum). Oral fluids were also collected to assess their role in SVA detection. Samples were collected at 1, 3, 7, 10, 14, 21, 28, 35, 42, and 48 days post-inoculation (dpi). Sera samples were not collected at 1, 42, and 48 dpi.

Oral swabs were collected by inserting the swabs into the oral cavity towards the caudal part of the mouth, between the cheek and teeth. Rectal swabs were collected by inserting the swabs into the rectum for saturation with feces. Tonsil swabs were collected using a mouth speculum and rubbing it against the tonsils of the soft palate. Tonsil scrapings were collected immediately after the collection of the tonsil swabs—while the speculum was still keeping their mouths open—and a disposable plastic spoon was used to scrape the tonsils in a back-and-forth motion (80), attempting to extract cells and mucus from the tonsillar crypts. A swab was then used to remove the collected material from the spoon. All swabs used in this study were rayon-tipped swabs with Stuart's medium (BBL CultureSwab™ liquid, Stuart single plastic applicator; Becton, Dickinson and Com. Sparks, MD, USA). Blood samples were collected from the external jugular vein into red-top BD Vacutainer® tubes.

Oral fluids were collected using the cotton rope technique (81). Briefly, a cotton rope was hanged in an accessible part of the pen for pigs to chew on it. At 1 and 3 dpi, the ropes were left hanging for 24 hours since young piglets do not promptly chew on the rope. From 7 dpi onward, the ropes were suspended for approximately 30 minutes.

4.3.4. Laboratory testing

All samples were submitted for testing at the University of Minnesota's Veterinary Diagnostic Laboratory immediately after being collected. Sera samples were tested by IFA for IgG antibodies, as previously described (58), and by SVA qRT-PCR to assess viremia. All swabs and oral fluid samples were tested by SVA qRT-PCR for the presence of SVA RNA, as previously described (13). Samples were considered positive when the cycle threshold (Ct) value was below 36.

All collected samples were tested individually up to 35 dpi. At 42 and 48 dpi, oral swab samples were tested in pools of four, while all other samples were still tested individually. At 48 dpi, rectal swabs were also tested in pools of four, while the tonsil swabs and tonsil scrapings were still tested individually.

4.3.5. Statistical Analysis

Descriptive statistics were used for sera qRT-PCR and IFA results and oral fluids qRT-PCR. Differences in the qualitative swab results by sample type and collection day for each SVA isolate were compared using the Cochran Q test and pairwise McNemar test with Bonferroni correction.

A multivariable mixed effects logistic regression was fit to evaluate the association between all swabs and tonsil scraping sample types and the probability of testing positive by SVA qRT-PCR. The test result (positive/negative) was the outcome. Sample type and number of dpi (as an integer variable) were modeled as the predictors. One interaction term was added between sample type and dpi. Individual pig information

(ear tag identification number) was added as a random effect to account for the longitudinal sampling of animals.

Kaplan-Meier survival curves were plotted to visualize time-to-negativity in oral, rectal, and tonsil swabs and tonsil scrapings over time after inoculation in each SVA isolate group. Median survival times for each sample type and SVA isolate group are also reported.

4.4. Results

All animals were confirmed to be SVA-negative by qRT-PCR in the pooled oral swabs and IgG negative by IFA upon arrival at the research facility. The NC group remained SVA-free throughout the study, as all samples yielded negative results. On the other hand, no animals from the C or H groups developed clinical signs compatible with a vesicular disease in this study after SVA inoculation. One pig from group C died on day 39 pi due to an SVA-unrelated cause.

4.4.1. Oral fluids qRT-PCR results

OF testing consistently detected SVA RNA in both groups up until 35 dpi. Ct values at 1 dpi were in the low 20s and above 30 for both SVA isolate groups at 35 dpi (Table 4.1).

4.4.2. Serum IFA results

The first IgG-positive samples were detected at 10 dpi in group C and 14 dpi in group H in one out of 12 and five out of 12 animals, respectively. Group H achieved 100% positivity (12 positives out of 12 animals) at 28 dpi, but group C only had 11 out of

12 IgG-positive pigs at this point. Group C achieved 100% positivity (12 positives out of 12 animals) at 42 dpi when the last pig tested IgG-positive by IFA.

4.4.3. Individual pig oral, rectal, and tonsil swabs, tonsil scrapings, and sera qRT-PCR results

Individual pig test results are shown in Figure 4.1. All pigs from both C and H groups had qRT-PCR positive oral and fecal swabs by 3 and 7 dpi. The positivity rate declined earlier in the oral swabs, with nine out of 12 animals from group H and six out of 12 from group C yielding positive qRT-PCR results at 14 dpi, with the last detections in three out of 12 group H pigs and one out of 12 group C pigs on days 21 and 28, respectively. All pigs from both groups had qRT-PCR positive rectal swabs at 21 dpi, with the last positive detections in one out of 12 group H and five out of 12 group C pigs at 28 and 35 dpi, respectively. Tonsil swabs also consistently tested positive for SVA, with the last positive results in two out of 12 group H and one out of 12 group C animals at 28 and 35 dpi, respectively. Tonsil scraping was the only sample type that detected SVA RNA in animals from both groups throughout the entire study period. Seven out of 12 group H and two out of 11 group C pigs had SVA-positive tonsil scraping results by qRT-PCR at 48 dpi. Serum qRT-PCR results were positive in 12 out of 12 pigs from group H and 11 out of 12 pigs from group C at 3 dpi. Viremia was detected up until 28 and 14 dpi in one and two animals from groups H and C, respectively.

4.4.4. Differences between individual sample types

Cochran's Q test indicated differences between the proportions of positive test results of the individual sample types for several days for both SVA isolate groups ($p <$

.05). In groups H and C, significant differences were seen at 7, 10, 14, 21, 28, 35, and 48 dpi. However, the Bonferroni-adjusted pairwise McNemar test revealed no differences between the sample types at 7, 28, 35, and 48 dpi in group H and 7, 14, 28, 35, 42, and 48 in group C. In group H, there was a statistically significant difference between the proportions of positive serum and tonsil scraping samples ($p=0.044$) at 10 dpi; serum/tonsil scraping ($p=0.044$) and serum/tonsil swab ($p=0.015$) at 14 dpi; and serum/rectal swab ($p=0.015$) at 21 dpi. In group C, statistically significant differences were found between serum and all other individual sampling methods ($p=0.017$) at 10 dpi; serum/rectal swab ($p=0.015$), and rectal swab/tonsil swab ($p=0.026$) at 21 dpi.

4.4.5. Multivariable mixed effects logistic regression

Significant associations were found for all variables and interaction terms (Table 4.2). The odds of testing positive by oral, rectal, and tonsil swabs significantly decreased over time after inoculation compared to tonsil scrapings ($p<0.001$). The odds of testing positive in the initial 7-10 days after inoculation were higher in oral, rectal, and tonsil swabs when compared to tonsil scrapings (Table 4.3). However, when compared to tonsil scrapings, the odds of testing positive in oral, rectal, and tonsil swabs gradually became lower as the dpi increased (Table 4.3).

4.4.6. Kaplan-Meier survival curves

Figure 4.2 shows the Kaplan-Meier survival curves for the contemporary and historical strain groups.

The median survival times for the oral swabs, rectal swabs, tonsil swabs, and tonsil scrapings were 17.5 dpi, 35 dpi, 21 dpi, and 38.5 dpi, respectively, in the contemporary SVA isolate group.

The median survival times for the oral swabs, rectal swabs, and tonsil swabs were 21 dpi, 28 dpi, and 28 dpi, respectively, in the historical SVA isolate group. The median survival time for the tonsil scrapings was not estimated because seven out of 12 pigs (~58%) were still positive in this sample type at 48 dpi.

4.5. Discussion

Vesicular disease outbreaks associated with SVA are constantly reported in different parts of the world. This virus is also responsible for the record-high numbers of foreign animal disease investigations in the United States (6) due to its clinical similarities with FMD. Therefore, identifying the best sample type to develop a robust sampling, monitoring, and surveillance strategy is critical to help stop SVA's spread. Furthermore, developing effective elimination plans will also depend on a robust herd monitoring plan based on the most sensitive sample type. Our results deliver novel information regarding individual and aggregate-level samples that can be directly applied to swine operations.

This is the first study reporting the use of tonsil swabs and tonsil scrapings to detect SVA. Tonsil swab testing had the same duration of SVA detection as rectal swabs (Figures 4.1 and 4.2). Conversely, tonsil scrapings was the only sample matrix where SVA RNA was successfully detected by qRT-PCR throughout the entire study period, regardless of the isolate. This sample type has been suggested as a prominent sample

matrix for detecting swine pathogens. Its use has been indicated for the early detection of classical swine fever virus (82). For porcine reproductive and respiratory syndrome virus, it may be a sample of choice during later stages of infection (83). In the case of SVA, tonsil scrapings are a sample to consider when investigating persistently infected pigs long after the resolution of clinical signs but when animals are likely still IgG-positive by IFA.

Identifying SVA persistently infected pigs may be necessary for adequately controlling this disease since one asymptomatic pig carrying SVA may act as a source of virus to other naïve animals. This is a known possible scenario in FMDV persistently-infected cattle and the reason why these animals are identified by collecting probang samples(17). In the case of SVA, it is unknown how long an infected pig may harbor the virus in their tonsils or other tissues and whether this virus will still be infectious. Still, SVA RNA has been detected in the tonsils and testicles of a boar up to 156 days after an SVA outbreak on a farm(76), and infectious SVA was isolated from the tonsils of pigs 60 days after infection (16). Due to this prolonged period when pigs may still be infectious to other naïve animals, it is highly recommended to monitor previously exposed herds by including tonsil scrapings qRT-PCR testing if an SVA-negative status is desired.

However, other sample types may be better suited for SVA testing and detection during earlier stages of infection. Cochran's Q test results indicated the presence of statistically significant differences between SVA detection rates in oral, rectal, and tonsil swabs, tonsil scrapings, and sera at most time-points after 7 dpi for both SVA isolate groups (Section 3.2). However, very few differences in the frequencies of positive results

between sample types were detected with the pairwise McNemar test after the Bonferroni correction. Most of the pairwise differences were between serum and other samples at 10, 14, and 21 dpi in animals infected with the historical strain and between serum and other samples at 10 and 21 dpi in animals infected with the contemporary SVA strain. The differences in the frequencies of SVA-positive results between the sample types were due to most serum samples testing negative after 10 dpi. There was a statistically significant difference between rectal and tonsil swabs at 21 dpi in group C animals due to all 12 group C pigs being positive in the fecal swab while most were negative in the tonsil swab samples. This higher positivity rate in fecal swabs at 21 dpi may be beneficial in field conditions due to the ease of sample collection compared to tonsil swabs, as the latter will require animal restraining and mouth speculums.

The results from the multivariable model further demonstrate how other sample types may be better suited for SVA detection during earlier disease stages. The odds of testing positive by oral, rectal, and tonsil swabs were higher than testing positive by tonsil scrapings in the first days post-inoculation ($p<0.01$). However, the odds of testing positive by oral, rectal, and tonsil swabs decreased by 21%, 13%, and 24%, respectively, for each additional dpi ($p<0.01$) (Table 4.2). Therefore, the probability of tonsil scraping yielding a positive SVA qRT-PCR result was significantly higher ($p<0.01$) during the late stages of infection (i.e., after 14 dpi) (Table 4.3). The Kaplan-Meier survival curves also illustrate graphically how rectal swabs successfully identified all SVA-positive animals up until 21 dpi, while tonsil scraping was the only sample type still detecting positive animals at 48 dpi. The median survival time (i.e., the time at which half the pigs tested

negative) was 35 dpi in rectal swabs and 38.5 dpi in tonsil scrapings from animals infected with the contemporary strain, and 28 dpi in rectal swabs from animals infected with the historical strain. Therefore, these findings suggest that even though tonsil scrapings may be the sample matrix of choice to detect persistently-infected animals, other sample types, such as rectal swabs, are likely the better choice to detect SVA in earlier stages of infection.

It is also noteworthy that OF qRT-PCR testing successfully detected SVA RNA in both groups of infected pigs until 35 dpi (Table 4.1), likely due to the high levels of virus shedding on oral secretions and feces. Collecting this sample is easier, inexpensive, animal-welfare friendly, and safe for farm personnel, making them an excellent alternative for the population-level assessment of pathogens such as SVA.

From a serology standpoint, SVA IgG detection obtained in our study are compatible with what has been previously reported (47). However, despite serological tests being useful when assessing past pathogen exposures, it is not suited to detect persistently-infected pigs. Molecular diagnostics are needed to detect SVA's genetic material in an infected pig's tonsils or other tissues. It is currently unknown whether immune animals can still become persistently infected with SVA after being re-exposed—such as in the case of FMDv-immune cattle after FMDv re-exposure (17,18).

Limitations of this study include the small sample size and the fact that all animals were inoculated with SVA simultaneously. A larger sample size could have permitted a better description of the differences between sample types. Due to all pigs being inoculated at the same time, 100% disease prevalence was achieved among the pigs

in the research biocontainment rooms. This is an implausible scenario in pig barns, which likely affected the probabilities of SVA detection over the different time points.

4.6. Conclusions

This study highlights the use of different sampling strategies to detect SVA throughout various stages of infection. Our findings suggest that a tonsil scraping sample is the best matrix to help detect SVA RNA during the later stages of infection when animals may be persistently infected. However, other sample types may be the best option during the earlier stages of infection, such as rectal swabs, which are also easier and less stressful to collect. Oral fluids can also be used for population-level SVA diagnosis due to the high virus shedding in oral secretions and feces. This novel information is essential for developing efficacious and robust SVA monitoring programs to minimize disease transmission between pig populations.

Chapter 5: First assessment of time-to-negative processing fluids in breeding herds after a Senecavirus A outbreak

This chapter is in preparation to be submitted to a peer-reviewed journal

5.1. Summary

Senecavirus A (SVA) causes a swine vesicular disease and is responsible for a rampant increase in the yearly number of foreign animal disease investigations conducted in the United States. Diagnostic investigations for SVA are performed by sampling animals individually by collecting individual-animal samples, which is labor-intensive and stressful. Developing an alternative aggregate sampling method would facilitate the detection of this virus at the population level. In a preliminary study, SVA was detected in processing fluids (PF) collected in a breeding herd before and after outbreak detection. The objective of this study was to estimate the average number of SVA-positive weeks in PF after an SVA outbreak. Ten farrow-to-wean breeding herds volunteered to participate in this study, longitudinally collecting PF samples after an SVA outbreak was detected in each farm for qRT-PCR testing. The PF samples from the 10 farms were SVA-positive for an average of 11.8 weeks after an outbreak. Testing of PF may be a cost-effective method to detect SVA presence and help halt its spread in SVA-endemic regions.

5.2. Introduction

Senecavirus A (SVA) has been responsible for swine vesicular disease outbreaks in different parts of the world (5,9,36–39), causing concern due to its clinical similarity to foot-and-mouth disease (FMD). Since all vesicular disease cases in pigs require a differential diagnosis from FMD—a World Organization for Animal Health-listed disease—SVA can be costly for governmental animal health agencies and the local swine industry. In the United States (U.S.), SVA has been knowingly responsible for a rampant increase in the yearly number of foreign animal disease investigations (FADI) since 2016

(6). Aside from vesicular lesions, the virus has also been associated with increased neonatal mortality and diarrhea in piglets (5,9,36). However, several aspects of this disease's epidemiology, pathogenesis, immunology, and production impact are still unclear.

Diagnostic investigations for SVA are performed by sampling animals individually by collecting oral, nasal, and rectal swabs (11), vesicular fluids, blood (9), and other tissue samples (9,47). However, individually sampling animals is labor-intensive and stressful; therefore, not optimal for disease monitoring and surveillance. The development of alternative aggregate sampling methods, such as oral fluids and processing fluids (PF) sampling, has facilitated the detection of pathogens at the population level (19,22). This sample type comprises the serosanguinous fluid recovered from piglet processing (i.e., castration and tail docking) during the first week after birth (84). This new sampling methodology is used for the detection of porcine reproductive and respiratory syndrome virus (PRRSV) (20,22), and its use has been suggested to help establish the breeding herd status in herds undergoing the elimination of wild-type viruses (85). Currently, there is scarce information regarding the presence of SVA in PF after an outbreak.

A preliminary study reported the presence of SVA RNA in PF 11 days before farm staff detected clinical signs suggestive of vesicular disease. The last SVA-positive PF sample was seen over 50 days after the outbreak was detected in one breeding farm (24). This sustained detection of SVA RNA in processing fluids may be potentially linked to long-term disease transmission. Therefore, characterizing the detection of the

virus in PF is necessary to advance the epidemiological knowledge of this disease. This can lead to the creation of a system for time-to-stability estimation, similar to what is currently done with PRRSV.

The objective of this study was to estimate the average number of SVA-positive weeks in PF after an SVA outbreak. As a secondary objective, we also aimed to assess the production losses associated with an SVA outbreak.

5.3. Materials and methods

5.3.1. Study design and breeding herd eligibility criteria

A cohort of 10 breeding sow farms undergoing an SVA outbreak was conveniently selected to participate in this study. Sow farms from pig-producing companies or managed by veterinary clinics in the U.S. were invited to participate in this study. Farm enrollment was dependent on their acceptance.

5.3.2. Sample size calculation

The sample size of 10 sow farms was determined to have 95% confidence for estimating the average number of weeks-to-negative processing fluids with a margin of error of 1.25 weeks, considering a standard deviation of 2 weeks.

5.3.3. Sample and data collection

All samples were collected by farm personnel based on pre-existing disease surveillance and monitoring programs. Farms were asked to collect four PF samples, with a maximum of 50 sows/litters being represented per PF in any given week. Farms were asked to collect PF every two weeks for the first four months after the outbreak was detected. For months 5 and 6 after the outbreak, farms were asked to collect one sample

per month for a total of 10 sampling weeks per farm. PF samples collected before outbreak detection were also requested for testing when available. All samples were shipped to the University of Minnesota's Veterinary Diagnostic Laboratory (UMN VDL) for testing.

All farms were also requested to summarize if herds underwent a planned exposure process and also share their production records electronically data for analysis.

5.3.4. Laboratory testing and classification of SVA status

All PF samples were tested for the presence of SVA RNA by quantitative reverse transcription PCR (qRT-PCR). RNA was extracted using a commercial extraction kit (Ambion MagMAX-96 viral RNA isolation kit; Life Technologies) and a magnetic particle processor (MagMAX Express-96 magnetic particle processor; Applied Biosystems), following the manufacturer's guidelines. Even though samples are usually considered positive for qRT-PCR when the cycle threshold (Ct) values are up to 35.99 and suspects between 36. and 40 (13), we considered any week as being positive if at least one sample had a Ct value under 40. Due to the decrease in sensitivity caused by aggregating multiple litters in one PF sample, suspect samples were treated as positives since SVA could be present at a lower prevalence.

5.3.5. Statistical analysis

Descriptive statistics from PF testing and the mean number of weeks-to-negativity with a 95% confidence interval were calculated. The 95% confidence interval for the number of weeks-to-negativity was calculated with a one-sample t-test using the

statistical software R (66) and mosaic package (86). Production data shared by participating farms was graphically and descriptively summarized.

5.4. Results

5.4.1. General farm information, characteristics, and outbreak occurrence date

A total of 10 farrow-to-wean breeding herds volunteered to participate in our study; seven of them belonged to one production system, one from a second production system, and the remaining two from veterinary clinics (Table 5.1).

One sow farm detected the SVA outbreak in June/2019; five farms in August/2020; two farms in September/2020; one in October/2020; and the last in November/2020 (Table 5.1). The month of SVA outbreak detection was defined as the month when vesicular disease signs were first seen, and local animal health regulatory authorities conducted an FADI to rule out FMD.

5.4.2. PF testing results

A total of 310 PF samples were tested from all 10 participating farms, with the number of tested PF samples per farm ranging between 15 and 85. Farms 1 and 2 had an average of 4.3 and 2.1 PF samples tested per tested week, while all others had only one tested PF sample per tested week (Table 5.1). The follow-up time in all farms ranged from 16 to 30 weeks, with an average of 22.5 weeks (Table 5.2).

Five sow farms had SVA-positive PF even before clinical signs were evident to the farm staff, with the earliest detection up to three weeks before the FAD investigation (Figures 5.1 and 5.2). The number of weeks post-outbreak when SVA-positive PF

samples were found ranged from 1 to 21, with an average of 11.8 (95% CI – 8.1, 15.5) weeks across all ten sow farms.

A wide range (1 to 10 weeks) of consecutively negative weeks between positives was seen (Table 5.2 and Figure 5.1). There was also a wide range of weeks with negative results after the last positive, ranging from 2 to 18.

5.4.3. Farm-level interventions to control SVA

All farms except one (Farm 2) did not report any interventions in response to the outbreak besides the FAD investigation. In the case of the one farm in which post-outbreak interventions were made towards the elimination of the virus, mass exposure was attempted eight weeks after the outbreak detection, followed by herd closure aiming to elicit herd immunity and decrease within-herd transmission. Briefly, the farm veterinarian collected the vesicular fluid from SVA-affected animals and mixed it with 500mL of phosphate-buffered saline (PBS) solution. The solution was kept at -20°C, and an aliquot was sent to the UMN VDL for SVA qRT-PCR testing, which had a positive result with a Ct value of 17. The SVA solution was then thawed, and sows, gilts, and heat-check boars were intranasally exposed vial nasal cannula using 0.1mL of the solution. All animals that did not develop vesicular lesions or did not share the same pen with an affected animal were inoculated. The remaining SVA solution was used to spray the pen area where the gilts were kept.

5.4.4. Impact of the SVA outbreaks on production parameters

The only marked difference in production parameters was the weekly pre-weaning mortality (PWM) (proportion of pigs that died in each cohort of weaned piglets)

during the four weeks following the outbreak in Farm 1. The mean PWM for the 52 weeks preceding the outbreak was 13.9%; however, this parameter increased from 9.1 to 18.1 and 23% during the initial three weeks after clinical signs were identified. The PWM peaked during the fourth week at 42.7%. PWM values returned to similar values from before the outbreak after five weeks of outbreak detection.

Farms 3, 4, 5, 6, 7, 8, and 9 also shared their production data, but no marked differences were seen after the onset of the outbreaks. Previous health issues may have masked the effects of SVA in the production parameters for these farms. Production data from Farms 2 and 10 was not available.

5.5. Discussion

The use of aggregate samples for pathogen herd-level assessment is often promoted as a tool to aid during the control, monitoring, and disease elimination efforts. This study provides evidence that testing PF samples for the presence of SVA is a valuable and efficient tool for monitoring, surveillance, and determining herd-level SVA status. To the authors' knowledge, this is the first study to report the use of PF samples to detect SVA and estimate the number of weeks where PF samples remain positive during an outbreak.

We detected SVA-positive PF samples up to three weeks before any clinical signs were observed on five farms. Interestingly, it would be expected that clinical signs would be seen much sooner after the first week of SVA positivity if we consider that clinical signs usually develop four days after an animal is exposed (11,14,15,87). It can be hypothesized that this delay in detecting vesicular disease signs could be due to a low

disease prevalence in a large population of sows in the weeks preceding the outbreak, making it harder to see vesicle-affected animals. In addition, clinical signs may not be apparent in all cases, which adds complexity when farm workers are walking the barns assessing health. The decreasing trend of the Ct values from weeks -3 to 0 in Farm 1, as shown in Figure 5.2, could be caused by a combination of increasing viremia levels of recently infected sows and the prevalence of SVA-affected litters, which would increase the concentration of SVA RNA in the pooled PF samples. Conversely, the continuous increase in Ct values in the following weeks could be due to the decrease in viremia levels and the prevalence of SVA-affected litters, diluting the total SVA RNA within the pooled PF samples. However, it is essential to note that no fixed amount of litters is represented in each PF sample tested in this study.

It is currently unknown how the dilution effect due to litter aggregation and pooling affects SVA qRT-PCR results and sensitivity. One previous study reported the detection of PRRSv in PF when only one PRRSv-positive pig was present in an aggregate sample of 50 litters or approximately 600 pigs (21). Another study estimated the probabilities of PF samples testing positive to PRRSv to be 43%, 80%, and 95% when a single PRRSv-positive piglet was present among 784, 492, and 323 PRRSv-negative piglets, respectively (20). Even though such findings support the high sensitivity of the currently available molecular diagnostic tools, no similar study has been published with SVA. Further investigation should be performed to assess this issue since this can affect results interpretation from farms in later stages of infection or previous recent history of SVA exposure, where the proportions of positive litters are expected to be lower.

The average number of weeks until the last SVA-positive PF was detected was 11.8 (95% CI – 8.1, 15.5) and ranged from 1 to 21 weeks (Farm 4 and Farm 2, respectively). It is unknown whether previous SVA exposures may have affected the reported results. It is possible that previous SVA exposure elicits some level of herd immunity that could potentially shorten virus transmission and the time-to-negative PF. It was not possible to ascertain previous SVA exposure in any of the tested farms. Interestingly, only one farm (Farm 2) attempted mass exposure and herd closure to eliminate the disease eight weeks after the outbreak was detected. There is a strong possibility that the mass exposure contributed to the SVA transmission chain, which may explain the prolonged detection of SVA in PF on this farm. The larger number of sows in inventory also may have contributed to this prolonged SVA detection, as the time needed for SVA to transmit to the majority of animals could be longer on larger farms. However, Farm 1 had the same number of sows in inventory, tested a larger number of weekly samples than all other farms, and did not have as consistently positive weekly results as Farm 2. Therefore, more extended shedding periods may be expected when SVA elimination is attempted through mass exposure methods.

It is still unclear what number of consecutive SVA qRT-PCR-negative weeks are needed to achieve optimal levels of confidence that SVA is not being transmitted within the farrowing room. Sow farms in this study had a varying number of consecutive negative weeks between positive results, ranging from 1 to 10 weeks. The sporadic detection of a pathogen during weekly monitoring using PF samples has also been reported for PRRSV, in which the pathogen was detected for up to 11 consecutive weeks

after obtaining consistently negative PF results (88). These findings should be considered if the weaning of consistently SVA-negative piglets is desired.

The number of weeks that farms tested negative after the last positive ranged from 2 weeks on two farms (Farms 2 and 7) to 18 weeks on Farm 4 (Figure 5.1), with an average value of 7.3 (Table 5.2). However, Farm 4 showed atypical results as the last SVA-positive PF was detected at week 1 after the outbreak. It is unknown why this herd behaved this way, and a possibility is that previous herd immunity could have played a role, as it has been reported for PRRSV (89). Based on our results, 10 consecutive negative weeks may not be enough to consider a herd PF-negative.

Despite eight tested farms sharing the production data before and after the SVA outbreak, only Farm 1 had marked differences. PWM from this farm reached 42.7% in the third week after outbreak detection, quickly returning to similar values from before the outbreak. This result is consistent with what has been previously reported. Neonatal mortality is seen in some farms during an SVA outbreak, with young piglets showing clinical signs of lethargy, weakness, diarrhea, and sudden death, with reported proportions of dead piglets as high as 70% (4,5,7,32,36,56). The mechanisms that cause the reported mortalities are still unknown.

Our study has limitations in that only 10 farms were monitored. It is also important to note that not all farms could sample and test with the same frequency throughout the entire study period, which may have impacted our findings.

5.6. Conclusions

This is the first study reporting the use of PF samples to detect and monitor SVA. Practicing veterinarians may expect sow farms to have SVA-positive PF samples for an average of 11.8 weeks after an outbreak. Sow farms should be monitored with PF until at least ten consecutive qRT-PCR-negative weeks are accumulated if weaning SVA-negative piglets is desired. However, further studies need to be performed to identify other sampling strategies to implement after PF-negativity and have high confidence in weaning SVA-negative piglets. Testing of PF may be a cost-effective method to detect SVA presence and help halt its spread in SVA-endemic regions.

General discussion and conclusions

6.1. General discussion

Senecavirus A (SVA) emerged as an important swine pathogen in late 2014 and has been responsible for multiple vesicular disease outbreaks worldwide (3–5,36–42). Every SVA-related vesicular disease outbreak needs to be investigated due to its clinical similarities to other high-consequence vesicular diseases, such as foot-and-mouth disease (FMD). In the United States (U.S.), SVA is responsible for a rampant increase in the total number of foreign animal disease investigations (FADI) since 2015-2016 (6) (Figure 1.1). Despite the costs associated with conducting FADI investigations and the potential risk of confusion SVA can cause in the event of FMD being introduced into an FMD-free country, not much research has been done to further the knowledge about SVA's transmission, spread, distribution, and to develop methods for SVA monitoring and surveillance.

The risk of SVA introduction into naïve farms has been associated with several factors, including farm size, number of employees, carcass disposal procedures, entry of replacement animals, and biosecurity gaps (7). Feed ingredients have also been suggested as potential virus carriers into susceptible populations (8,43,50). At the animal level, SVA transmission is believed to depend on the shedding of SVA by infected animals. The virus is released in large quantities in the fluid of vesicles that rupture around 5-6 days after infection (11,12) and in oral/nasal secretions and feces up to 28 days after infection (11). Pigs may be exposed to SVA at very early ages since SVA can be successfully detected in processing fluids during outbreaks in sow farms (24). The persistent infection state characterized by the late SVA detection in the tonsils (14–16)

may also play a role in disease transmission from persistently infected to susceptible animals.

However, data about SVA transmission, prevalence among pig farms, and factors associated with SVA positivity are lacking. The current knowledge about SVA shedding provides critical insights into how SVA spreads and potentially gets transmitted. Still, there is a critical knowledge gap on the potential methods to monitor and survey this virus. There is a need to address these knowledge gaps to provide scientific evidence for developing monitoring and surveillance programs and controlling SVA in pig farms. Therefore, the goals of this thesis were to 1) estimate the prevalence of SVA-exposed pig farms at the national U.S. level and the risk factors associated with SVA-positivity, 2) assess how SVA may transmit indirectly between pig populations through fomites, 3) compare different sample types to detect SVA in animals at different stages of infection, and 4) better understand the use of processing fluids to monitor SVA at the population level on sow farms after an outbreak.

Even though there is a high incidence of swine-vesicular FAD investigations in the U.S (6)., the prevalence of SVA-exposed sow and growing-pig farms appears to be relatively low. The prevalence of sow and growing pig farms with SVA IgG antibodies were estimated at 17.3% (95%CI – 10.4, 26.3%) and 7.4% (95% CI – 3, 14.6%), respectively. Despite sow farms having 2.64 (95% CI - 1.04, 6.69) times higher odds of SVA seropositivity when compared to growing pig farms in the study from chapter 2, there is not enough evidence to conclude that the seroprevalence is higher among sow farms than in growing pig farms as the confidence intervals for the prevalence estimates

are overlapping. However, the continuous flow nature of sow farms may contribute to the persistence of this virus in pig populations. The dynamic nature of sow farm populations, where older animals are constantly being replaced by introducing younger animals, may create the optimal condition for the transmission between infected to susceptible animals.

Conversely, growing pig farms work as an all-in/all-out system, where all the pigs enter the farm at a similar age, and they all leave to the point that the farm is emptied. This allows the farm to be cleaned, disinfected, and re-populated with SVA-free pigs in the following groups. Therefore, this may explain the differences between the seroprevalences in both farm types. One important conclusion from this study is that the prevention of SVA spread should be a priority. A low SVA seroprevalence means that most farms are still susceptible to SVA infection. The high proportion of susceptible farms can potentially lead to even more FADI investigations if the transmission is not properly prevented.

Unsurprisingly, implementing a higher number of biosecurity measures seemed to make sow farms have 0.2 (95% CI – 0.1, 0.99) times lesser odds of being SVA seropositive (Table 2.2). Additionally, the carcass disposal method was also a significant predictor of SVA seropositivity, with sow farms that have rendering trucks coming in to pick up dead animals having 9.2 (95% CI – 2.5, 33.7) higher odds of being seropositive (Table 2.2). Biosecurity is known to reduce the risk of pathogen entry to pig farms (68), and the practice of rendering is linked to increased risk of other pathogens in pigs (69,70) and poultry (71,72). These associations can help producers, companies, and veterinarians make better-informed decisions to prevent the introduction of SVA in pig farms.

Ensuring farm personnel compliance with biosecurity measures and avoiding having a rendering truck visit several farms to pick up dead animal carcasses may help reduce the risk of SVA introduction to pig farms. However, further studies are needed to understand these risks since the current study was mainly intended to estimate disease prevalence rather than the factors associated with SVA positivity.

The transmission of SVA between pig populations was further studied in the third chapter of this thesis. Two SVA-naïve pigs were infected with SVA after being placed in direct contact with 6 infected animals showing no vesicular lesions, which evidences the role of SVA shedding through oro-nasal secretions and feces on its transmission. The transmission of SVA between asymptomatic pigs can also potentially happen in farms, making it significantly harder to detect.

Personal protective equipment (PPE), such as boots, gloves, and coveralls worn by animal caretakers, were also involved in the indirect transmission of SVA between pig populations. The virus was detected on surfaces of either boots, gloves, or coveralls worn by personnel after they were in direct contact with SVA-infected animals (Table 3.4), and infectious virus was isolated from swab samples taken from boots and coveralls of personnel after contacting SVA-infected pigs. The same contaminated PPE was worn in 3 different rooms housing susceptible pigs, resulting in their infection (Figures 3.2 and 3.4, and Table 3.2). Transmission and infection appeared to have been prevented by having study personnel change their PPEs—with or without showering—after interacting with infected pigs and before moving into the rooms where the susceptible pigs were housed. These findings confirm the already established knowledge about the benefits of on-farm

biosecurity measures on swine health (68). They may also partially explain the protective association between having a higher number of biosecurity measures and SVA seropositivity in sow farms, as reported in chapter 2 (Table 2.2). One limitation of this study was the low number of infected animals serving as the source of contamination to study personnel, which may explain the lack of SVA detection in their nostrils. The presence of SVA in humans' oral and nasal cavities should be further investigated after contact with a higher number of infected animals since exposure to a higher amount of the virus could lead to detectable levels in humans. The presence of SVA RNA on the exposed skin of personnel was not assessed. It is possible that SVA can survive on exposed skin and pose a risk of being indirectly transmitted to susceptible pigs. Therefore, strict on-farm biosecurity procedures should be implemented and enforced with showering facilities with clean/disinfected clothes and PPEs always available.

Persistently-infected pigs have been shown to resume shedding after stressful conditions, and infectious SVA has been isolated from the tonsils of pigs 60 days after infection (16). The risk of persistently infected pigs transmitting SVA to susceptible pigs needs further evaluation, even though the susceptible pigs from the tonsil homogenate bioassay described in chapter 3 remained negative. The risk of transmission may be significantly increased under field conditions, considering the high number of pigs of different SVA statuses in constant interaction in large farm operations.

The development of monitoring and surveillance tools is critical to help control SVA’s spread and design effective elimination plans. In this thesis's fourth and fifth chapters, novel information is presented regarding the individual pig and aggregate-level

samples in which SVA can be detected, and that can be directly applied in swine operations. Tonsil scrapings were shown to be a viable sample matrix for detecting SVA up to 48 days after the infection of pigs. Tonsil scrapings can be used as one sample type to help identify persistently-infected pigs, which may asymptomatically carry this virus and potentially act as sources of infection to susceptible animals—hindering the effectivity of SVA elimination attempts.

Other individual-pig sample types may be better suited for detecting SVA at earlier stages of infection. Oral and rectal swabs are easier to collect in pigs, especially in the case of rectal swabs, which may be taken without the need to restrain the animal. These two sample types had higher odds of testing positive by SVA qRT-PCR on the initial 7-10 days of infection than tonsil scrapings (Table 4.3).

Aggregate sampling strategies have been proven cost-effective, less labor-intensive, and less stressful for animals and personnel to detect swine pathogens (19,20,22,90,91). Processing fluids (PF) and oral fluids (OF) samples are two types of aggregate sample types commonly used in the swine industry, and both were shown to be effective at detecting SVA. In the case of OF, SVA was detected for up to 35 days post-infection under experimental conditions (Table 4.1). However, this detection was done in two groups of pigs inoculated simultaneously with a historical and a contemporary SVA strain, as described in chapter 4. The time of SVA-positivity in OF after exposure may be longer under field conditions, given that farm animals get infected and transmit the virus at different time points; thus, SVA is likely to be shed by different animals for an extended period. PF samples were shown to test positive for an average of 11.8 weeks

after SVA outbreak detection under field conditions, after the longitudinal testing of 10 different sow farms described in chapter 5 (Table 5.2). However, the number of consecutively negative weeks by PF testing among the 10 farms ranged from 1 to 10 weeks (Table 5.2 and Figure 5.1). The sporadic pathogen detection during weekly PF monitoring was also reported with porcine reproductive and respiratory syndrome virus (PRRSV) (88). These findings may serve as the basis for developing a comprehensive SVA status classification system for farms that wish to eliminate this virus, similar to what is currently used for PRRSV (85).

Many aspects of SVA's epidemiology, transmission, and detection are still unknown. SVA is a relatively new disease for the swine industry, considering that the first large-scale outbreaks were only reported after 2014-2015. This may explain why epidemiologic information about this virus is scarce. This thesis provides novel information about SVA's presence in a national herd, risk factors associated with exposure, its persistence, and transmission, as well as different sampling strategies that may be used for its detection at individual and population levels. It serves as the foundation for developing more studies or strategies for disease monitoring, surveillance, and control.

6.2. General conclusions

This thesis revealed that despite being present in a relatively low prevalence in the U.S., SVA poses a significant risk for most pig farms that are still susceptible. The role of persistently-infected pigs in perpetuating this virus must be addressed, and better monitoring and surveillance strategies must be developed. Fortunately, several sampling

strategies can be used for its detection. The thesis' main findings that support these conclusions are:

- Seroprevalences of 17.3% and 7.4% were estimated among U.S. breeding and growing pig farms.
- Rendering is a common practice in the U.S. swine industry and was significantly associated with SVA seropositivity. However, having a higher number of on-farm biosecurity measures in a farm was significantly associated with seronegativity.
- Contaminated fomites play an essential role from an indirect transmission standpoint.
- Tonsil scrapings seem to be the best matrix to help detect SVA during the later stages of infection when animals are persistently-infected. However, other sample types, such as rectal swabs, are easier to collect and have higher chances of detecting SVA in the early stages of infection.
- Aggregate samples can also be used to detect SVA. OF samples were positive for up to 35 days after the experimental infection of pigs, while PF samples were SVA-positive by qRT-PCR for an average of 11.8 weeks after outbreak detection.

Illustrations: Tables

Table 2.1 Demographic characteristics of the 193 United States (U.S.) pig farms participating in the National Senecavirus seroprevalence and risk factors study

	Total samples n=193	**Breeding farms n=98**	**Growing-pig farms n=95**
Responded to survey	155 (80%)	77 (79%)	78 (82%)
Farm size[1]			
Range (Minimum-Maximum)	----	120 – 9600	800 – 55194
Median	----	2752	3600
Mean (S.D.)	----	3147 (1884)	4922 (6518)
Companies[2]			
Number of participating companies	19	19	17
Median number of sampled farms per company	10	4	5
Mean number of sampled farms per company (SD)	10 (7)	5 (3)	6 (4)
U.S. States			
Number of participating states	17	16	11
Median number of sampled farms per State	5	4	6
Mean number of sampled farms per State (SD)	10 (10)	5 (5)	7 (6)

[1]Data from 75 and 76 participating breeding and growing-pig farms, respectively.

[2]Data from all participating production companies, veterinary clinics, and two private practitioners.

Table 2.2 Univariable and multivariable logistic regression analysis of the risk factors associated with SVA seropositivity in U.S. breeding farms

	Univariable			Multivariable		
Characteristic	OR	95%CI	p-value	OR	95%CI	p-value
Carcass disposal method						
Composting, burying, or incinerating	---	---	---	---	---	---
Rendering	7.9	2.4 – 26.7	<0.001	9.2	2.5 – 33.7	<0.001
Biosecurity measures in place*						
Four or less	---	---	---	---	---	---
Five or six	0.3	0.1 – 1.1	0.06	0.2	0.1 – 0.99	0.49
At least one employee works on another farm						
No	---	---	---	---	---	---
Yes	0.2	0.0 – 1.7	0.1	---	---	---
Type of manure storage						
Uncovered lagoon	---	---	---	---	---	---
Deep pits	0.2	0.1 – 0.8	0.02	---	---	---
Water treatment						
No	---	---	---	---	---	---
Yes	0.2	0.0 – 0.9	0.03	---	---	---
Cull sows and weaned piglets use the same ramp when truck loading						
No	---	---	---	---	---	---
Yes	0.4	0.1 – 1.4	0.16	---	---	---

*Farms were categorized as having "four or less" or "five or six" biosecurity measures in place if they responded to having any combination of <=4 or >=5 biosecurity measures in place, respectively, from a list of six biosecurity measures included in the survey

Table 2.3 Univariable logistic regression analysis of the risk factors associated with SVA seropositivity in U.S. growing pig farms

Characteristic	OR	95%CI	p-value
External pig-loading crew[1]			
No	---	---	---
Yes	9.3	1.03 – 84.9	0.047
Direct access to pigs in the barn after loading a truck[2]			
No	---	---	---
Yes	0.1	0.01 – 0.8	0.035
Hires more than one external crew[3]			
No	---	---	---
Yes	3.3	0.6 – 19.2	0.188
Single-sourced pigs[4]			
No	---	---	---
Yes	0.3	0.05 – 1.6	0.158
All trucks come clean and disinfected[5]			
No	---	---	---
Yes	0.3	0.05 – 1.7	0.165

[1] Market pigs are loaded into trucks by an external pig-loading crew. [2] People who load pigs into trucks have direct access to pigs in the barn after loading a truck. [3] More than one external crew is hired to work at the farm. [4] All pigs on the farm are sourced from a single breeding farm. [5] All trucks that arrive on the farm are cleaned and disinfected.

Table 3.1 Animal sampling scheme with the dates of feces and blood collections for the Infected and Low, Medium, and High Biosecurity groups

Day post-inoculation	2	3	4	5	6	7	8	9	10	14	18	21	24	28	35	38	42	45	52	57
Feces from the infected group	X	X	X	X	X	X	X	X	X		X	X		X	X	X	X	X	X	X
Blood from the infected group	X		X		X		X		X	X										
Feces from biosecurity groups		X	X	X	X	X	X	X	X	X		X	X							
Sera from biosecurity groups		X		X		X		X		X		X	X							

Table 3.2 SVA qRT-PCR and IFA results from all pigs at necropsy

Pig ID	Group	Necropsy day - dpi	Tonsil qRT-PCR Ct value	IFA SVA IgG titer	Pig ID	Group	Necropsy day - dpi	Tonsil qRT-PCR Ct value	IFA SVA IgG titer
147	INF	57	26	1:80[1]	**148**	M.B.2	24	(-) pool[4]	(-)
180	INF	57	27	1:80[1]	**185**	M.B.2	24	(-) pool	(-)
186	INF	57	26	1:80[1]	**187**	M.B.2	24	(-) pool	(-)
191	INF	57	24	1:80[1]	**275**	M.B.2	24	(-) pool	(-)
196	INF	57	27	1:80[1]	**178**	M.B.3	24	(-) pool	(-)
200	INF	57	28	1:80[1]	**182**	M.B.3	24	(-) pool	(-)
141	INF - Sentinel	57	26	1:80[1]	**183[2]**	M.B.3	7[2]	(-)	
188	INF - Sentinel	57	31	1:80[1]	**273**	M.B.3	24	(-) pool	(-)
179	L.B.1	24	22	1:40	**140**	H.B.1	24	(-) pool	(-)
192	L.B.1	24	31	1:80	**149**	H.B.1	24	(-) pool	(-)
194	L.B.1	24	38	(-)	**266[3]**	H.B.1	67[3]	(-)	(-)
198	L.B.1	24	27	(-)	**268**	H.B.1	24	(-) pool	(-)
135[2]	L.B.2	11[2]	17		**137[3]**	H.B.2	67[3]	(-)	(-)
181	L.B.2	24	24	1:80	**139**	H.B.2	24	(-) pool	(-)
189[2]	L.B.2	14[2]	15		**144**	H.B.2	24	(-) pool	(-)
269	L.B.2	24	29	1:80	**146**	H.B.2	24	(-) pool	(-)
138	L.B.3	24	22	1:80	**142[2]**	H.B.3	10[2]	(-)	
143	L.B.3	24	22	1:80	**267**	H.B.3	24	(-) pool	(-)
145	L.B.3	24	22	1:80	**272**	H.B.3	24	(-) pool	(-)
271	L.B.3	24	25	1:80	**274**	H.B.3	24	(-) pool	(-)
134	M.B.1	24	(-) pool	(-)	**136[3]**	NC	67[3]	(-)	(-)
150	M.B.1	24	(-) pool	(-)	**184[3]**	NC	67[3]	(-)	(-)
195	M.B.1	24	(-) pool	(-)	**197[3]**	NC	67[3]	(-)	(-)
263	M.B.1	24	(-) pool	(-)	**262[3]**	NC	67[3]	(-)	(-)

[1] INF pigs were tested for the presence of SVA IgG antibodies at day 28 pi. Pigs from all other groups were tested for the presence of IgG antibodies at necropsy. Pigs that were euthanized due to non-SVA-related issues were not tested by IFA.

[2] Euthanized on different days due to non-SVA-related problems.

[3] Animals that participated in the SVA bioassay portion of the study.

[4] Tonsils from M.B.1, M.B.2, M.B.3, H.B.1, H.B.2, and H.B.3 groups were tested by pooling all tonsils from the same group replicate together. The tonsils from pigs necropsied at different dates due to early euthanasia or the animal participating in the bioassay portion of the study were tested individually.

Table 3.3 Senecavirus A qRT-PCR results from the pooled fecal and sera samples tested from the Low (L.B.), Medium (M.B.), and High (H.B.) biosecurity treatment groups

Day post-inoculation (pi)	**3**	**4**	**7**	**9**	**14**	**21**	**24**
Movement day (Mv)	**2**	**3**	**6**	**8**			
Day post-movement (PMv)					**4**	**11**	**14**
Feces L.B.1	Negative	Negative	35.71	37.12	Negative		
Feces L.B.2	Negative	Negative	Negative	36.78			
Feces L.B.3	Negative	Negative	Negative	37.46	26.74		
Feces M.B.1	Negative				Negative	Negative	Negative
Feces M.B.2	Negative				Negative	Negative	Negative
Feces M.B.3	Negative				Negative	Negative	Negative
Feces H.B.1	Negative				Negative	Negative	Negative
Feces H.B.2	Negative				Negative	Negative	Negative
Feces H.B.3	Negative				Negative	Negative	Negative
Serum L.B.1	Negative	Negative	Negative	Negative	35.19		
Serum L.B.2	Negative	Negative	Negative	26.78			
Serum L.B.3	Negative	Negative	Negative	Negative	30.67		
Serum M.B.1	Negative				Negative	Negative	Negative
Serum M.B.2	Negative				Negative	Negative	Negative
Serum M.B.3	Negative				Negative	Negative	Negative
Serum H.B.1	Negative				Negative	Negative	Negative
Serum H.B.2	Negative				Negative	Negative	Negative
Serum H.B.3	Negative				Negative	Negative	Negative

Table 3.4 Senecavirus A qRT-PCR cycle threshold results from fomites after contact with infected pigs and upon entering the low, medium, and high biosecurity biocontainment rooms

Movement day (Mv) and room	L.B.1	L.B.2	L.B.3	M.B.1	M.B.2	M.B.3	H.B.1	H.B.2	H.B.3
Mv 1 – Leaving INF	Boot (33)[(1)]				Boot (35)		Boot (34)	Boot (33)	
Mv 1 – Entering the biocontainment room	Same as when leaving INF								
Mv 4 – Leaving INF	Boot (31), Glove (35)	Boot (32)	Boot (29)	Boot (32), Coverall (34), Glove (34)	Boot (32), Coverall (35)	Boot (32)	Boot (32)	Boot (32)	Boot (33), Coverall (32), Glove (33)
Mv 4 – Entering the biocontainment room	Same as when leaving INF	Same as when leaving INF	Same as when leaving INF						
Mv 7 – Leaving INF	Boot (31), Coverall (29)	Boot (32)	Boot (32), Glove (34)	Boot (31)	Boot (31)	Boot (30)	Boot (32), Coverall (34)	Boot (31)	Boot (33)
Mv 7 – Entering the biocontainment room	Same as when leaving INF	Same as when leaving INF	Same as when leaving INF						

Table 4.1 SVA qRT-PCR cycle threshold (Ct) positive values (<36) in oral fluid (OF) samples over time after SVA inoculation

Days post-inoculation (dpi)	**SVA isolate groups Ct values**	
	Historical	Contemporary
1 dpi	22.63	20.49
3 dpi	26.32	25.27
7 dpi	27.83	26
10 dpi	29.25	24.85
14 dpi	31.15	30.89
21 dpi	34.5	34.61
28 dpi	29.91	29.18
35 dpi	31.97	33.19
42 dpi	Negative	Negative
48 dpi	Negative	Negative

Table 4.2 Odds ratios (OR) of detecting a positive pig by sample type controlling for SVA isolate group, day post inoculation (DPI), and interactions between sample type/DPI and SVA isolate group/DPI with tonsil scrapings and contemporary isolate group as the references

Variable	ß[1]	SE[2]	OR (95% CI)	*p*-value
Tonsil scraping	—	—	—	—
Oral swab	2.48	0.77	11.95 (2.63 – 54.22)	0.001
Rectal swab	3.02	0.80	20.47 (4.30 – 97.44)	<0.001
Tonsil Swab	4.41	1.11	82.10 (9.28 – 726.14)	<0.001
DPI	-0.08	0.01	0.92 (0.90 – 0.94)	<0.001
Tonsil scraping : DPI	—	—	—	—
Oral swab : DPI	-0.23	0.05	0.79 (0.72 – 0.87)	<0.001
Rectal swab : DPI	-0.14	0.03	0.87 (0.82 – 0.92)	<0.001
Tonsil swab : DPI	-0.27	0.05	0.76 (0.69 – 0.85)	<0.001

[1]Estimated regression coefficients. [2]Standard error of the estimated regression coefficients.

Table 4.3 Odds ratios of pigs testing SVA qRT-PCR-positive by sample type, using the tonsil scrapings as the reference

DPI*	1	3	7	10	14	21	28	35	42	48
Tonsil scraping	—	—	—	—	—	—	—	—	—	—
Oral swab	8.76	4.71	1.36	0.54	0.16	0.02	0.002	0.00	0.00	0.000
Rectal swab	16.44	10.59	4.39	2.27	0.94	0.20	0.04	0.00	0.00	0.00
Tonsil swab	57.97	28.79	7.10	2.48	0.61	0.05	0.005	0.00	0.00	0.00

DPI = days post-inoculation. Results shown in this table were calculated using the output from the mixed-effects logistic model shown in Table 4.1.

Table 5.1 Senecavirus A affected farms characteristics and information on the number of processing fluid (PF) samples and weeks tested

Farm ID	Farm Size (No. of sows)	Month of SVA outbreak	No. of collected samples	No. of weeks tested before the outbreak	No. of weeks tested after the outbreak	Follow-up time (No. of weeks)	No. of weeks tested	Average No. of samples per tested week
1	6,000	Jun-19	85	3	26	30	20	4.3
2	6,000	Sep-20	64	6	23	30	30	2.1
3	2,000	Aug-20	22	2	19	22	22	1.0
4	2,000	Aug-20	19	2	19	22	19	1.0
5	2,000	Aug-20	22	3	18	22	22	1.0
6	2,000	Aug-20	22	2	19	22	22	1.0
7	2,000	Aug-20	21	3	17	21	21	1.0
8	2,000	Sep-20	22	5	16	22	22	1.0
9	2,000	Oct-20	18	1	16	18	18	1.0
10	2,600	Nov-20	15	0	18	16	15	1.0
Total	—	—	310	—	—	—	—	—
Average	—	—	—	2.7	19.1	22.5	22.1	1.4

Table 5.2 Summary statistics of the number of tested weeks and SVA status in processing fluids over time for 10 sow farms undergoing an outbreak

Statistic	Minimum value	First Quartile	Median	Third Quartile	Maximum value	Average	Standard Deviation
Total follow-up time in weeks	16	21.2	22	22	30	22.5	4.5
No. of followed weeks before outbreak detection	0	2	2.5	3	6	2.7	1.8
No. of followed weeks after outbreak detection	16	17.2	18.5	19	26	19.1	3.1
Last positive week after outbreak detection	1	9.5	11.5	14.7	21	11.8	5.2
Number of negative weeks between positive weeks	1	1.2	2	3	10	2.9	3
Number of negative weeks after the last positive week	2	4.2	6	8.5	18	7.3	5.2

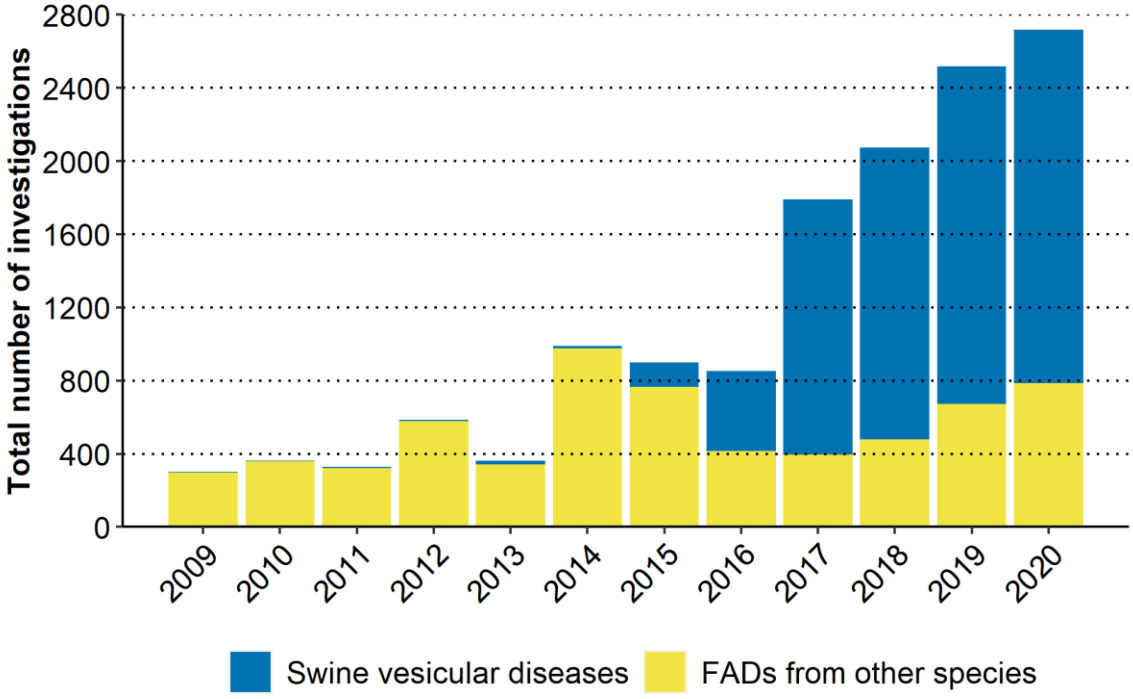

Figure 1.1 Total number of foreign animal disease (FAD) investigations carried out by the United States Department of Agriculture from 2009 to 2020, split by swine vesicular disease complaints and all other FADs from all species

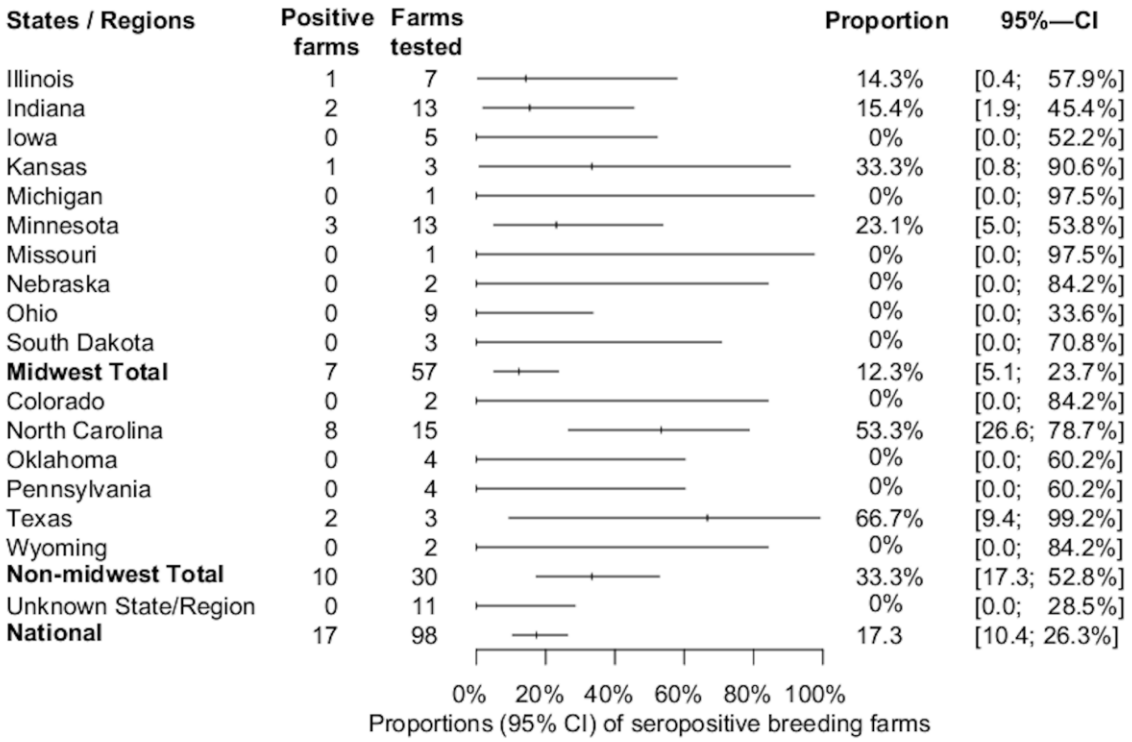

Figure 2.1 Estimated proportions and 95% confidence intervals of SVA-seropositive breeding farms by state, region, and national estimate

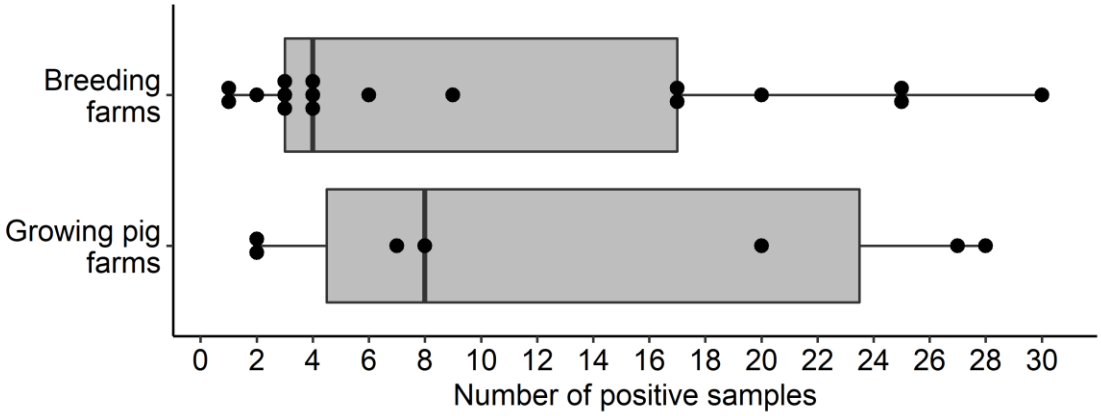

Figure 2.2 Box and whisker plot of the number of SVA IFA-positive samples by pig farm type in the U.S

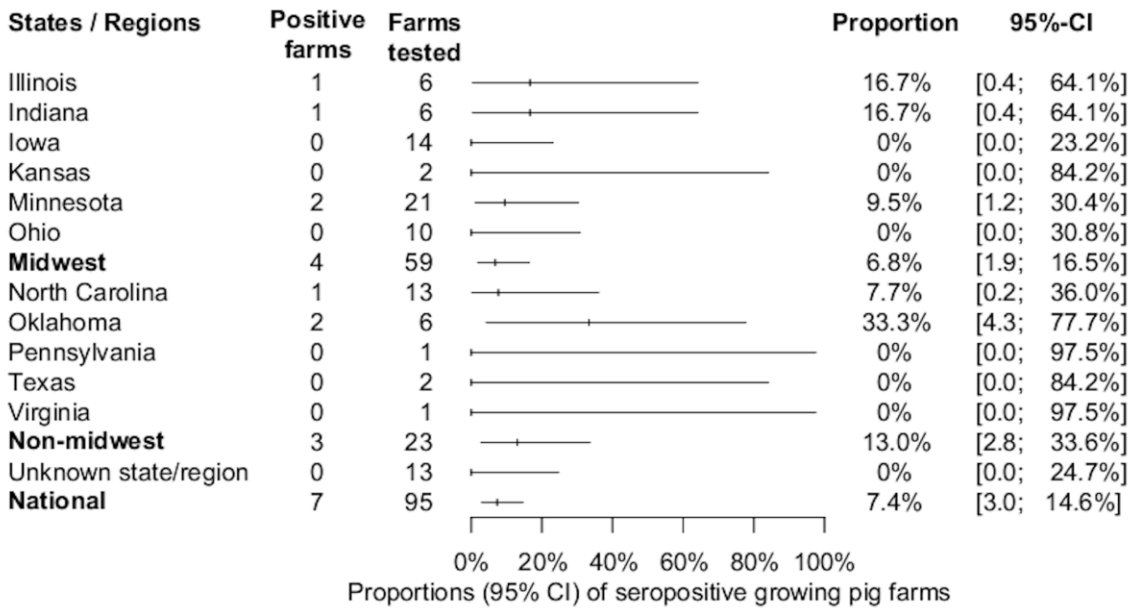

Figure 2.3 Estimated proportions and 95% confidence intervals of SVA-seropositive growing-pig farms by state, region, and national estimate

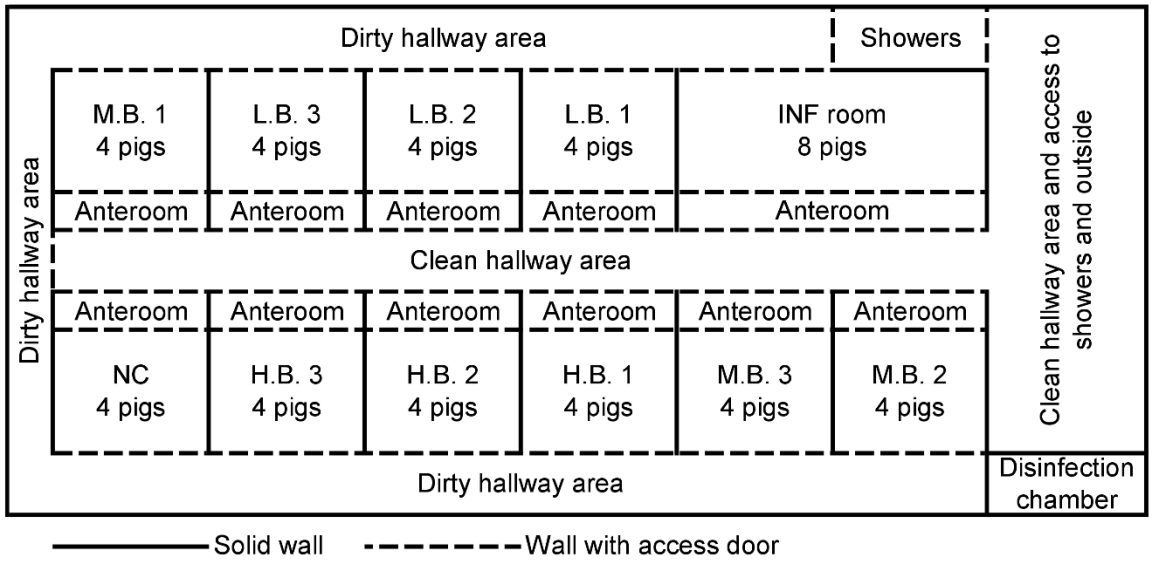

Figure 3.1 Biocontainment rooms layout

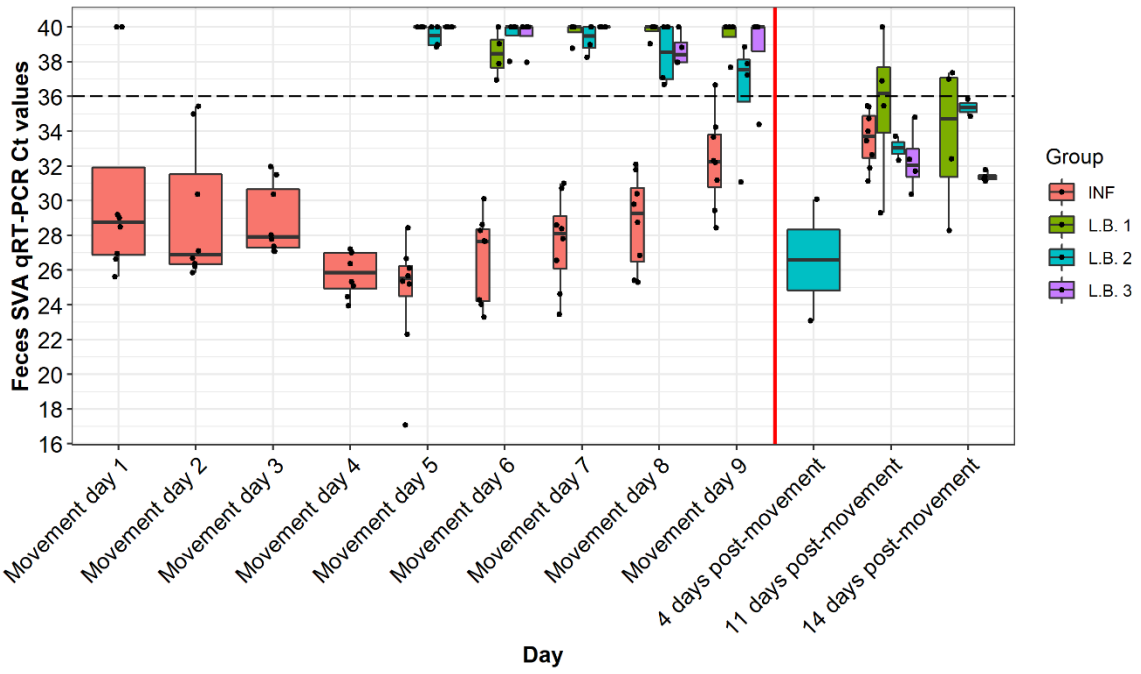

Figure 3.2 Individual SVA qRT-PCR results from feces collected from the Infected (INF), Low (L.B.), Medium (M.B.), and High biosecurity (H.B.) groups over time

The horizontal dashed line represents the threshold Ct value to consider a positive SVA qRT-PCR result (<36 when positive and >=36 when negative). The vertical solid red line splits the data between test results from feces collected during the personnel movement period (to the left of the red vertical line) and test results from feces collected 11 and 14 days after the movement period had ended.

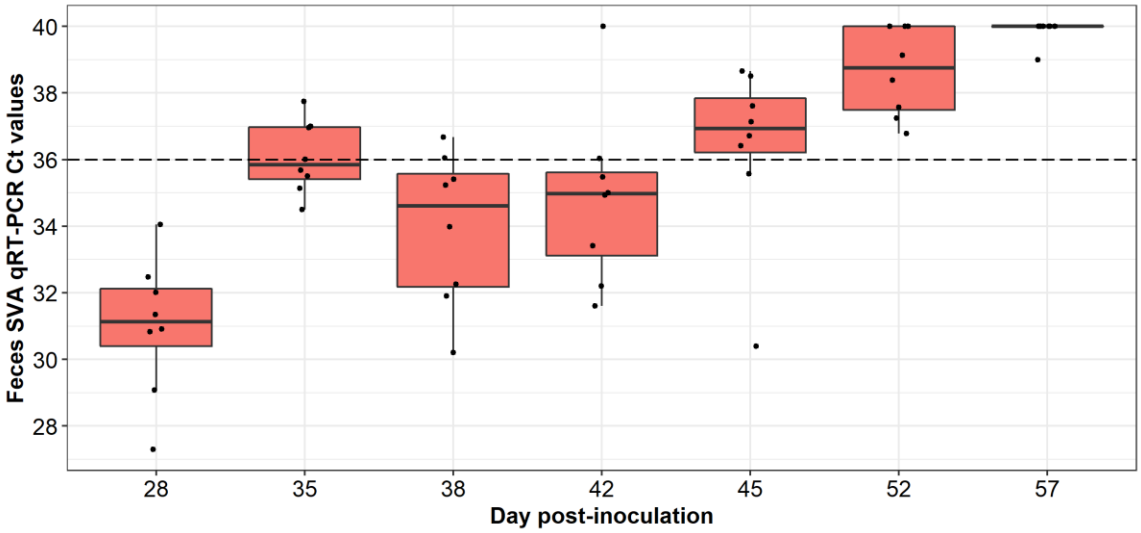

Figure 3.3 Individual SVA qRT-PCR results from feces collected from the infected group from day 28 post-inoculation (pi) until necropsy at day 57 pi

The dashed line represents the threshold Ct value to consider a positive SVA qRT-PCR result (<36 when positive and >=36 when negative).

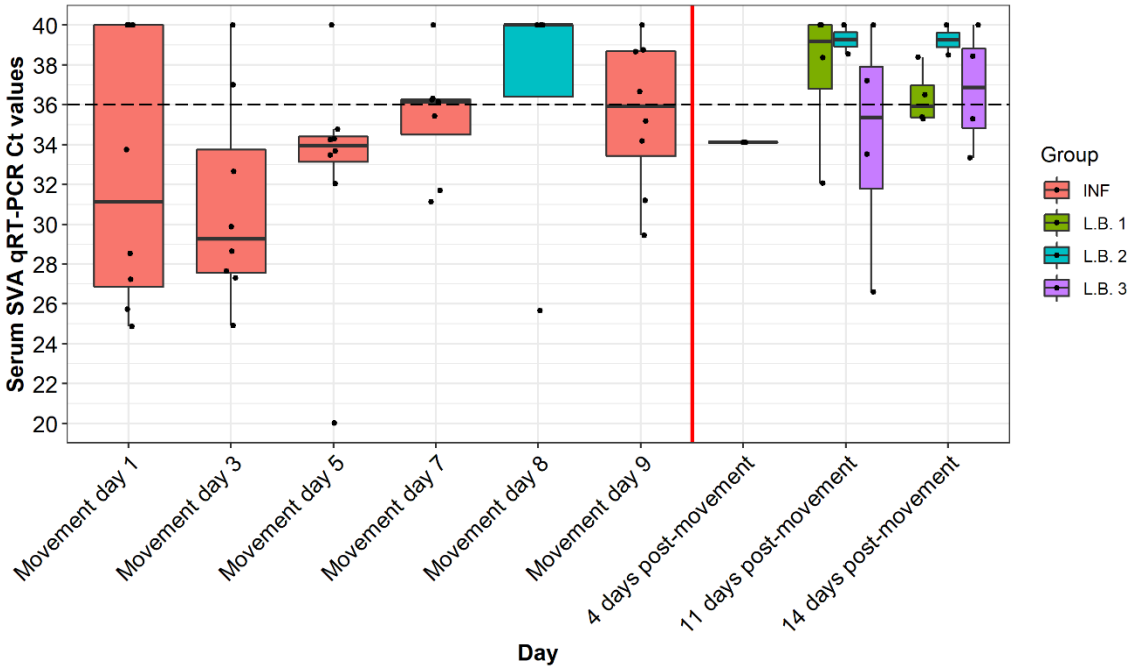

Figure 3.4 Individual SVA qRT-PCR results from serum collected from the Infected (INF) and Low biosecurity (L.B.) groups over time

The dashed line represents the threshold Ct value to consider a positive SVA qRT-PCR result (<36 when positive and >=36 when negative). The vertical solid red line splits the data between test results from serum samples collected during the personnel movement period (to the left of the red vertical line) and test results from serum samples collected 11 and 14 days after the movement period had ended.

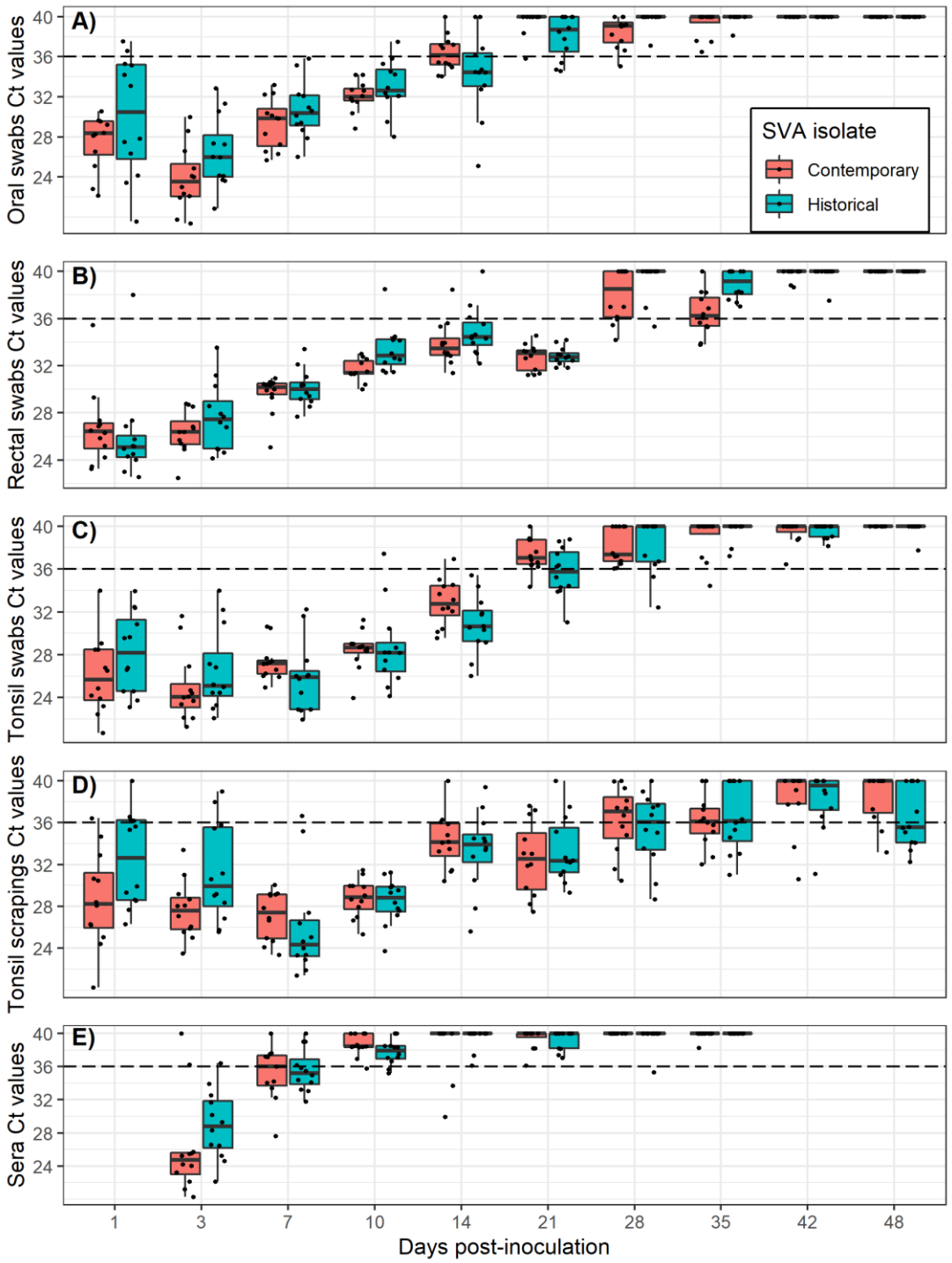

Figure 4.1 Box and whisker plots of Senecavirus A qRT-PCR cycle threshold (Ct) values (y-axis) over time after experimental inoculation (x-axis) of 12 pigs with either a contemporary or historical strain by sample type A) oral swabs, B) rectal swabs, C) tonsil swabs, D) tonsil scrapings, and E) sera by SVA isolate group. Horizontal dashed line at Ct = 36 represents the threshold for SVA qRT-PCR positivity: Ct <36 = positive samples, Ct >=36 negative samples. Jittered points were added to help visualize the distribution of data, where each point represents the Ct value of a singular test result

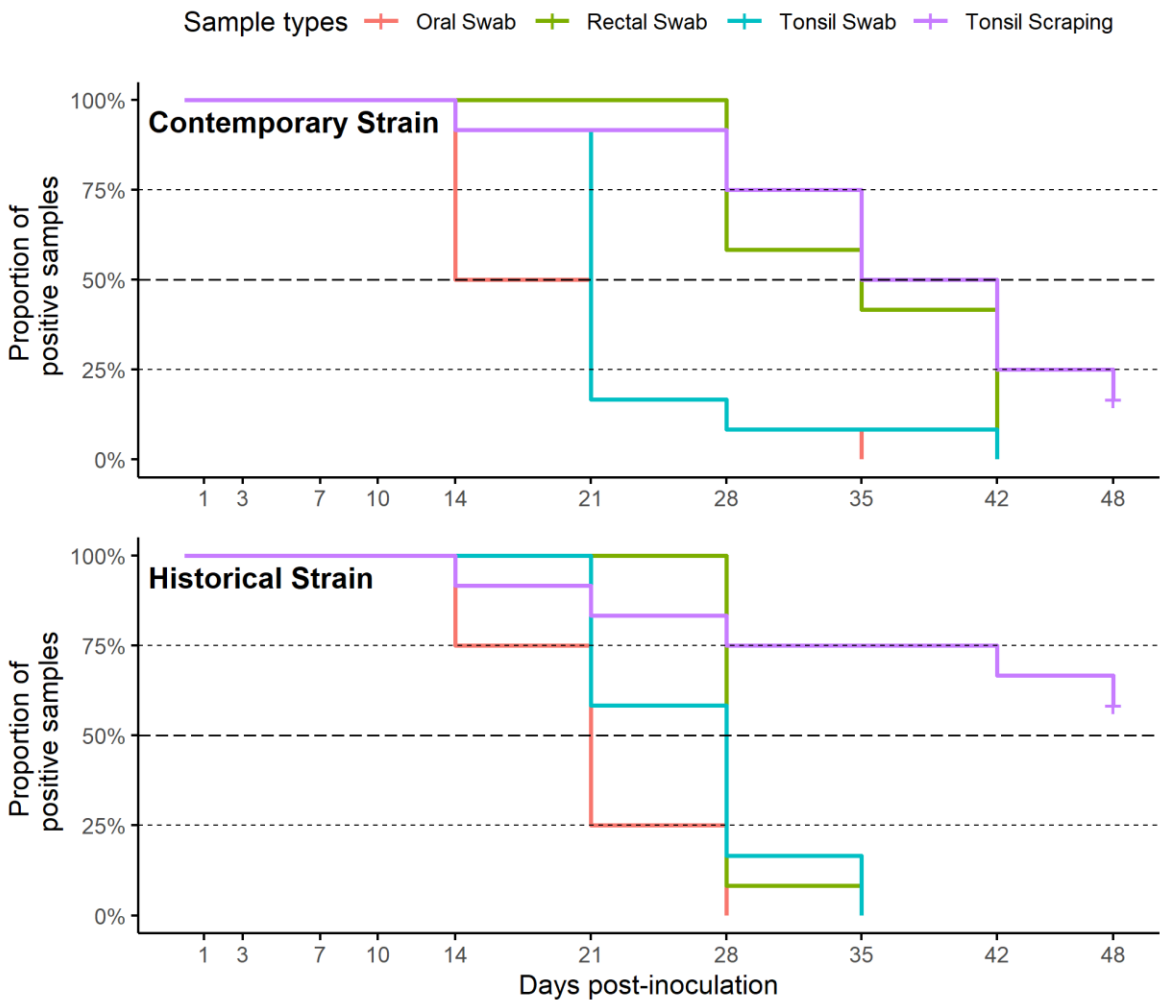

Figure 4.2 Kaplan-Meier survival curve for the time-to-negativity in oral, rectal, and tonsil swabs and tonsil scrapings over time after SVA inoculation in the contemporary and historical SVA strain groups

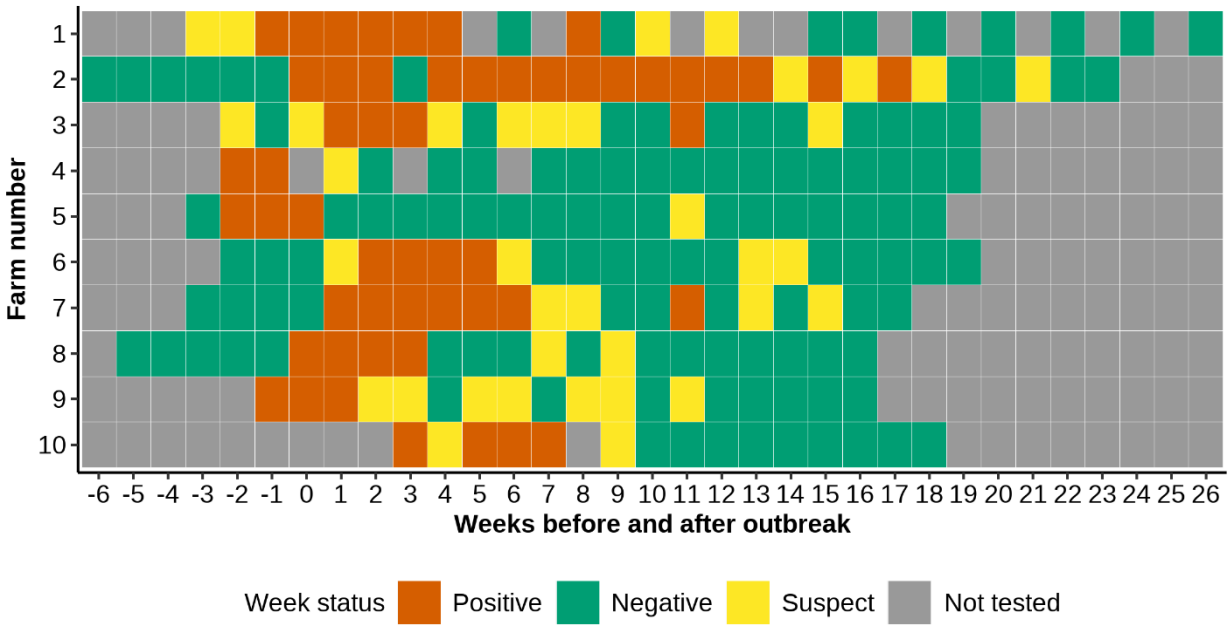

Figure 5.1 Weekly SVA status by PF testing in 10 sow farms before and after outbreak detection. Red = At least one PF sample had a qRT-PCR Ct value below 36. Yellow = At least one PF sample had a qRT-PCR Ct from 36 to 40. Green = All tested samples were negative. Grey = No samples were tested. The suspect results were considered positive in this study, but they are shown here to visualize the variation in results over time.

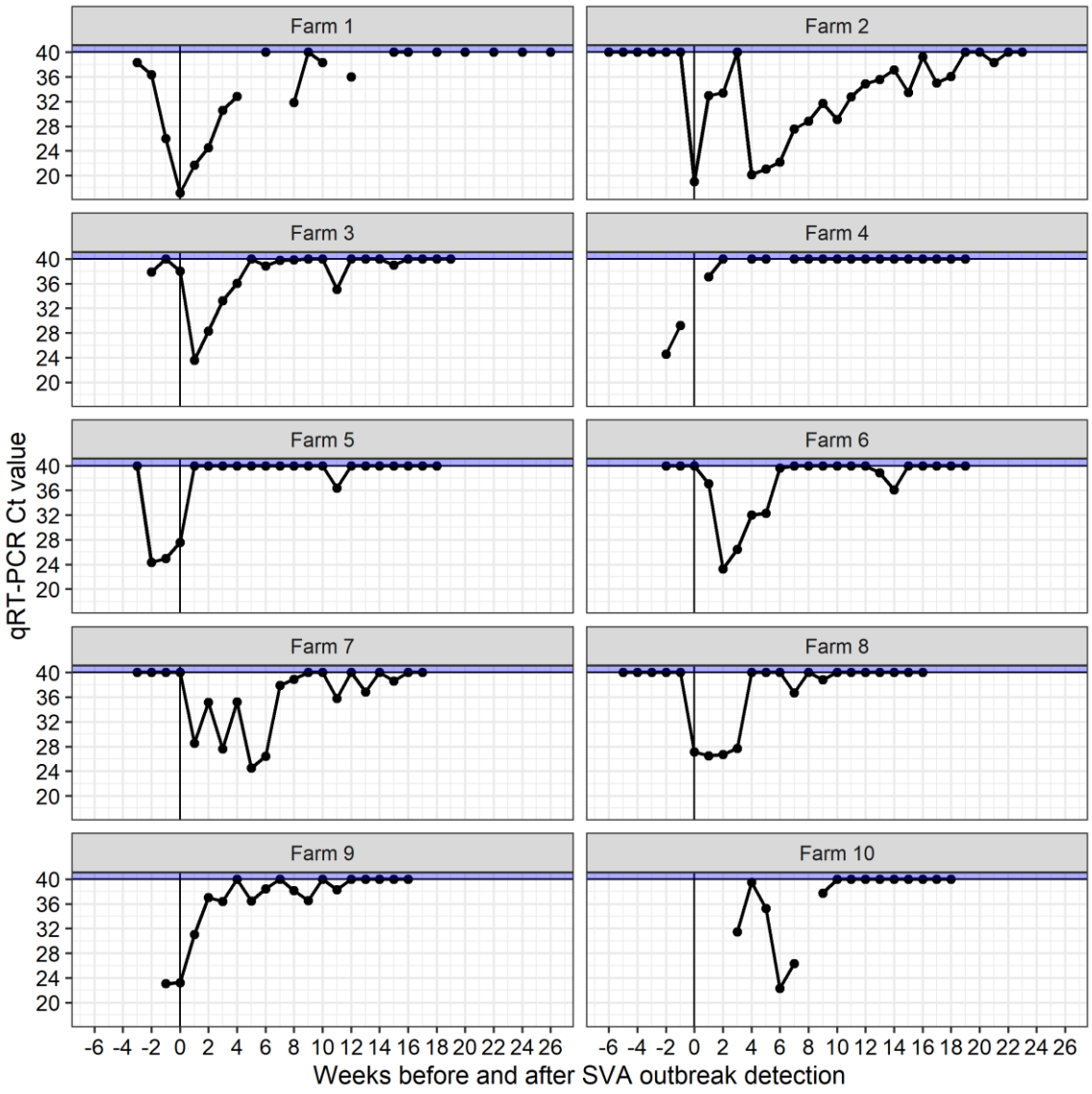

Figure 5.2 Weekly processing fluids SVA qRT-PCR results by farm, before and after SVA outbreak detection. The reported Ct values are the results from the positive sample with the lowest Ct value for any given week. Samples with a Ct = 40 (blue shaded area) represent a week where all tested processing fluid samples were negative.

Bibliography

1. Hales LM, Knowles NJ, Reddy PS, Xu L, Hay C, Hallenbeck PL. Complete genome sequence analysis of Seneca Valley virus-001, a novel oncolytic picornavirus. *J Gen Virol* (2008) **89**:1265–1275. doi:10.1099/vir.0.83570-0
2. Jamal SM, Belsham GJ. Foot-and-mouth disease : past , present and future. (2013)1–14.
3. Leme RA, Zotti E, Alcântara BK, Oliveira M V., Freitas LA, Alfieri AF, Alfieri AA. Senecavirus A: An Emerging Vesicular Infection in Brazilian Pig Herds. *Transbound Emerg Dis* (2015) **62**:603–611. doi:10.1111/tbed.12430
4. Gimenez-Lirola LG, Rademacher C, Linhares D, Harmon K, Rotolo M, Sun Y, Baum DH, Zimmerman J, Piñeyro P. Serological and Molecular Detection of Senecavirus A Associated with an Outbreak of Swine Idiopathic Vesicular Disease and Neonatal Mortality. *J Clin Microbiol* (2016) **54**:2082–2089. doi:10.1128/jcm.00710-16
5. Canning P, Canon A, Bates JL, Gerardy K, Linhares DCL, Piñeyro PE, Schwartz KJ, Yoon KJ, Rademacher CJ, Holtkamp D, et al. Neonatal Mortality, Vesicular Lesions and Lameness Associated with Senecavirus A in a U.S. Sow Farm. *Transbound Emerg Dis* (2016) **63**:373–378. doi:10.1111/tbed.12516
6. United States Department of Agriculture. Calendar Year 2020 Update: FAD Investigation Report. Riverdale, MD (2021). Available at: https://www.aphis.usda.gov/animal_health/emergency_management/downloads/summary-fad-investigations-cy2020.pdf
7. Baker KL, Mowrer C, Canon A, Linhares DCL, Rademacher C, Karriker LA,

Holtkamp DJ. Systematic Epidemiological Investigations of Cases of Senecavirus A in US Swine Breeding Herds. *Transbound Emerg Dis* (2017) **64**:11–18. doi:10.1111/tbed.12598

8. Dee S, Havas K, Spronk G. Detection of Senecavirus A in pigs from a historically negative national swine herd and associated with feed imports from endemically infected countries. *Transbound Emerg Dis* (2022)1–3. doi:10.1111/tbed.14684
9. Joshi LR, Mohr KA, Clement T, Hain KS, Myers B, Yaros J, Nelson EA, Christopher-Hennings J, Gava D, Schaefer R, et al. Detection of the Emerging Picornavirus Senecavirus A in Pigs, Mice, and Houseflies. *J Clin Microbiol* (2016) **54**:1536–1545. doi:10.1128/jcm.03390-15
10. Bai J, Fan H, Zhou E, Li L, Li S, Yan J, Jiang P. Pathogenesis of a senecavirus a isolate from swine in shandong Province, China. *Vet Microbiol* (2020) **242**:108606. doi:10.1016/j.vetmic.2020.108606
11. Joshi LR, Fernandes MHV, Clement T, Lawson S, Pillatzki A, Resende TP, Vannucci FA, Kutish GF, Nelson EA, Diel DG. Pathogenesis of senecavirus a infection in finishing pigs. *J Gen Virol* (2016) **97**:3267–3279. doi:10.1099/jgv.0.000631
12. Montiel N, Buckley A, Guo B, Kulshreshtha V, VanGeelen A, Hoang H, Rademacher C, Yoon KJ, Lager K. Vesicular disease in 9-week-old pigs experimentally infected with senecavirus A. *Emerg Infect Dis* (2016) **22**:1246–1248. doi:10.3201/eid2207.151863
13. Resende TP, Marthaler DG, Vannucci FA. A novel RNA-based in situ hybridization to detect Seneca Valley virus in neonatal piglets and sows affected

with vesicular disease. *PLoS One* (2017) **12**:1–10. doi:10.1371/journal.pone.0173190

14. Buckley A, Montiel N, Guo B, Kulshreshtha V, van Geelen A, Hoang H, Rademacher C, Yoon KJ, Lager K. Dexamethasone treatment did not exacerbate Seneca Valley virus infection in nursery-age pigs. *BMC Vet Res* (2018) **14**:352. doi:10.1186/s12917-018-1693-8

15. Fernandes MHV, Maggioli MF, Joshi LR, Clement T, Faccin TC, Rauh R, Bauermann F V., Diel DG. Pathogenicity and cross-reactive immune responses of a historical and a contemporary Senecavirus A strains in pigs. *Virology* (2018) **522**:147–157. doi:10.1016/j.virol.2018.06.003

16. Maggioli MF, Fernandes MH V., Joshi LR, Sharma B, Tweet MM, Noll JCG, Bauermann F V., Diel DG. Persistent Infection and Transmission of Senecavirus A from Carrier Sows to Contact Piglets. *J Virol* (2019) **93**:1–19. doi:10.1128/jvi.00819-19

17. Alexandersen S, Zhang Z, Donaldson AI. Aspects of the persistence of foot-and-mouth disease virus in animals - The carrier problem. *Microbes Infect* (2002) **4**:1099–1110. doi:10.1016/S1286-4579(02)01634-9

18. McVicar JW, Sutmoller P. The epizootiological importance of foot-and-mouth disease carriers. *Arch Gesamte Virusforsch* (1969) **26**:217–224. doi:10.1007/bf01242374

19. Turlewicz-Podbielska H, Włodarek J, Pomorska-Mól M. Noninvasive strategies for surveillance of swine viral diseases: a review. *J Vet Diagnostic Investig* (2020) **32**:503–512. doi:10.1177/1040638720936616

20. López WA, Gauger PC, Harmon KM, Holtkamp DJ, Cano JP, Macedo N, Zhang M, Silva GS, Angulo J, Zimmerman JJ, et al. Probability of PRRS virus detection in pooled processing fluid samples. *Vet Microbiol* (2021) **261**:2019–2022. doi:10.1016/j.vetmic.2021.109190

21. Vilalta C, Baker J, Sanhueza J, Murray D, Sponheim A, Alvarez J, Sylvia F, Polson D, Torremorell M, Corzo C, et al. Effect of litter aggregation and pooling on detection of porcine reproductive and respiratory virus in piglet processing fluids. *J Vet Diagnostic Investig* (2019) **31**:625–628. doi:10.1177/1040638719852999

22. Vilalta C, Sanhueza J, Alvarez J, Murray D, Torremorell M, Corzo C, Morrison R. Use of processing fluids and serum samples to characterize porcine reproductive and respiratory syndrome virus dynamics in 3 day-old pigs. *Vet Microbiol* (2018) **225**:149–156. doi:10.1016/j.vetmic.2018.09.006

23. Holtkamp DJ, Torremorell M, Corzo CA, L Linhares DC, Almeida MN, Yeske P, Polson DD, Becton L, Snel-son H, Donovan T, et al. Proposed modifications to porcine reproductive and respiratory syndrome virus herd classification. *J Swine Heal Prod* (2021) **29**:261–270.

24. Preis G, Benjamin N, Corzo C. Detection of Senecavirus A and IgG antibodies over time after an outbreak in a breeding herd: A Case Report. in *2020 Allen D. Leman Swine Research Abstracts* (Saint Paul, MN). Available at: https://sites.google.com/a/umn.edu/leman-swine-conference/home/2020/2020-research-abstracts

25. Venkataraman S, Reddy SP, Loo J, Idamakanti N, Hallenbeck PL, Reddy VS.

Crystallization and preliminary X-ray diffraction studies of Seneca Valley Virus-001, a new member of the Picornaviridae family. *Acta Crystallogr Sect F Struct Biol Cryst Commun* (2008) **64**:293–296. doi:10.1107/S1744309108006921

26. Adams MJ, Lefkowitz EJ, King AMQ, Bamford DH, Breitbart M, Davison AJ, Ghabrial SA, Gorbalenya AE, Knowles NJ, Krell P, et al. Ratification vote on taxonomic proposals to the International Committee on Taxonomy of Viruses (2015). *Arch Virol* (2015) **160**:1837–1850. doi:10.1007/s00705-015-2425-z
27. Knowles NJ, Hales LM, Jones BH, Landgraf JG, House JA, Skele KL, Burroughs KD, Hallenbeck PL. Epidemiology of Seneca Valley Virus: Identification and Characterization of Isolates from Pigs in the United States. *XIVth Meet Eur Study Gr Mol Biol Picornaviruses* (2006)G2. Available at: https://www.aasv.org/SVV/SVVUS.pdf
28. Burke M. Oncolytic Seneca Valley Virus: past perspectives and future directions. *Oncolytic Virotherapy* (2016) **Volume 5**:81–89. doi:10.2147/ov.s96915
29. Luo D, Wang H, Wang Q, Liang W, Liu B, Xue D, Yang Y, Ma B. Senecavirus A as an Oncolytic Virus: Prospects, Challenges and Development Directions. *Front Oncol* (2022) **12**:1–13. doi:10.3389/fonc.2022.839536
30. Rudin CM, Poirier JT, Senzer NN, Stephenson J, Loesch D, Burroughs KD, Reddy PS, Hann CL, Hallenbeck PL. Phase I clinical study of Seneca Valley Virus (SVV-001), a replication-competent picornavirus, in advanced solid tumors with neuroendocrine features. *Clin Cancer Res* (2011) **17**:888–895. doi:10.1158/1078-0432.CCR-10-1706
31. Schenk EL, Mandrekar SJ, Dy GK, Aubry MC, Tan AD, Dakhil SR, Sachs BA,

Nieva JJ, Bertino E, Lee Hann C, et al. A Randomized Double-Blind Phase II Study of the Seneca Valley Virus (NTX-010) versus Placebo for Patients with Extensive-Stage SCLC (ES SCLC) Who Were Stable or Responding after at Least Four Cycles of Platinum-Based Chemotherapy: North Central Cancer Tre. *J Thorac Oncol* (2020) **15**:110–119. doi:10.1016/j.jtho.2019.09.083

32. Segalés J, Barcellos D, Alfieri A, Burrough E, Marthaler D. Senecavirus A: An Emerging Pathogen Causing Vesicular Disease and Mortality in Pigs? *Vet Pathol* (2017) **54**:11–21. doi:10.1177/0300985816653990

33. USDA - APHIS. Foot-and-Mouth Disease Factsheet. (2013).

34. Pasma T, Davidson S, Shaw SL. Idiopathic vesicular disease in swine in Manitoba. *Can Vet J = La Rev Vet Can* (2008) **49**:84–85. Available at: https://pubmed.ncbi.nlm.nih.gov/18320985

35. Singh K, Corner S, Clark SG, Scherba G, Fredrickson R. Seneca valley virus and vesicular lesions in a pig with idiopathic vesicular disease. *J Vet Sci Technol* (2012) **3**:3–5. doi:10.4172/2157-7579.1000123

36. Vannucci FA, Linhares DCL, Barcellos DESN, Lam HC, Collins J, Marthaler D. Identification and Complete Genome of Seneca Valley Virus in Vesicular Fluid and Sera of Pigs Affected with Idiopathic Vesicular Disease, Brazil. *Transbound Emerg Dis* (2015) **62**:589–593. doi:10.1111/tbed.12410

37. Wu Q, Zhao X, Bai Y, Sun B, Xie Q, Ma J. The First Identification and Complete Genome of Senecavirus A Affecting Pig with Idiopathic Vesicular Disease in China. *Transbound Emerg Dis* (2017) **64**:1633–1640. doi:10.1111/tbed.12557

38. Sun D, Vannucci F, Knutson TP, Corzo C, Marthaler DG. Emergence and whole-

genome sequence of Senecavirus A in Colombia. *Transbound Emerg Dis* (2017) **64**:1346–1349. doi:10.1111/tbed.12669

39. Saeng-chuto K, Rodtian P, Temeeyasen G, Wegner M, Nilubol D. The first detection of Senecavirus A in pigs in Thailand, 2016. *Transbound Emerg Dis* (2018) **65**:285–288. doi:10.1111/tbed.12654

40. Arzt J, Bertram MR, Vu LT, Pauszek SJ, Hartwig EJ, Smoliga GR, Palinski R, Stenfeldt C, Fish IH, Hoang BH, et al. First Detection and Genome Sequence of Senecavirus A in Vietnam. *Microbiol Resour Announc* (2019) **8**:17–18. doi:10.1128/mra.01247-18

41. Ministerio de Agricultura/Gobierno de Chile. Informar sobre deteccion Virus Seneca en Chile. Santiago, Chile (2022). Available at: https://ceropapel.sag.gob.cl/validar/?key=121105740&hash=8cc10

42. CPA/SENASICA Gobierno de Mexico. AVISE 21 Boletin Informativo de la CPA. Ciudad de Mexico, Mexico (2022). Available at: https://dj.senasica.gob.mx/Contenido/files/2022/marzo/FEBRERO2022_ff602385-52bf-485c-a39d-00c5dd9986b3.pdf

43. Dee SA, Bauermann F V., Niederwerder MC, Singrey A, Clement T, De Lima M, Long C, Patterson G, Sheahan MA, Stoian AMM, et al. Survival of viral pathogens in animal feed ingredients under transboundary shipping models. *PLoS One* (2018) **13**:1–18. doi:10.1371/journal.pone.0194509

44. Miles LA, Burga LN, Gardner EE, Bostina M, Poirier JT, Rudin CM. Anthrax toxin receptor 1 is the cellular receptor for Seneca Valley virus. *J Clin Invest* (2017) **127**:2957–2967. doi:10.1172/JCI93472

45. Platt H. Phagocytic activity in squamous epithelia and its role in cellular susceptibility to foot-and-mouth disease. *Nature* (1961) **190**:1075–1076. doi:10.1038/1901075a0

46. Guo B, Piñeyro PE, Rademacher CJ, Zheng Y, Li G, Yuan J, Hoang H, Gauger PC, Madson DM, Schwartz KJ, et al. Novel senecavirus a in swine with vesicular disease, United States, July 2015. *Emerg Infect Dis* (2016) **22**:1325–1327. doi:10.3201/eid2207.151758

47. Maggioli MF, Lawson S, de Lima M, Joshi LR, Faccin TC, Bauermann F V., Diel DG. Adaptive Immune Responses following Senecavirus A Infection in Pigs. *J Virol* (2017) **92**:1–17. doi:10.1128/jvi.01717-17

48. Sharma B, Fernandes MHV, de Lima M, Joshi LR, Lawson S, Diel DG. A Novel Live Attenuated Vaccine Candidate Protects Against Heterologous Senecavirus A Challenge. *Front Immunol* (2019) **10**:1–17. doi:10.3389/fimmu.2019.02660

49. Joshi LR, Mohr KA, Gava D, Kutish G, Buysse AS, Vannucci FA, Piñeyro PE, Crossley BM, Schiltz JJ, Jenkins-Moore M, et al. Genetic diversity and evolution of the emerging picornavirus Senecavirus A. *J Gen Virol* (2020) **101**:175–187. doi:10.1099/jgv.0.001360

50. C. Caserta L, Jessica JC, Singrey A, Niederwerder MC, Dee S, Nelson EA, Diel DG. Stability of Senecavirus A in animal feed ingredients and infection following consumption of contaminated feed. *Transbound Emerg Dis* (2022) **69**:88–96. doi:10.1111/tbed.14310

51. Tousignant SJP, Bruner L, Schwartz J, Vannucci F, Rossow S, Marthaler DG. Longitudinal study of Senecavirus a shedding in sows and piglets on a single

United States farm during an outbreak of vesicular disease. *BMC Vet Res* (2017) **13**:1–5. doi:10.1186/s12917-017-1172-7

52. Billinis C, Paschaleri-Papadopoulou E, Psychas V, Vlemmas J, Leontides S, Koumbati M, Kyriakis SC, Papadopoulos O. Persistence of encephalomyocarditis virus (EMCV) infection in piglets. *Vet Microbiol* (1999) **70**:171–177. doi:10.1016/S0378-1135(99)00137-6

53. Fowler VL, Ransburgh RH, Poulsen EG, Wadsworth J, King DP, Mioulet V, Knowles NJ, Williamson S, Liu X, Anderson GA, et al. Development of a novel real-time RT-PCR assay to detect Seneca Valley virus-1 associated with emerging cases of vesicular disease in pigs. *J Virol Methods* (2017) **239**:34–37. doi:10.1016/j.jviromet.2016.10.012

54. Dall Agnol AM, Miyabe FM, Leme RA, Oliveira TES, Headley SA, Alfieri AA, Alfieri AF. Quantitative analysis of senecavirus A in tissue samples from naturally infected newborn piglets. *Arch Virol* (2018) **163**:527–531. doi:10.1007/s00705-017-3630-8

55. Feronato C, Leme RA, Diniz JA, Agnol AMD, Alfieri AF, Alfieri AA. Development and evaluation of a nested-PCR assay for Senecavirus A diagnosis. *Trop Anim Health Prod* (2018) **50**:337–344. doi:10.1007/s11250-017-1436-z

56. Leme RA, Oliveira TES, Alcântara BK, Headley SA, Alfieri AF, Yang M, Alfieri AA. Clinical manifestations of senecavirus a infection in neonatal pigs, Brazil, 2015. *Emerg Infect Dis* (2016) **22**:1238–1241. doi:10.3201/eid2207.151583

57. Dvorak CMT, Akkutay-Yoldar Z, Stone SR, Tousignant SJP, Vannucci FA, Murtaugh MP. An indirect enzyme-linked immunosorbent assay for the

identification of antibodies to Senecavirus A in swine. *BMC Vet Res* (2017) **13**:1–6. doi:10.1186/s12917-017-0967-x

58. Goolia M, Vannucci F, Yang M, Patnayak D, Babiuk S, Nfon CK. Validation of a competitive ELISA and a virus neutralization test for the detection and confirmation of antibodies to Senecavirus A in swine sera. *J Vet Diagnostic Investig* (2017) **29**:250–253. doi:10.1177/1040638716683214

59. Saporiti V, Fritzen JTT, Feronato C, Leme RA, Lobato ZIP, Alfieri AF, Alfieri AA. A ten years (2007–2016) retrospective serological survey for Seneca Valley virus infection in major pig producing states of Brazil. *Vet Res Commun* (2017) **41**:317–321. doi:10.1007/s11259-017-9697-6

60. Singh A, Mor SK, Aboubakr H et al. Efficacy of three disinfectants against Senecavirus A on five surfaces and at two temperatures. *J Swine Heal Prod* (2017) **25**:64–68.

61. Vannucci FA. Experiences with SVA elimination in sow farms. in *Allen D. Leman Swine Conference* (Saint Paul, MN). Available at: https://www.youtube.com/watch?v=Jbrg5tKkGa0

62. Leme RA, Alfieri AF, Alfieri AA. Update on senecavirus infection in pigs. *Viruses* (2017) **9**:1–15. doi:10.3390/v9070170

63. Dohoo I, Martin W, Stryhn H. *Methods in epidemiologic research.* , ed. S. M. McPike Charlottetown: VER Inc (2012).

64. Christensen J, Gardner IA. Herd-level interpretation of test results for epidemiologic studies of animal diseases. *Prev Vet Med* (2000) **45**:83–106. doi:10.1016/S0167-5877(00)00118-5

65. Sergeant E. Epitools Epidemiological Calculators. *Ausvet* (2018) Available at: http://epitools.ausvet.com.au

66. R Core Team. R: A language and environment for statistical computing. (2022) Available at: http://www.r-project.org/

67. Houston E, Giménez-Lirola LG, Magtoto R, Mora-Díaz JC, Baum D, Piñeyro PE. Seroprevalence of Senecavirus A in sows and grower-finisher pigs in major swine producing-states in the United States. *Prev Vet Med* (2019) **165**:1–7. doi:10.1016/j.prevetmed.2019.01.012

68. Alarcón LV, Alberto AA, Mateu E. Biosecurity in pig farms: a review. *Porc Heal Manag* (2021) **7**:1–15. doi:10.1186/s40813-020-00181-z

69. Rose N, Madec F. Occurrence of respiratory disease outbreaks in fattening pigs: Relation with the features of a densely and a sparsely populated pig area in France. *Vet Res* (2002) **33**:179–190. doi:10.1051/vetres

70. Lambert MÈ, Arsenault J, Poljak Z, D'Allaire S. Epidemiological investigations in regard to porcine reproductive and respiratory syndrome (PRRS) in Quebec, Canada. Part 2: Prevalence and risk factors in breeding sites. *Prev Vet Med* (2012) **104**:84–93. doi:10.1016/j.prevetmed.2011.11.002

71. Garber L, Bjork K, Patyk K, Rawdon T, Antognoli M, Delgado A, Ahola S, McCluskey B. Factors Associated with Highly Pathogenic Avian Influenza H5N2 Infection on Table-Egg Layer Farms in the Midwestern United States, 2015. *Avian Dis* (2016) **60**:460–466. doi:10.1637/11351-121715-Reg

72. McQuiston JH, Garber LP, Porter-Spalding BA, Hahn JW, Pierson FW, Wainwright SH, Senne DA, Brignole TJ, Akey BL, Holt TJ. Evaluation of risk

factors for the spread of low pathogenicity H7N2 avian influenza virus among commercial poultry farms. *J Am Vet Med Assoc* (2005) **226**:767–772. doi:10.2460/javma.2005.226.767

73. Houston E, Temeeyasen G, Piñeyro PE. Comprehensive review on immunopathogenesis, diagnostic and epidemiology of Senecavirus A. *Virus Res* (2020) **286**:198038. doi:10.1016/j.virusres.2020.198038

74. Allerson MW, Cardona CJ, Torremorell M. Indirect Transmission of Influenza A Virus between Pig Populations under Two Different Biosecurity Settings. *PLoS One* (2013) **8**:2–10. doi:10.1371/journal.pone.0067293

75. Kim Y, Yang M, Goyal SM, Cheeran MCJ, Torremorell M. Evaluation of biosecurity measures to prevent indirect transmission of porcine epidemic diarrhea virus. *BMC Vet Res* (2017) **13**:1–9. doi:10.1186/s12917-017-1017-4

76. Sturos MJ, Murray D, Johnson L, Preis G, Corzo CA, Rossow S, Vannucci FA. Persistence and shedding of senecavirus A in naturally infected boars. *J Vet Diagnostic Investig* (2022)104063872210840. doi:10.1177/10406387221084054

77. Buckley A, Lager K. Infectious dose of Senecavirus A in market weight and neonatal pigs. *PLoS One* (2022) **17**:1–12. doi:10.1371/journal.pone.0267145

78. Sellers RF, Donaldson AI, Herniman KAJ. Inhalation, persistence and dispersal of foot-and-mouth disease virus by man. *J Hyg (Lond)* (1970) **68**:565–573. doi:10.1017/S0022172400042492

79. Hierholzer JC, Killington RA. "Virus isolation and quantitation," in *Virology Methods Manual*, eds. B. W. J. Many, H. O. Kangro (Amsterdan: Elsevier), 25–46.

80. USDA. Classical Swine Fever Surveillance GOT TONSIL? Available at: https://www.aphis. [Accessed May 3, 2019]

81. Prickett J, Simer R, Christopher-Hennings J, Yoon KJ, Evans RB, Zimmerman JJ. Detection of Porcine reproductive and respiratory syndrome virus infection in porcine oral fluid samples: A longitudinal study under experimental conditions. *J Vet Diagnostic Investig* (2008) **20**:156–163. doi:10.1177/104063870802000203

82. Donahue BC, Petrowski HM, Melkonian K, Ward GB, Mayr GA, Metwally S. Analysis of clinical samples for early detection of classical swine fever during infection with low, moderate, and highly virulent strains in relation to the onset of clinical signs. *J Virol Methods* (2012) **179**:108–115. doi:10.1016/j.jviromet.2011.10.008

83. Walker HL, Bowman AS, Ferreira JB, Nelson SW, Pairis-Garcia M, Arruda AG. Tonsil scrapings for porcine reproductive and respiratory syndrome virus detection in growing pigs under field conditions. *J Swine Heal Prod* (2021) **29**:72–80. Available at: https://www.aasv.org/shap/issues/v29n2/v29n2p72.html

84. Lopez WA, Angulo J, Zimmerman JJ, Linhares DCL. Porcine reproductive and respiratory syndrome monitoring in breeding herds using processing fluids. *J Swine Heal Prod* (2018) **26**:146–150.

85. Holtkamp DJ, Torremorell M, Corzo CA, L Linhares DC, Almeida MN, Yeske P, Polson DD, Becton L, Snel-son H, Donovan T, et al. Proposed modifications to porcine reproductive and respiratory syndrome virus herd classification. *J Swine Heal Prod* (2021) **29**:261–270. Available at: http://www.aasv.org/shap.html.

86. Pruim R, Kaplan DT, Horton NJ. The mosaic Package: Helping Students to “Think

with Data" Using R. *R J* (2017) **9**:77–102. Available at: https://journal.r-project.org/archive/2017/RJ-2017-024/index.html

87. Hole K, Ambagala T, Nfon C. Vesicular disease in pigs inoculated with a recent canadian isolate of senecavirus A. *Can J Vet Res* (2019) **83**:242–247.

88. de Almeida MN, Corzo CA, Zimmerman JJ, Linhares DCL. Longitudinal piglet sampling in commercial sow farms highlights the challenge of PRRSV detection. *Porc Heal Manag* (2021) **7**:1–10. doi:10.1186/s40813-021-00210-5

89. Linhares DCL, Cano JP, Torremorell M, Morrison RB. Comparison of time to PRRSv-stability and production losses between two exposure programs to control PRRSv in sow herds. *Prev Vet Med* (2014) **116**:111–119. doi:10.1016/j.prevetmed.2014.05.010

90. Rotolo ML, Main RG, Zimmerman JJ. Herd-level infectious disease surveillance of livestock populations using aggregate samples. *Anim Heal Res Rev* (2018) **19**:53–64. doi:10.1017/S1466252318000038

91. Vilalta C, Sanhueza JM, Schwartz M, Kikuti M, Torremorell M, Corzo CA. Assessing the litter level agreement of RT-PCR results for porcine reproductive and respiratory syndrome virus in testicles, tails and udder wipes diagnostic samples relative to serum from piglets. *Prev Vet Med* (2021) **186**:105211. doi:10.1016/j.prevetmed.2020.105211