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SURVEILLANCE AND EVALUATION OF MANURE TREATMENT PRACTICES FOR MITIGATION OF THE PORCINE EPIDEMIC DIARRHEA VIRUS (PEDV) IN A COMMERCIAL SWINE FARM SETTING

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by

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SURVEILLANCE AND EVALUATION OF MANURE TREATMENT PRACTICES FOR MITIGATION OF THE PORCINE EPIDEMIC DIARRHEA VIRUS (PEDV) IN A COMMERCIAL SWINE FARM SETTING

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The emergence of the porcine epidemic diarrhea virus (PEDv) in the United States in 2013 resulted in billions of dollars in annual losses for the U.S. swine industry. Infection with PEDv causes severe diarrhea and vomiting in pigs, spreads rapidly through ingestion of infected manure, and produces nearly 100% mortality in pre-weaned piglets. Because swine manure slurry is a valuable crop nutrient source, concerns about virus persistence in stored manure remain a major barrier to proper manure management. Proper manure handling and application practices are necessary to control the risk of pathogen re-infection at affected production sites or infecting new sites through virus-contaminated manure handling equipment. alkaline stabilization managed to control potential infection from manure sources. Alkaline stabilization of manure with hydrated lime to pH 10 for at least one hour was proven an effective treatment to render PEDV-positive swine manure slurry non-infective as confirmed via live pig bioassays. This treatment goal can be achieved with a dosing rate of 22.7 kg (50 lbs) of quicklime per 3,785 L (1,000 gal) of swine manure. Ammonia loss from simulated storage pit and manure tanker settings were approximately 30% and 15%, respectively, representing a potential loss in nutrient value from volatilized ammonia nitrogen. To minimize the

potential for any disease outbreak, strict biosecurity planning and implementation of biosecure practices is essential. A field study was conducted to assess the effectiveness of industry recommended biosecurity practices to eliminate PEDv at critical control points (CCPs) on three commercial swine farms in the midwestern U.S. following disease outbreaks. Sampling of CCPs on each farm was conducted immediately following confirmation of PEDv on the farms, immediately following disinfection and at 6, 12 and 18 months post-disinfection. We conclude, based on survey results, that practices used to control PEDv in commercial swine farm settings are effective at eliminating the virus and preventing reinfection of the herd with the same viral strain.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I.

REVIEW OF LITERATURE

INTRODUCTION

This is a comprehensive literature review describing (i) commercial swine production in the United States and the associated management of swine manure; (ii) a brief history of the emergence of swine enteric coronavirus diseases (SECD) in the United State swine industry, with specific emphasis on the porcine epidemic diarrhea virus (PEDv); and (iii) an overview of the epidemiology of PEDv and potential mechanisms for mitigating the environmental persistence of the virus.

The porcine epidemic diarrhea virus (PEDv), an enteric disease of swine, first appeared in the United States in 2013. Having only been previously seen in Asia and Europe, the virus quickly became recognized as a worldwide threat to the health and production of swine as it spread rapidly throughout the U.S. swine population and other North American swine industries. The PED virus affects the gastrointestinal tract of pigs causing severe diarrhea, dehydration, and vomiting 12 to 36 hours after exposure to the virus.

High morbidity and mortality are associated with the virus; nearly 100% mortality results among pre-weaned piglets exposed to the disease while older pigs experience production delays. As such, the economic impact of the virus to the United States was estimated at $8 billion in the first two years after the emergence (Stevensen, 2014). While

many factors were considered in this estimate – including increased fixed capital costs and negative impacts on trade (Paarlberg, 2014) – the deaths of over 8 million pigs contributed heavily to the significant economic impact of PEDv.

In addition to the virus being very deadly, it is also extremely infectious. Experts in veterinary medicine have speculated that a thimble – with a capacity of about 1 mL – could feasibly contain about 100 million PED virions, which is enough to infect the entire pig population in the state of Iowa. This represents about one-third of the pig population in the United States, or 20 million live hogs.

Related viral diseases of swine, including porcine delta corona virus (PDCoV) and swine delta corona virus (SDCV), were discovered during the PEDv outbreak and, collectively, the illnesses caused by these viruses are referred to as swine enteric coronavirus diseases, or SECDs. While not designated as foreign animal diseases (FADs), PEDv and other SECDs have been designated "transboundary diseases" due to their rapid translocation and transmission. This characteristic demonstrates the need for immediate and effective action to mitigate the spread of these disease.

United States Swine Production

As of September 2018, the U.S. swine population was 75.5 million head (USDA-NASS, 2018). Within this population are approximately 6.33 million head of breeding inventory (USDA-NASS, 2018), the sector of swine production where PEDv-related mortality is greatest due to the concentrated population of newborn piglets. Immediately prior to the PEDv outbreak in the United States in 2013, the swine inventory was near 67

million head but dropped sharply following the PEDv outbreak to just over 61 million head (USDA-NASS, 2018) as illustrated in Figure 1.1.

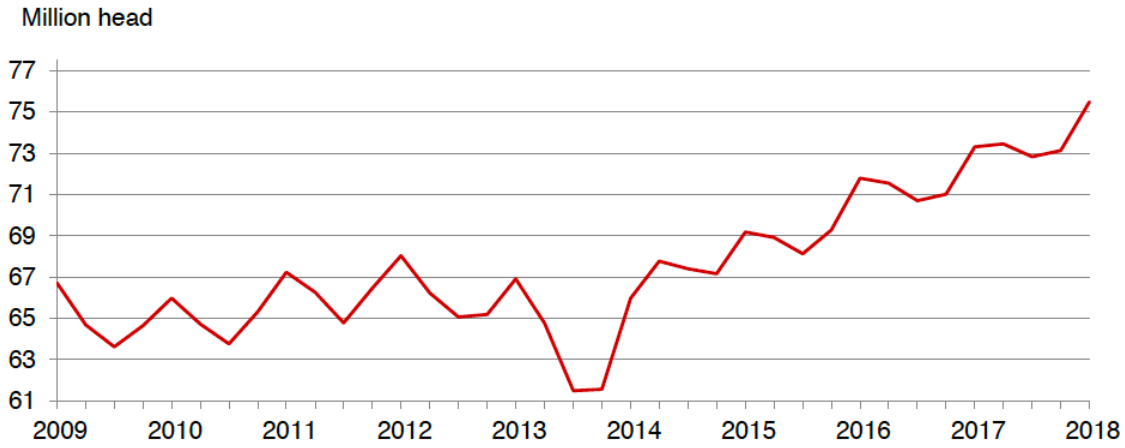

Figure 1.1. U.S. Quarterly Hogs and Pigs Inventory: September 2018 (USDA-NASS, 2018)

Swine manure production varies by animal type and weight; sows produce up to 3.33 gal d^{-1} when lactating, while nursery and grow-finish pigs produce about 0.30 and up to 1.79 gal/d, respectively (MWPS-18, 2004). While an exact estimate of total nationwide manure production on an annual basis is difficult to determine, the current swine inventory in the U.S. could feasibly produce up to 35 million gallons of manure each year. Because PEDv is spread among animals via the fecal-oral route, the management of infected manure is recognized as being a critical factor in controlling transmission of the disease.

Swine Manure Management

On modern U.S. farms where pigs are raised inside climate-controlled buildings, swine manure is commonly collected by allowing excreted urine and feces to drop into a pit below the animals via narrow openings in concrete floors (referred to as "slatted floors") or expanded metal floors. Once in the collection pit, the manure may be stored as a slurry in a deep pit beneath the production building or discharged via gravity flow or hydraulic flushing through below-ground piping into an anaerobic treatment lagoon outside the production facility.

Deep pit slurry storages are reinforced concrete structures located beneath the flooring in the animal production area (Figure 1.2) designed with enough capacity to store up to 365 days of manure and process wastewater generated by the animals housed in the production building. While slurry storages do not offer any form of manure treatment, they are regarded as a much better storage option than lagoons if nutrient retention in the manure is a major goal. Generally, slurry pits are sized for 1.0 cubic foot of storage per 1,000 pounds of live pig per day. Nebraska regulations require that manure storage systems for totally housed operations provide a minimum storage period of 180 days (Nebraska Administrative Code, Title 130). Therefore, for a 2,400-head grow-finish facility, this minimum storage period would require volume to contain approximately 800,000 gallons of manure.

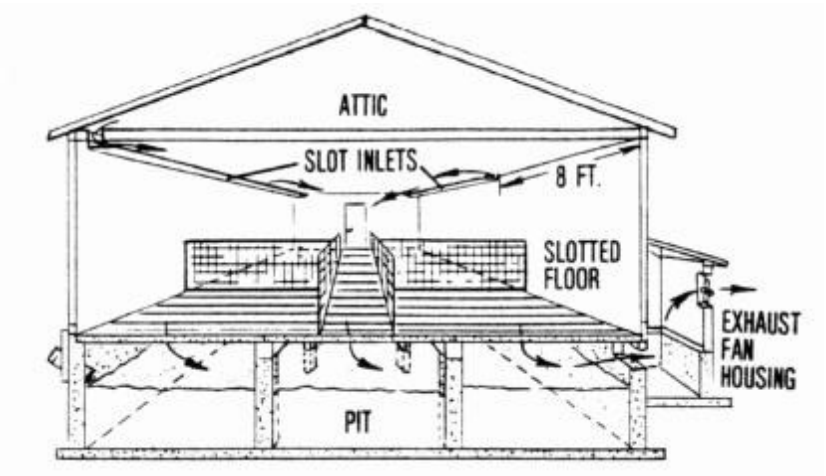

Figure 1.2. Swine production building with slotted floors and deep pit slurry manure storage

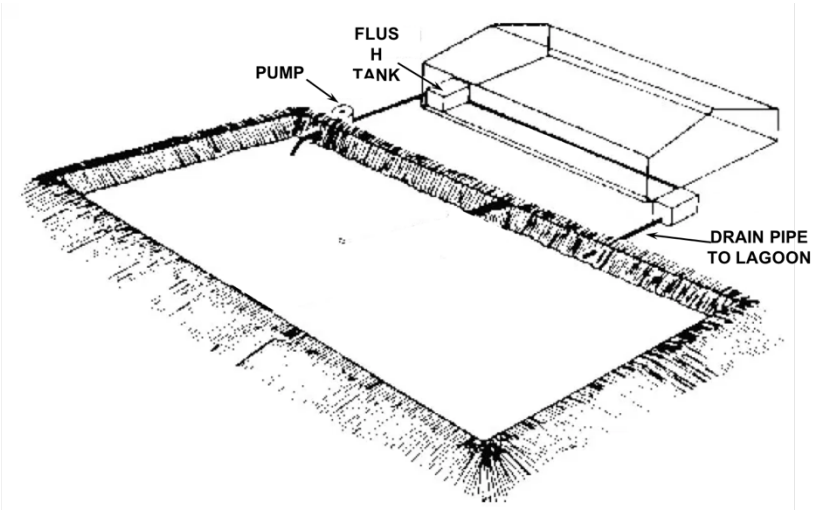

Figure 1.3. Anaerobic treatment lagoon with recycled effluent for flushing and gravity drained conveyance of manure to the lagoon

Anaerobic lagoons are typically constructed as an earthen basin designed to store and treat manure while also having adequate volume for sludge storage, net rainfall, and anaerobic treatment (Figure 1.3). Manure can be collected from the production area in a number of ways to facilitate transport to an anaerobic lagoon, but partially or fully slatted floors are a common method for collection. Narrow slots in the flooring allow feces and urine to fall into a shallow storage pit below the floor of the production area where the manure may be stored for a period of days or frequently removed and transferred to the lagoon. Gravity flow or mechanical flush systems that utilize fresh water or recycled lagoon effluent to move material from the building to the lagoon via hydraulic flow are common manure conveyance methods.

Anaerobic lagoons are commonly designed to provide six to twelve months of storage and treatment capacity to accommodate the available land application seasons in their location (Dickey, 1980). Treatment is accomplished through the actions of anaerobic bacteria in the storage that break down volatile solids present in the waste stream, forming gases, liquids and sludge in the process. Therefore, along with the manure and process wastewater generated in the livestock production area and precipitation deposited in the storage, anaerobic lagoons contain a permanent “treatment volume” to maintain the necessary biological capacity to treat the incoming waste stream and a “sludge storage volume” to accommodate settled solids from the manure, neither of which are removed during seasonal pumping of the storage.

Regardless of storage method, the ultimate destination for manure collected on livestock and poultry operations is as a fertilizer input to a crop production system. The

value of manure lies in its nutrient content – primarily nitrogen, phosphorous, and potassium, along with several micronutrients – organic matter, and microbes. While manure can contribute to environmental pollution when not properly managed, recommended methods and rates of application to meet the agronomic needs of agricultural crops are established and accompanied by recognized guidelines, or best management practices (BMPs), that provide a basis for developing economically and environmentally sound manure management (Dickey, 1980).

Emergence and Epidemiology of PEDv

Porcine epidemic diarrhea virus (PEDv) was first documented in the United Kingdom in 1971 as a swine disease resembling transmissible gastroenteritis (Pensaert, 1978). In 1978, the etiologic agent of PEDv was identified in Belgium as a new coronavirus and was designated as PEDv, prototype strain CV777 (Pensaert, 1978). For the next 20 years, PEDv was reported in several other European countries, including Hungary, Italy, Germany, France, Switzerland and the Czech Republic (Song, 2012). In Asia, PEDV was first identified in 1982 and was considered an endemic, causing substantial economic losses to pork producers in China, South Korea, Thailand and Vietnam (Song, 2012). However, it was not until 2010 that massive PEDv outbreaks were reported in China. The outbreaks have been characterized by an 80 to 100% illness incidence among infected swine herds and a 50 to 90% mortality rate among infected suckling piglets (Valasova, 2014).

North America was first impacted by PEDv in the United States in April 2013 (Stevensen, 2013). In a matter of months, the disease spread rapidly across the U.S. and Canada causing high rates of mortality among piglets and substantial economic losses for the industry. As of March 2018, 39 states have confirmed at least one case of PEDv (AASV, 2018).

While new cases of the virus continue to be reported five years after its initial detection in the U.S., a decreased incidence of new case reports over time is evident (Figure 1.4). Following the initial outbreak that began in spring 2013, case report quantities by week remained relatively steady until about October, 2013, after which the quantity of new case reports by week increased sharply until peaking in late February or early March, 2014, and then steadily decreased until October, 2014. Beginning in October, 2014, weekly case report quantities again began increasing until peaking in March, 2015. A consistent and similar trend in new case reports is evident throughout the remainder of time for which data is illustrated. The data appears to demonstrate that colder temperatures are preferential for virus survival and transmission. Accordingly, the lower incidence of new cases during summer months further supports a temperature effect on virus survival.

Another trend illustrated by the data in Figure 1.4 is a steady decline in new case reports with each subsequent year beyond the initial virus emergence in 2013. One explanation for this trend may be the development of natural acquired active immunity among surviving pigs following initial exposure and infection with PEDv. This immunity likely protected some pigs from reinfection during later exposure to the same or different

strains of the virus. However, repeated outbreaks have occurred in an unspecified number of U.S. swine herds, suggesting that not all pigs develop immunity to the virus once infected. As such, biosecurity measures to prevent virus persistence within a farm boundary appear to be as critical as measures intended to prevent viral transmission between farms.

Research has been done on the sensitivity of PEDv in production settings to help limit the infectivity via porcine plasma. Research by Quist-Rybachuk (2015) demonstrated that a heat-alkalinity-time (HAT) pasteurization procedure at ≥40°C and pH 9.2 for 30 min inactivated the virus during industrial processing of porcine plasma. Several studies have been conducted on pH and temperature controls, but one study researched the persistence of the virus on inanimate objects routinely used on swine farms. Styrofoam, rubber, plastic, coveralls, and other equipment were tested under different temperature conditions (Kim, 2007).When exposed to a controlled temperature environment at 4°C, the virus was detected up to 15 days after inoculation on Styrofoam, aluminum, Tyvek® coverall, cloth, and plastic. However, when the environment was held at room temperature (40°C) the virus could not be detected after 1 day post inoculation on all materials (Kim, 2007).

In addition to studies assessing the survivability of the virus on surfaces of various materials, limited research has been published to describe potential airborne transmission of the virus. In 2015, a study was conducted testing the risk of PEDv infection to swine facilities via predominate wind direction from up-wind PEDv positive facilities (Beam, 2015). This study suggests that wind could potentially direct the spread

of the disease but other factors were not considered, such as direct and indirect transmission via transportation trailers, personnel, and feed (Beam, 2015). Another study measured the size of particulate matter on which the virus could be carried. This study found that the virus could be detected via RT-PCR on particle sizes ranging from $1.3x10^6$ RNA copies-m^{-3} (0.4 to 0.7 µm) to $3.5x10^8$ RNA copies-m^{-3} (9.0 to 10.0 µm) (Alonso, 2015). This study concluded that the virus can be transmitted in the air via a wide range of particulate matter sizes. Although they can persists on different particles, the particle size determines the viability of the virus (Alonso, 2015). PEDv can develop a transient nasal epithelium infection, carrying dendritic cells allowing the virus to be transferable to $CD3^+$T cells via virological synapses. Another way of infection is direct cell-to-cell contact after infected $CD3^+$T cell have reached the intestine through blood circulation (Li, 2018).

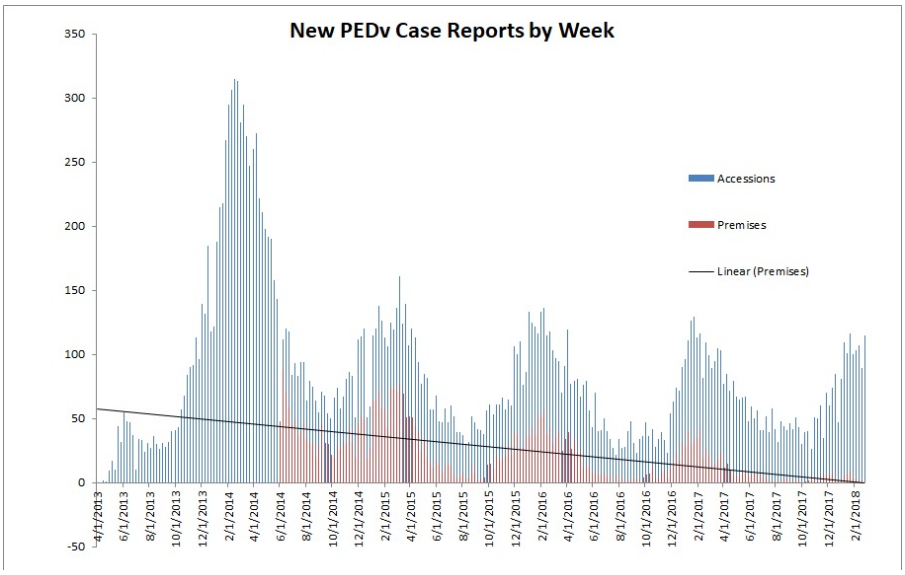

Figure 1.4. New PEDv case reports by week, April 2013 to present (AASV 2018)

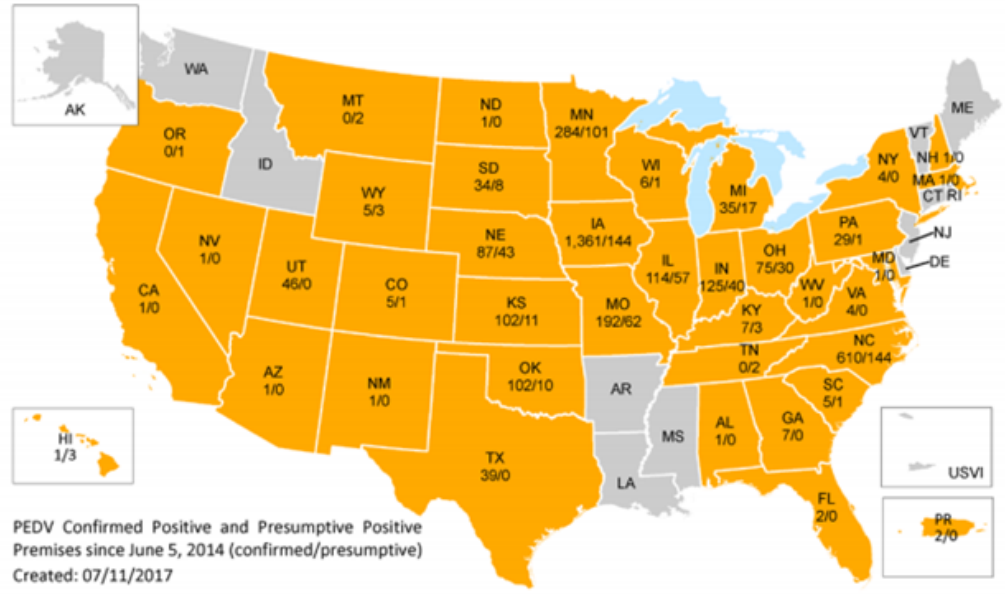

Figure 1.5. Cumulative confirmed and presumptive PEDv-positive premises since June 2014 (USDA, 2017)

Because PEDv is an enteric virus, infectious material is excreted in the manure of PEDv-infected animals and transmitted to healthy pigs via the fecal-oral route. Research focused on PEDv persistence in manure was initiated shortly after the virus emerged in the US. Unpublished research funded by the National Pork Board was conducted to assess viability of the PED virus in manure storages throughout the midwestern U.S. Five swine lagoons were sampled in three states following confirmed infection of pigs on each of the farms. While all five storages were reportedly positive for PEDv by PCR, only a single sample produced infection during a swine bio assay.

A study was conducted in Manitoba, Canada, to describe the survivability of PEDv in earthen manure storages (EMS), a common storage system in Canada. The

authors concluded that the virus could survive in a lagoon environment for up to nine months following a PEDv infection on the farm. While PED viral load in a studied lagoon averaged 1.1 x 10^5 copies mL^{-1}, the loads differed by depth leading the authors to suggest that ultraviolet (UV) sunlight exposure may be responsible for destruction of infectious virus in the surface layer of the system.

Structure and Function of PEDv

PEDv is an alphacoronavirus in the *Coronaviridae* family and, like other coronaviruses, possesses a large positive-sense RNA genome of more than 28 kilo-base pair (kbp) (Jaru-Ampornpan, 2016). There are different clades of the virus recorded, including the most virulent, CV777 PEDv.

The PED virus genome is composed of two overlapping open reading frames (ORF) encoding two polyproteins and five other ORFs that make up the genome structure (Figure 1.6). The first two-thirds of the genome contains ORF1a and ORF1b, which encode the replicase/transcriptase proteins; this is how the virus replicates and copies inside a host. The remaining one-third of the genome encodes five structural proteins: spike (S), envelope (E), membrane (M), nucleocapsid (N), and proteins (not displayed in Figure 1.6). The polyproteins are processed into individual, non-structural proteins by virally encoded proteases: papain-like proteases (PLP1/PLP2; nsp3) and 3-Chymotrypsin-like protease (3Cpro; nsp5) (Jaru-Ampornpan, 2016). The PEDv nucleocapsid protein plays a key role in organizing the viral genome by viral RNA binding and self-multimerization. Exclusively, the PEDv replicates in the cytoplasm but

the nucleocapsid has been shown to localize in the nucleus of infected cells and have both nuclear localization and export signals for its nucleo-cytoplasmic shuttling. The nucleocapsid can also aid in the manipulation of other cells along with PEDv pathogenesis (Jaru-Ampornpan, 2016).

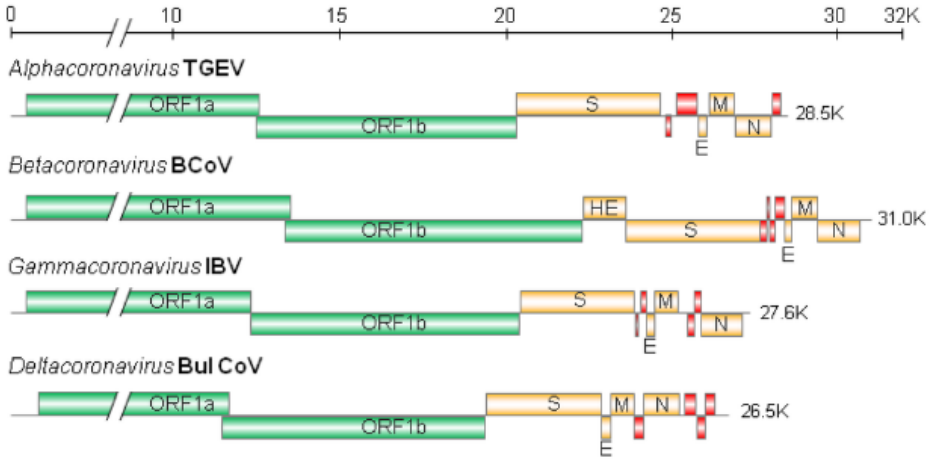

Figure 1.6. Genome organization of PED virus (Ujike, 2015)

The S protein of PEDv is the major envelope glycoprotein of the virion (Figure 1.7); it interacts with the cellular receptor during virus entry and stimulates neutralizing antibodies in the natural host (Fehr and Perlman, 2015). The PEDv S protein in known to be an appropriate viral gene for determining the genetic relatedness among PEDv isolates and for developing diagnostic assays and also vaccines (Fehr and Perlman, 2015). The M protein is the most abundant component of the envelope and it is required for the assembly process and can also aid in the production of protective antibodies with virus-neutralizing activity (Fehr and Perlman, 2015). The E protein which is a relatively small

envelope plays an important role during coronavirus budding; the expression of the E and M proteins can form spike-less virions. PEDv E and N proteins are found in the endoplasmic reticulum where they independently reduce the stress because they are located between the intercellular membrane compartments of the endoplasmic reticulum and the Golgi complex. N proteins of coronaviruses interact with the viral genomic RNA and interact with other N protein molecules in order to protect the viral genome. This then serves as the critical basis for the helical nucleocapsid during the coronavirus assembly. The PEDv N protein also deflects antiviral responses by antagonizing interferon production as part of the immune evasion strategy. The production of ORF3, the only accessory gene in PEDv, is thought to function as an ion channel influencing virus production (Fehr and Perlman, 2015). Some *beta-coronaviruses* have an additional hemagglutinin-esterase (HE) gene (Figure 1.8). To make the genome of each genus or species unique, they each have a set of unique accessory proteins shown in red on Figure 1.7.

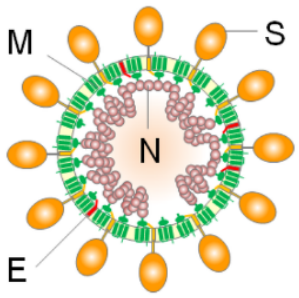

Figure 1.7. Schematic diagram of coronavirus virions (Ujike, 2015)

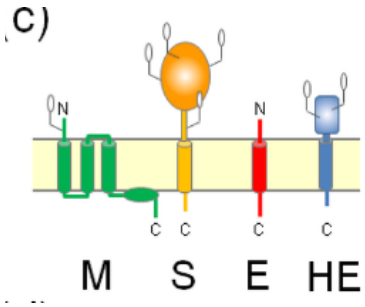

Figure 1.8. Topology of four structural envelope proteins (Ujike, 2015)

The PEDv nucleocapsid protein plays a key role in organizing the viral genome by viral RNA binding and self-multimerization. Exclusively, the PEDv replicates in the cytoplasm but the nucleocapsid has been shown to localize in the nucleus of infected cells and have both nuclear localization and export signals for its nucleo-cytoplasmic shuttling. The nucleocapsid has also been shown to aid in the manipulation of other cells along with PEDv pathogenesis (Jaru-Ampornpan, 2016). Coronaviruses attach to specific cellular receptors via the spike protein triggering a conformational change in the spike, which then mediates fusion between the viral and cell membranes causing the release of the nucleocapsid into the cell. Upon entry into the cell, the 5′ end of the genome RNA, ORFs 1a and 1b, are translated into pp1a and pp1ab; pp1ab is translated via a frameshift mechanism (Chen, 2014). ORF 1a encodes one or two papain-like proteases (PLpro or PLP) and a picornavirus 3C-like protease (3CLpro), which function to process pp1a and pp1ab into the mature replicase protein (Chen, 2014). PED virus is an enveloped coronavirus so entry into the host cell can occur directly after binding to the receptor or after internalization via endocytosis with fusion taking place in the endosomal

compartment. Conformational changes of the spike protein drives the fusion of viral membranes with the host membranes. Over time coronaviruses have modified their spike proteins leading to the diversity of triggers used to activate their fusion. These changes can be initiated by receptor binding by additional triggers such as pH acidification or proteolytic activation (Ma, 2013). Figure 1.9 is the three-dimensional predicted structure of the SARS-CoV spike protein which would be similar to the PEDv coronavirus. The S1 and S2 domains as well as the cleavage sites and putative fusion peptide are highlighted. A visual representation from GeneBank is shown in figure 1.9.

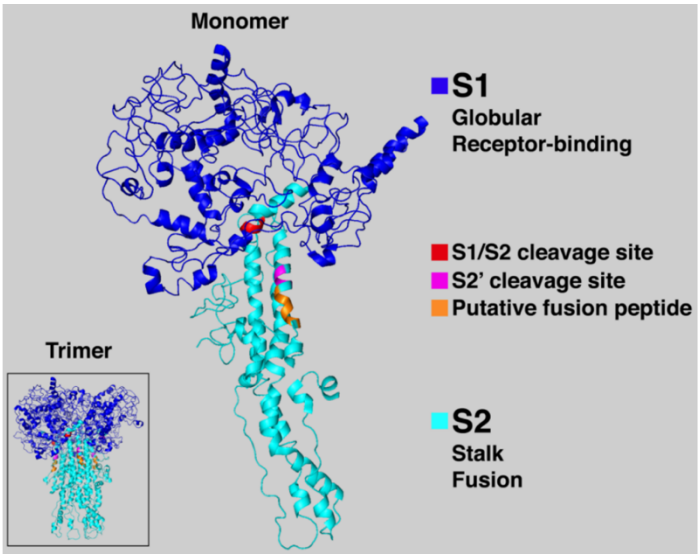

Figure 1.9. 3D represented structure of SRS-CoV protein (GeneBank)

A negatively stained sample in an electron microscopy analysis revealed the presence of medium-sized viral particles of approximately 80-120 nm in diameter (Figure

1.10). Some of the virions surface projections is a characteristic of the coronaviruses and can be seen in Figure1.11 (Pan, 2012).

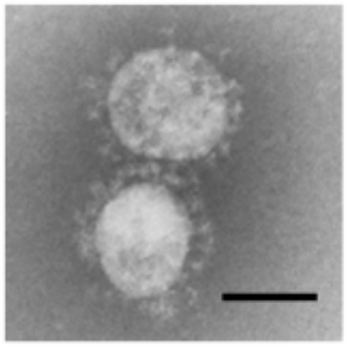

Figure 1.10. Electron micrograph of SCoV. Bar: 100 nm. (EM image courtesy of Dr. Nagata at National Institute of Infectious Diseases). (Ujike, 2015)

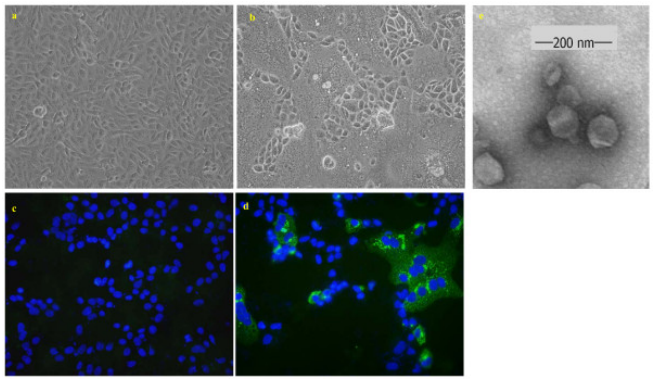

Figure 1.11. CHGD-01 strain of PEDv isolate by optical microscopy, IFA assay and electron microscopy (Pan, 2012)

Critical Control Points (CCPs) for PEDv

In a livestock production setting, biosecurity procedures are intended to mitigate the transmission of disease-causing organisms that may adversely impact the health of the animals. Many vectors for disease transfer exist on livestock farms (Figure 1.13). In fact, it is often said that "If it moves, it can carry disease." Each vector can present multiple biosecurity challenges as animals, vehicles and other fomites move among various points on and off the farm.

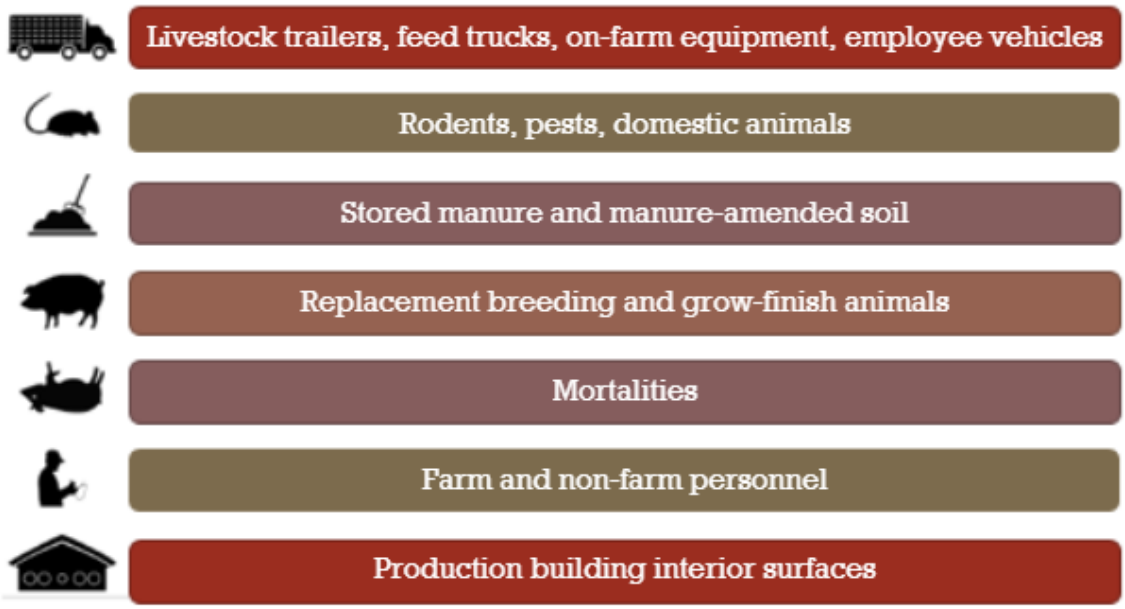

Figure 1.12 Evidence-based techniques for decontamination and control of PEDV at critical control points (CCPs) within the swine farm setting.

Studies have been conducted on controlling the spread of PEDv via livestock transport trucks that could transfer infectious material among animals and production

sites (Thomas, 2015). Time and temperature combinations were studied to identify heat exposure periods that could inactivate the PED virus on metal surfaces. Results demonstrated that heating of the commercial livestock trailer to 71°C (160°F) for 10 minutes or allowing the trailer to sit for 7 days at 20°C (68°F) was sufficient to limit the infectivity of PEDv in organic residues. Similar research has been focused on using heated water for virus inactivation (Zentkovich, 2016). Viable PEDv was not recovered after a ten second or longer treatment with water heated to at least 76°C, though PEDv nucleic acid was still detectable in the treatment. This could serve as a decontamination practice if chemical practices are not an option.

Contaminated feed ingredients were identified as a culprit for the introduction of the virus into North America initially. As such, an experiment was performed to evaluate a standardized protocol to sanitize a feed manufacturing facility following PED virus exposure (Huss, 2017). While equipment samples that were collected after handling contaminated feed were positive for the virus, as expected, other surface samples that did not come directly in contact with the feed were also PEDv positive indicating that dust could serve a vital role in the transmission of the virus. After sanitation protocols were performed, the amount of PEDv viral genomes were reduced, but not completely eliminated (Huss, 2017).

Hard lines of separation are another on-farm practice being promoted for improved biosecurity in response to PEDv. Lines of separation are physical demarcations between two areas of potentially different disease status. The entry gate to a farm, shower-in/shower-out facilities, and a simple tape line on the floor between the gestation

and farrowing areas of a sow farm are all examples of lines of separation. While many of these lines of separation existed prior to the emergence of PEDv in the U.S., others have been established or improved through more explicit procedures or more secure barriers.

Alkaline Stabilization of Biological Wastes

The use of lime as a treatment for human septage is approved by the Environmental Protection Agency (EPA) for human protection against pathogens and other biological agents (40 CFR Part 503). The federal code states that alkali stabilization of the pH of the domestic septage so that it remains at pH 12 or greater for at least 30 minutes before land applying is a proven practice for treatment. Treatment of biological wastes with lime is based on several chemical reactions. Calcium hydroxide is an alkaline compound that can increase pH as high 12.4. As pH exceeds 12 and temperature increases, cell membranes of harmful pathogens are lysed.

Because lime has low solubility in water, lime molecules persist in biosolids to prevent regrowth of pathogens. When quicklime (CaO) is used with water, an exothermic reaction occurs. As heat is released, the temperature of the biological waste can increase to 70ºC, which can provide effective pasteurization.

OBJECTIVES

Because PEDv is a highly infectious and deadly disease transmitted through infected fecal material, virus persistence in manure could potentially infect naïve animals introduced to a production facility that has been infected by the PED virus. Land

application of manure on crop production fields provides nutrients and other inputs that benefit crop production, but could serve as a transmission vector for PEDv if the virus persists in soil following manure application. Because alkaline stabilization of human septage using lime is approved by the EPA for human health protection, we hypothesized that using lime for alkaline stabilization of PEDv-positive manure would produce a similar positive effect of reducing the pathogenicity of the virus in manure. Likewise, decontamination and disease transmission prevention practices that are effective throughout the swine production setting numerous; however, no specific recommendations have been issued regarding practices to include in a herd management plan following a positive PEDv accession on a farm. Furthermore, we know of no previous assessment of the effectiveness of multiple biosecurity practices implemented within a farm for disease prevention. This thesis project, therefore was designed to address three primary objectives, which are presented in two manuscripts as described:

Manuscript I: Alkaline Stabilization of Manure Slurry Inactivates Porcine Epidemic Diarrhea Virus

1) Assess PEDv persistence and infectivity in swine manure slurry following alkaline treatment with lime; and
2) Quantify ammonia volatilization losses during lime treatment of manure.

Manuscript II: Porcine Epidemic Diarrhea Virus (PEDv) Assessment in Swine Production Facilities

1) Assess the effectiveness of industry-recommended biosecurity practices to mitigate and prevent PEDv in commercial swine production settings.

During the completion of the objective addressed in Manuscript II, another laboratory study was completed with a separate objective:

1) Assess persistence of PEDv in manure amended soil to determine potential transmission risk following manure application.

THESIS PRESENTATION

This thesis is written in manuscript form. Chapter II is written as a manuscript titled, "Alkaline Stabilization of Manure Slurry Inactivates Porcine Epidemic Diarrhea Virus," and formatted for publication in *Journal of Swine Health and Production*. This chapter presents research on the persistence of PEDv in swine manure. The effect of manure slurry pH on virus pathogenicity as impacted by hydrated lime addition is presented as a treatment practice for PEDv-infected swine manure. This information is intended to help determine the effects of lime on infected manure, assess the practice for potential utilization on commercial swine farms, and provide a basis for recommending lime dosing. The information also provides a basis for best and safe practices to use when lime is the treatment source.

Chapter III is written as a manuscript titled, "Porcine Epidemic Diarrhea Virus (PEDv) Assessment on Three Swine Production Sites for Eighteen Months Following Disease Outbreak". This chapter presents data collected on three midwestern U.S. swine operations over a period of a year and a half to assess the effectiveness of industry recommended biosecurity practice on PED virus persistence in commercial swine farm settings. This information is intended to assess biosecurity practices and provide

information to guide swine producers and swine health specialists in selecting appropriate and effective practices to prevent and eliminate the virus in a commercial swine farm setting.

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CHAPTER II.
ALKALINE STABILIZATION OF MANURE SLURRY INACTIVATES PORCINE EPIDEMIC DIARRHEA VIRUS

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ABSTRACT

Hydrated lime manure treatment was evaluated to determine porcine epidemic diarrhea virus (PEDv) susceptibility to alkaline stabilization. At pH 10, PEDv decreased (quantitative polymerase chain reaction) and lost infectivity (swine bioassay). Although ammonium decreased above pH 9 (up to 25%), alkaline stabilization managed to control potential infection from manure sources.

Keywords. Swine, manure, porcine epidemic diarrhea virus, pH, hydrated lime

INTRODUCTION

The emergence of the porcine epidemic diarrhea virus (PEDv) in the United States in 2013 resulted in billions of dollars in annual losses in the US swine industry (Stevensen, 2013; Chen, 2014). Infection with PEDv causes severe diarrhea and vomiting in swine, spreads rapidly through ingestion of infected manure, and in naïve herds produces nearly 100% mortality in piglets less than one week old. Although the virus persists in feces for several days and may transport several miles from infected production sites as bioaerosol (Alonso, 2014; Chae, 2000), recent research indicates that management strategies can limit the virus' spread between production sites on transportation equipment (Thomas, 2015). However, concerns about virus persistence in various types of manure storage (i.e., deep pit, lagoon, or slurry tank) remain a major barrier to proper manure management.

Because swine manure slurry is a valuable source of nitrogen and phosphorus, manure typically is utilized in agricultural fields for crop production. Proper manure handling and application practices are necessary to control the risk of pathogen re-infection at affected production sites, or infecting new sites through virus-contaminated manure handling equipment. A variety of treatment options have been proposed and evaluated for their capacity to inactivate viruses in swine manure slurry (Turner, 1997). Hydrated lime [$Ca(OH)_2$] has been demonstrated to inactivate porcine enterovirus types 2 and 3 (Derbyshire, 1979), and alkaline stabilization is an approved treatment for septage prior to land application when a pH of 12 is maintained for at least 30 minutes (EPA, 2000). However, increasing manure slurry pH may decrease its value as a fertilizer, since ammonia losses through volatilization would be enhanced. It was

hypothesized that alkaline stabilization of manure would decrease infectious PEDv in swine production and in manure handling systems. Laboratory studies were conducted to assess the abundance and survival of PEDv in stored swine manure slurry treated with hydrated lime and to quantify potential ammonia volatilization losses during hydrated lime treatment.

MATERIALS AND METHODS

The experimental protocol was approved by the University of Nebraska-Lincoln Institutional Animal Care and Use Committee prior to the initiation of any research activity.

Manure for the first pH incubation study (conducted in 2015) was collected from swine that had been experimentally infected with PEDv strain CO/13 at the Life Sciences Annex at the University of Nebraska-Lincoln School of Veterinary Medicine and Biomedical Sciences (UNL VMBS). Manure for the second study was collected in 2016 at a commercial breed-to-wean operation in south central Nebraska. At the commercial location, freshly excreted swine manure solids were collected into sterile sample containers from the floor surfaces in four separate farrowing room sites showing clinical signs of suspected porcine epidemic diarrhea, and transported on ice to the University of Nebraska-Lincoln. Prior to use in incubation studies, manure samples were confirmed as PEDv-positive using a reverse transcriptase polymerase chain reaction protocol (RT-qPCR). The quantification cycle (Cq) value for these manure sources was 23, equivalent to approximately 10^5 virus genomes per PCR reaction. The first alkaline stabilization

incubation had triplicate manure slurries consisting of fresh manure (UNL VMBS) and deionized water (final composition: 18.5% solids content, 38.4% “volatile” by combustion loss at 550°C). The three slurries were mixed and sampled prior to any treatment (time=0 hours, no hydrated lime added). Each slurry was then distributed (250 mL) into two glass beakers (six total). Each pair received 1.5 g and 2.5 g of hydrated lime per L to achieve a final pH of 10 or 12, respectively. Aliquots (10 mL) were collected from each beaker at 1 and 12 h following hydrated lime addition, immediately neutralized with 10 mM HCl, and frozen at -80°C for subsequent analysis.

In the second alkaline stabilization incubation, manure samples were collected at four replicate sites at the commercial swine operation. To better mimic the typical consistency of stored manure slurry, each manure sample was mixed in equal portion with deionized water (1 kg manure: 1 L H_2O) prior to treatment (final composition: 21.6% total solids, 80.2% “volatile” by combustion loss). Each 250 mL replicate of slurry received stepwise (0.25 g) additions of hydrated lime with continuous stirring to gradually increase manure slurry pH to 12. After each addition of hydrated lime, pH was determined (FiveEasy Plus; Metter-Toledo AG, 8603 Scherzenbach, Switzerland) and duplicate 2 mL samples of each manure slurry were collected, immediately neutralized (10 mM HCl), and stored at -80°C for subsequent PEDv RNA copy enumeration and infectivity in a pig bioassay.

A PCR approach was used to quantify PEDv genomes in manure samples. The RNA in each manure slurry sample was extracted using TRIzol reagent following manufacturer’s suggested protocol for biological liquids and hard to lyse samples (Life

Technologies, Carlsbad, California). Bead mill homogenization using 0.1 mm glass beads in an Omni Bead Ruptor (Omni International, Kennesaw, Georgia) at 4.5 m s^{-1} for 45 s was included in the protocol to aid in cell lysis. An RT-PCR product was generated from RNA extracted from reference PEDv (CO/13) using primers and conditions as previously described. Run-off transcripts were generated from the T7 promoter on the PEDv forward primer using the MEGAshortscript T7 kit (Invitrogen, Carlsbad, California). Transcripts were quantified by RiboGreen fluorometry (Turner BioSystems, Sunnydale, California), and then 10-fold serial dilutions of the transcripts were prepared at concentrations ranging from $1x10^1$ to $1x10^6$ copies of PEDV (as RNA targets) per mL of subsequent RT-qPCR. Quantification of PEDv genomes in the purified manure slurry RNA extracts was accomplished using and Applied Biosystems StepOnePlus thermal cycler (ThermoFisher Scientific, Waltham, Massachusetts), primers, probes, and amplification conditions as previously described, with the exception that internal PCR probe contained both 3' Iowa Black fluorescence quencher (Integrated DNA Technologies, Coralville, Iowa) located nine bases from the 5' end. Briefly, one step RT-qPCR was carried out in a 20 mL reaction containing 1 mL of RNA extract or RNA standard, 0.1 µL of both PEDv forward and reverse primer, 0.25 µL of PEDv internal PCR probe, 12.5 µL of QIAGEN QuantiTech Probe reverse transcriptase mix and 5.8 µL of water. Thermal cycler conditions: initial reverse transcription at 50 °C for 30 min, followed by initial denaturation at 94 °C for 15 min 40 cycles of denaturation at 94 °C for 15 s, annealing at 60°C for 1 minute, and extension at 72 °C for 30 s. All RT-qPCR runs had reported efficiencies > 80% and R > 0.997.

Two swine bioassays were conducted with the alkaline stabilized and non-stabilized PEDV-infected manure slurry samples in order to relate RT-qPCR results with disease infectivity. For the first study, 15 pigs (approximately 21 days old) were sourced from a high-health facility whose dams tested negative for PEDv antibodies and virus by PCR. Pigs were each randomly assigned to individual housing in one of three BSL-2 animal rooms at the University of Nebraska-Lincoln Life Sciences Annex, grouped as follows, and allowed to acclimate for three days: control (three pigs), pH 10 manure (six pigs), and pH 12 manure (six pigs). Each pig was then administered a 10 mL oral gavage of diluted manure slurry from the first alkaline stabilization incubation (1 part manure slurry: 9 parts sterile buffer): three pigs in the control room each received one of the three un-limed slurry samples; six pigs in the pH 10 room received one of the six limed (pH 10) slurry samples (three limed for 1h and three limed for 12h); and six pigs in the pH 12 room received one of the six limed (pH 12) slurry samples (three limed for 1h and three limed for 12h). Pigs were monitored for fecal shedding for PEDV for 4 days until control animals began to demonstrate clinical signs of PEDV infection, at which time all pigs were humanly euthanized. Fecal swabs and ileum, jejunum, and mesenteric lymph node tissue samples were collected from each animal and fixed in formalin. Fecal and tissue samples were analyzed for the presence of PEDV by immunohistochemistry (IHC) and RT-qPCR (Cq only).

The second bioassay used a similar design, including pig source, history, age, housing, inoculation, and processing to assess PEDv infectivity in the various samples from the second incubation study. Manure slurry samples were selected from three of the manure slurries at points where pH was closest to 7, 8, 9, 10, or 11. Fifteen pigs were

housed in three rooms (five per room) with one animal in each receiving one of the five pH-diluted manure slurries by oral gavage. Pigs were monitored for signs of disease for a week prior to euthanasia. Fecal swabs and tissue samples were collected and tested for the presence of PEDv.

A third manure slurry incubation was conducted to assess changes in nitrogen content, since alkaline stabilization may enhance ammonia volatilization from treated manure during simulated storage in a deep pit or transport in a manure tank wagon. Fresh manure samples were collected from three replicate locations at the commercial site, diluted to create manure slurry (1 kg manure: 1 L H_2O), and distributed into ten 250 mL bottles. Five bottles were each randomly assigned to one of two treatments: simulated storage in a manure pit (PIT) or simulated transport in a manure tank wagon (TANK), and hydrated lime additions were randomly applied to each manure slurry (n=3) within PIT or TANK blocks to achieve one of five pH endpoints: 8.0, 8.5, 9.0, 9.5, and 10.0. To mimic deep pit storage at a swine production site, PIT bottles were left uncapped while the trail was conducted. To mimic storage in a tank, the TANK bottles were tightly capped during the experiment. PIT samples (1 mL) were sampled initially and 24h following hydrated lime application (simulated overnight treatment). Samples (1 mL) from the TANK block were collected initially and 2h following hydrating-lime application (simulated short-term treatment). All samples acidified with 20 µL of 10% sulfuric acid to adjust the pH to < 3 and refrigerated until analysis for ammonium using the Phenate method.

ANOVA (SAS version 9.2, SAS Institute, Cary, North Carolina) was used to analyze log-transformed PEDv abundance in the first two manure alkaline stabilization

incubation and to analyze ammonium percent-age increase or decrease ($1-C_{final} / C_{initial}$) in the third manure slurry incubation. For the first incubation, five treatments were compared (control, pH 10 for 1h, pH 10 for 12h, pH 12 for 1h, and pH 12 for 12h) with treatment as the main effect comparing log PEDv. In the second incubation, ANOVA was conducted using target pH (7, 8, 9, 10, and 11) as the main effect, comparing log PEDV. For the third incubation, manure storage and manure pH were the main effects, comparing the ammonium percentage increase or decrease.

RESULTS

In the first manure slurry incubation, RT-qPCR analysis of samples detected PEDV RNA sequences in all treatments (hydrated lime or untreated) except at pH 12 after a 12h incubation. A clear trend for lower PEDv abundance with hydrated-lime addition (pH 10 versus 12) and with increased hydrated-lime exposure time (1 versus 12h) was observed. In the swine bioassay, pigs receiving limed manure treatments (pH 10 or 12 incubated for 1 or 12h) via oral gavage displayed none of the clinical signs of PEDv infection (e.g., diarrhea, dehydration, or vomiting) and did not shed PEDv in the feces (as determined by PCR). All control pigs (n = 3) receiving un-limed manure displayed clinical signs of disease, tested positive for PEDv infection via IHC, and shed PEDv in the feces (i.e., had a low Cq by RT-PCR).

In the second manure slurry incubation, stepwise addition of hydrated lime gradually increased the pH of the manure slurries. Quantitative PCR analysis of samples revealed a rapid decline in the number of PEDv copies above pH 10, but no change in the abundance of PEDv targets below pH 10 (10^9 PEDv targets per gram of

manure slurry). Swine bioassay results on a subset of those samples were consistent with RT-qPCR results: IHC and RT-PCR detections of PEDv were observed only in pigs exposed to manure slurry when the pH was less than 10.

For the final manure slurry incubation, initial ammonium concentrations varied considerably between the three replicate locations at the commercial site (0.90 ± 0.06, 1.89 ± 0.17, and 2.49 ±0.24 g NH_4+ per L). Prior to statistical analysis, final concentrations were normalized to initial concentration for each manure slurry container yielding a percentage increase or decrease ($1-C_{final} / C_{initial}$). Of the two main effects (manure storage and manure pH) and their interaction term, only manure pH proved to be significant ($p < 0.05$). During manure storage, the average ammonium content increased by 6.6%. The largest differences in manure slurry ammonium content were found between low pH (8, 8.5, and 9) and high pH (9.5 and 10) manure samples ($P < .01$). Ammonium in the low pH group increased an average of 15.7% ± 3.9% relative to initial concentration. In comparison, ammonium in the high pH group decreased by 7.1% ± 3.5%.

DISCUSSION

Alkaline stabilization was achieved in manure initially containing 10^9 PEDv targets per gram of slurry at the above pH 10 (i.e., infectivity was eliminated). Comparing the pig bioassay results with RT-qPCR results, an interesting relationship emerges. Although reduced by more than 100-fold above pH 10, PEDv target genomes could still be detected at 10^5 to 10^7 per gram of slurry. Alkaline stabilization impeded virus infection but did not destroy all past evidence of the presence of the virus (i.e., some

remnant RNA persisted for a short period of time). Alkaline pH likely altered virus envelope integrity, which released PEDv RNA into the manure slurry where RNA was quickly hydrolyzed. Not all animals exposed to PEDv-contaminated manure slurry below pH 10 became infected with PEDv, particularly animals in the second study. It was noted that the pigs in the second manure slurry trial were slightly larger than those in the first trial, and this may account for the lower incidence of disease in pigs exposed to manure slurry below pH 10.

Ammonium increased by a substantial fraction in the third manure slurry incubation, particularly in the lower pH treatments (8.0, 8.5, and 9.0). Decomposition processes in the lower pH fresh manure (urea, hydrolysis and organic matter decomposition) likely accounted for the increase, while higher pH may have inhibited these decomposition processes. Additionally, in the manure samples of higher pH (9.5 and 10.0), the dissociation of ammonium to ammonia (pKa 9.25) would shift ammonium to ammonia, which is more easily lost via volatilization. Although simulated storage (PIT versus TANK) showed no difference, slurry pH had a dramatic effect on ammonium concentrations (up to 25% difference between low and high pH) after a short incubation period.

While “lime” is a term broadly used to describe calcium-containing inorganic materials, “quicklime” applies to the chemical compound calcium oxide (CaO), which is unstable and highly reactive to moisture. To reduce the reactivity of quicklime and make it more stable, water is often added to quicklime to convert all oxides of calcium and magnesium to hydroxides. The resulting compound, calcium hydroxide [$Ca(OH)_2$], is sold under a number of different names, including hydrated lime is commonly used

during the cleanup phase after a disease outbreak in livestock production systems. Despite being more stable than quicklime, hydrated lime is still caustic and quick to react with water, so it must be handled with care. Precautions should be taken to protect against inhalation or contact with skin and eyes. In addition to keeping arms and legs covered, gloves, safety goggles and a dust mask should be worn during handling.

To accomplish alkaline treatment of manure slurry at a swine production site using hydrated lime, a doing rate of approximately 23 kg (50 lb) of hydrated lime per 3800 L (1000 gal) of manure is recommended. At a cost of around $40 for a 50 lb bag, treatment of a full 5000-gal slurry tank spreader can be accomplished for approximately $200 (USD). Addition of the hydrated lime to the tank wagon prior to it being filled with slurry is recommended to facilitate mixing. Addition of lime while a slurry tank is being filled with manure is not recommended, since the concentration of ammonia gas emanating from the tank wagon fill port could be high enough to cause asphyxiation for the person adding lime at the tank port. While the research presented included an analysis of ammonia loss during treatment of stored slurry, the addition of hydrated lime to deep pit manure storages is not recommended. The substantial amount of ammonia gas generated during alkalization of an entire manure pit containing several thousand gallons of manure slurry may pose a significant health risk to workers and animals in and near the production facility.

IMPLICATIONS

1. Alkaline stabilization through hydrated lime addition to achieve a threshold pH 10 for 1h is sufficient to deactivate the porcine epidemic diarrhea virus in manure

slurry on the basis of bioassay outcomes. Although PEDV was still detectable above pH 10 by RT-qPCR (10^5 to 10^7 genomes per gram manure slurry), no disease risk was observed.

2. Important questions remain regarding the minimum treatment time needed for alkaline stabilization and whether longer treatment periods at < 10 pH are as efficacious as briefer, higher pH treatment.
3. Raising manure slurry pH above 9.25 will likely enhance ammonia losses by volatilization and decrease fertilizer nitrogen value. Alkaline stabilization of manure slurry could present a risk for ammonia asphyxiation during manure treatment and pumping if proper air flow is inadequate.

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APPENDIX

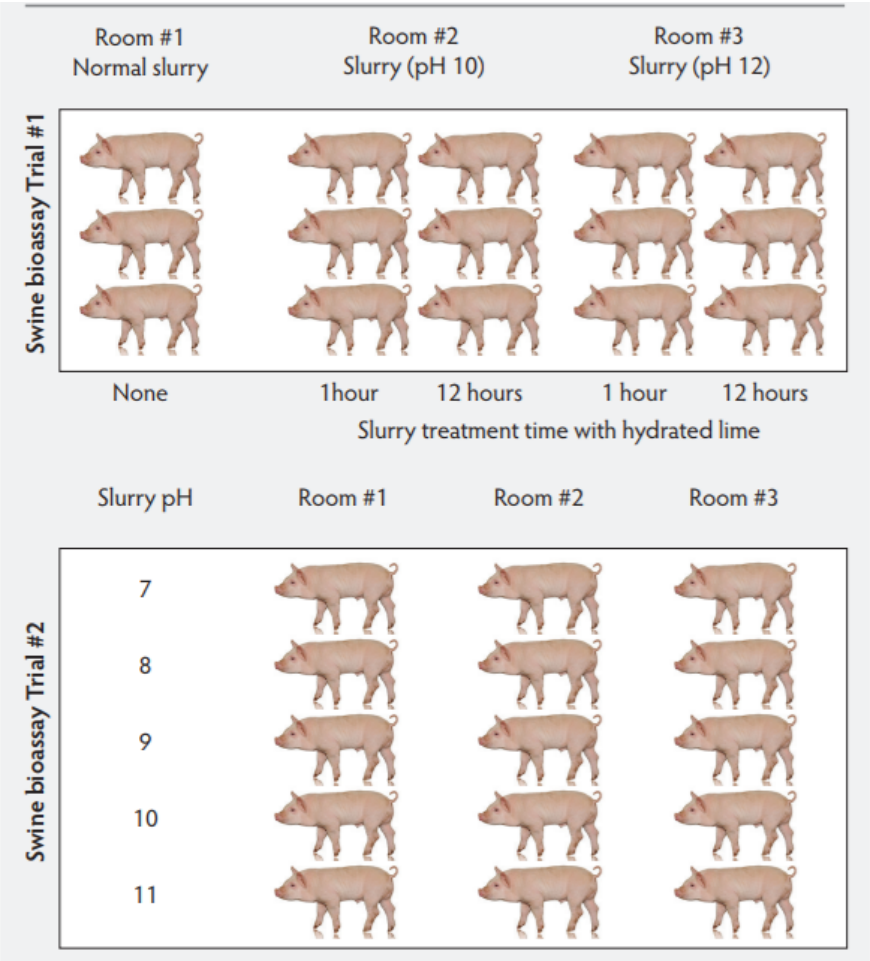

Figure 2.1. For the swine bioassays, pigs were randomly assigned to multiple rooms and housed in individual crates. The pigs were administered diluted, PEDV-positive manure slurry (untreated and treated with hydrated lime) and monitored for several days for signs of disease (including PEDv-specific PCR of fecal swabs). After euthanasia, additional gastrointestinal tissue samples were collected for PCR and immunohistochemistry test. PCR = polymerase chain reaction.

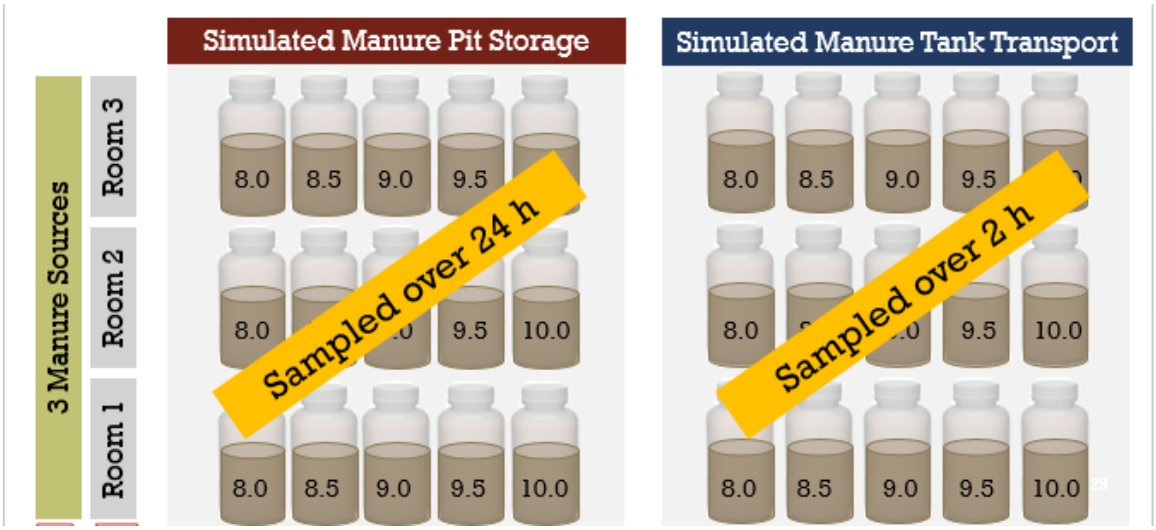

Figure 2.2. Reference of how the ammonia volatilization study was set up. Each 250 mL replicate of slurry received stepwise (0.25 g) additions of hydrated lime with continuous stirring to gradually increase manure slurry pH to 12. After each addition of hydrated lime, pH was determined

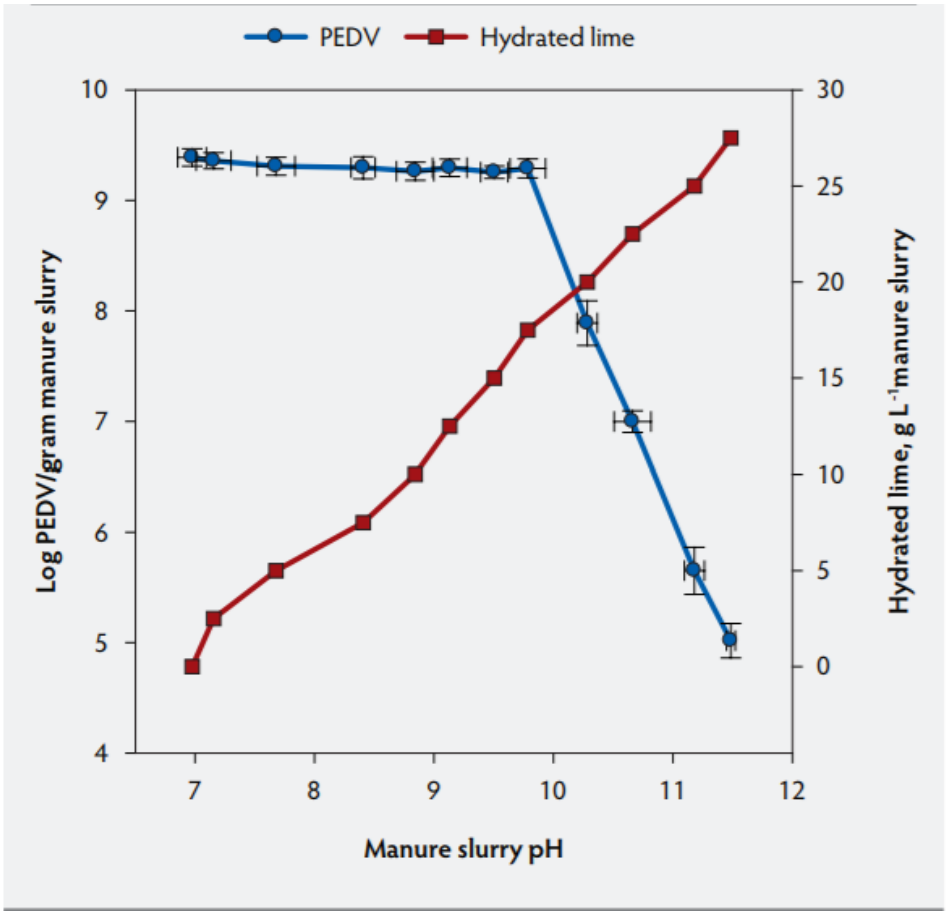

Figure 2.3. Effect of increasing hydrated lime amendment during alkaline stabilization on swine manure slurry pH and PEDv genome abundance assessed using reverse transcriptase quantitative PCR.

Error bars = 1 SE.

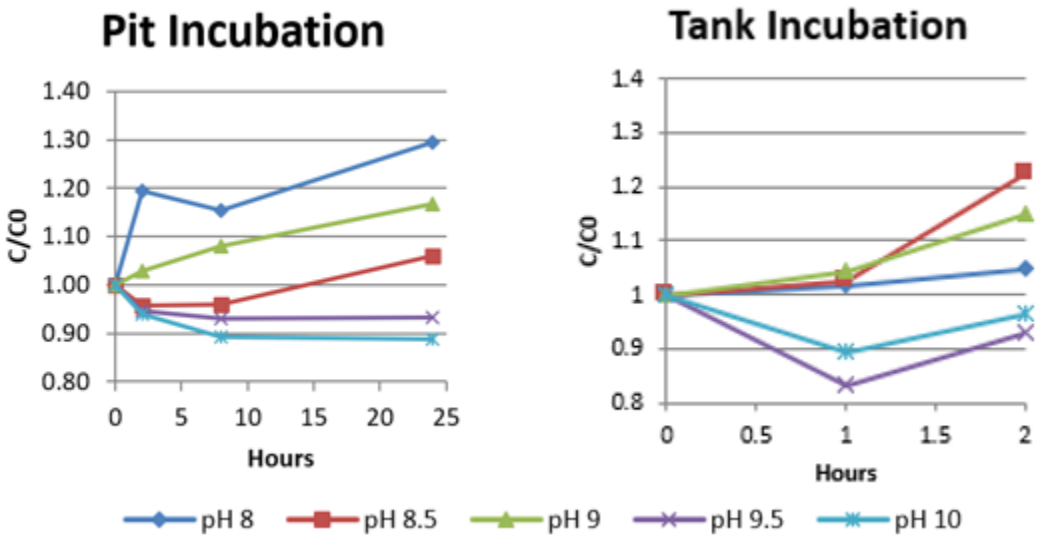

Figure 2.4. Final concentrations were normalized to initial concentration for each manure slurry container yielding a percentage increase or decrease ($1-C_{final} / C_{initial}$).

Table 2.1. Effect of hydrated lime manure treatment exposure (1 or 12 hours) at pH 10 or 12 on porcine epidemic diarrhea virus (PEDv) abundance and potential to cause disease

Treatment	Time (hours)	Slurry PEDV* log/gram	Pig bioassay†	
			IHC (%)	Rectal swab (Cq)
None	0	9.16 ± 0.02^{a}	3/3 (100)	20.4, 23.4, 20.7
pH 10	1	7.00 ± 0.36^{b}	0/3 (0)	All > 40
	12	5.38 ± 0.33^{c}	0/3 (0)	All > 40
pH 12	1	4.5 ± 0.01^{c}	0/3 (0)	All > 40
	12	BD	0/3 (0)	All > 40

* Log RNA targets/g wet manure slurry determined by reverse transcriptase quantitative PCR ± 1 SE; (Cq > 40 ≈ 10^4 per gram in manure slurry or 25 copies/PCR reaction).
† IHC was performed as previously described.[9] Cq = quantification cycle for rectal swab at necropsy. Cq values ≤ 35 were considered positive and > 35 were considered negative.
[abc] Values within a column with different superscripts are significantly different ($P < .05$; ANOVA).
BD = below detection; IHC = immunohistochemistry.

Table 2.2. Effect of incremental hydrated lime addition on manure slurry pH and PEDv abundance and potential to cause disease.

Hydrated lime (g/L)	Average pH	Slurry PEDV* log/g	Pig bioassay†	
			IHC (%)	Rectal swab (Cq)
0.0	6.92	9.36^{a}	1/3 (33)	> 40; 25.93; > 40
6.7	8.14	9.26^{a}	1/3 (33)	25.49; > 40; > 40
12.5	9.13	9.23^{a}	2/3 (67)	23.25; >40; 23.31
18.3	9.96	8.66^{a}	0/3 (0)	All > 40
24.2	11.07	6.15^{b}	0/3 (0)	All > 40

* Log RNA targets/g wet manure slurry determined by reverse transcriptase quantitative PCR.
† IHC performed as previously described.[9] Cq = quantification cycle for rectal swab at necropsy. Cq values ≤ 35 were considered positive and > 35 were considered negative.
[ab] Values within a column with different superscripts are significantly different ($P < .05$, ANOVA).
PEDV = porcine epidemic diarrhea virus; IHC = immunohistochemistry.

CHAPTER III.

PORCINE EPIDEMIC DIARRHEA VIRUS (PEDV) ASSESSMENT ON THREE SWINE PRODUCTION SITES FOR EIGHTEEN MONTHS FOLLOWING DISEASE

E. E. Boyles, A. M. Schmidt, D. N. Miller, and D. Loy

ABSTRACT

A field survey was conducted at three midwestern swine production facilities to assess the effectiveness of industry recommended practices to decontaminate after an incidence of porcine epidemic diarrhea virus (PEDv) at the facilities. Samples were collected inside and outside each production site before facility decontamination, after decontamination, and for 18 months after the outbreak from production area surfaces (alleyways, pen floors, and pen rails); office and breakroom surfaces; and vehicle and farm equipment surfaces. Animal fluid samples (rectal swabs and oral fluids), biological samples (mortality compost and leachate, stored manure, mortality holding areas, and others) and soils receiving manure application were also collected. At the initial time of the outbreak in each facility, PEDv was detected (qPCR) in 95% of the tested surface samples collected from pig production areas, in 60% of the break room and office area samples, and 0% of the samples collected outside the production facility. After facility decontamination, rectal swab samples collected at four weeks post-decontamination from

farrowing sows at NE-02 and IL-01 (breed-to-wean sow farms) were positive for PEDv. No positive results were reported for NE-01 (grow-finish pig farm) following decontamination. No PEDv was detected from any samples after four weeks post-decontamination, including soil samples. We conclude that current decontamination practices help control PEDv outbreaks and limit the potential for reinfection from sources initially contaminated during a previous outbreak.

Keywords. Swine, porcine epidemic diarrhea virus, PEDv, manure, soil, disinfection

INTRODUCTION

The porcine epidemic diarrhea virus (PEDv) first emerged in the United States (U.S.) in 2013 and spread rapidly throughout the country, resulting in billions of dollars in annual losses to the U.S. swine industry (Stevensen et al., 2013; Chen et al., 2014). Once infected with PEDv, pigs experience severe diarrhea and vomiting, producing infectious material that transmits the virus when infected manure is ingested. The virus yields nearly a 100% mortality rate in piglets less than three weeks old; weaned piglets and sows experience losses in productivity. While lactogenic immunity can develop among sows following initial infection with PEDv and be passed on to piglets, resistance to one strain of the virus does not guarantee reduced losses upon exposure to a genetically altered strain of the virus.

Research indicates that the virus persists in feces for days to months and may transport several miles from infected production sites as bioaerosol (Alonso et al., 2014; Tun et al., 2016). While alkaline stabilization of infected manure via hydrated lime has been shown to eliminate infectivity at pH 10 (Stevens et al., 2017) and other confirmed management strategies help limit virus transmission via transportation equipment (Thomas et al., 2015), little data is available to describe on-farm viral persistence within a facility and in soil treated with manure from infected pigs. Swine manure is typically applied to fallow agricultural soils as a nutrient source and soil amendment. Because manure slurry is a valuable source of nutrients for crop production, this reuse serves as an important method to recycle nutrients in agroecosystems. However, application of

infectious manure to soil could inadvertently create a reservoir for future PEDv outbreaks if the virus persists in soil after manure application.

Implementing internal and external biosecurity practices to prevent outbreaks and control transmission of PEDv has been promoted by the U.S. pork industry with substantial funding provided by the National Pork Board to investigate potential disinfection and mitigation practices. Evidence-based techniques to decontaminate a facility following infection with the PED virus include power washing to remove organic residue, disinfection of surfaces and fomites following organic material removal, and white-washing of building and equipment surfaces (Kihlstrom et al., 2001; Gallien et al., 2018). Additional practices on sow farms include depopulation of infected piglets, induced abortions in pregnant sows and feedback of contaminated material to unexposed breeding stock to generate an immune response (Goede et al., 2015; Clement et al., 2016). On grow-finish farms, it is uncommon to depopulate since mortality is much lower in older animals and animals are not retained over multiple production cycles as they are in breeding facilities. While effectiveness of these and other practices have been demonstrated at various critical control points (CCPs) within swine production systems, a substantial gap exists in understanding the effectiveness of integrating multiple industry recommended decontamination and disease prevention practices to remediate a farm prior to repopulation to prevent subsequent outbreaks. Therefore, the goal of this study was to assess the effectiveness of industry recommended decontamination and disease prevention practices applied collectively within commercial swine farm settings after an incidence of porcine epidemic diarrhea virus (PEDv). A laboratory study to confirm the

efficiency of the soil RNA extraction methods used for soil samples evaluated during this study is also presented.

MATERIALS AND METHODS

Site Description

The production facilities surveyed for this study were identified with the help of swine veterinarians, state swine associations, and other swine industry professionals throughout midwestern U.S. states upon confirmation of PEDV infection. Two study sites were located in Nebraska and one in Illinois. Characteristics of each farm are summarized in Table 3.1. Briefly, the Illinois site (IL-01) was a breed-to-wean sow farm with a designed capacity of approximately 4,000 sows that was confirmed PEDv-positive in March of 2016. The Nebraska sites were a grow-finish farm (NE-01) and a breed-to-wean sow farm (NE-02) with design capacities of about 4,000 grow-finish pigs and about 2,000 sows, respectively, both confirmed PEDv-positive in April 2016. None of the farms surveyed in this study had reported infection with the PED virus prior to the onset of the study.

At IL-01, farrowing sows were confined in individual crates in 27 rooms housing up to 24 sows and litters per room. Six farrowing rooms (numbered 5, 7, 8, 12, 17 and 24) were randomly selected for surveillance during the study.

NE-01 housed approximately 20,000 grow-finish pigs within 10 barns separated into 20 individual production areas via an enclosed hallway connecting all rooms. Each production area was comprised of two rows of up to 10 pens each with up to 50 animals

group-housed per pen. Rows of pens were separated along the center of each production area by a concrete alleyway. Pens were constructed with concrete flooring and walls with integrated metal gates for pig and worker movement. The majority of manure was deposited by the animals in concrete alleys along the outside walls of each building that were flushed with fresh water periodically throughout each day. Flushed manure was allowed to gravity drain to an anaerobic lagoon for storage and treatment. Six production areas (numbered 1, 5, 8, 13, 14, and 17) were randomly selected for surveillance during the study.

NE-02 housed approximately 2,000 sows with farrowing capacity for 432 sows in 18 rooms equipped with individual farrowing crates. Manure collected beneath slatted concrete or metal flooring from the gestation and farrowing rooms, respectively, was gravity drained to an anaerobic lagoon for storage and treatment. Six farrowing rooms (numbered 3, 4, 5, 5b, 6, and 18) were randomly selected for surveillance during the study with up to 24 sows and litters in each.

Sample Collection

Initial sampling at all sites was performed within two weeks of PEDV confirmation and additional sampling was performed during decontamination activities and at 6, 12 and 18 months post-decontamination (Figure 3.1). Additionally, rectal swabs were collected by farm personnel at IL-01 and NE-02 from farrowing sows at 2, 4 and 6 weeks after decontamination and shipped to the UNL campus in coolers with ice packs for analysis.

The types and frequencies of samples collected inside and outside production buildings at each site throughout the project are summarized in Table 3.2. Briefly, surface swabs were collected from six swine production rooms, the office, and employee breakroom at each facility. In each production room, swabs were collected from three surfaces – alleyway, pen floor and pen rail – at four random locations per room. All swab samples were collected using a sterile gauze pad (Covidien Curity^{TM}, Thermo Fisher Scientific, Inc., Bartlesville, OK) wetted with 10 mL of 0.01 M phosphate buffered saline (PBS) with pH 7.4 (Sigma-Aldrich, St. Louis, MO). Pen floor and alleyway samples were collected by wiping an area of approximately 0.09 m^2 (1 ft^2) as shown in Figure 3.2. For the pen rails, approximately one linear foot of rail was wiped with the wetted gauze pad.

In offices, the desk surface, computer keyboard and computer mouse were swabbed, as were the floor, tabletop(s) and refrigerator door handle in the employee break room. Swab samples were individually bagged in sterile Whirl-pak bags (Nasco, Fort Atkinson, WI) for storage on ice and transportation to the UNL campus in Lincoln, Nebraska. Negative control samples were collected inside each monitored production area, the office and breakroom by wetting a gauze pad with PBS and inserting it into a Whirl-pak bag.

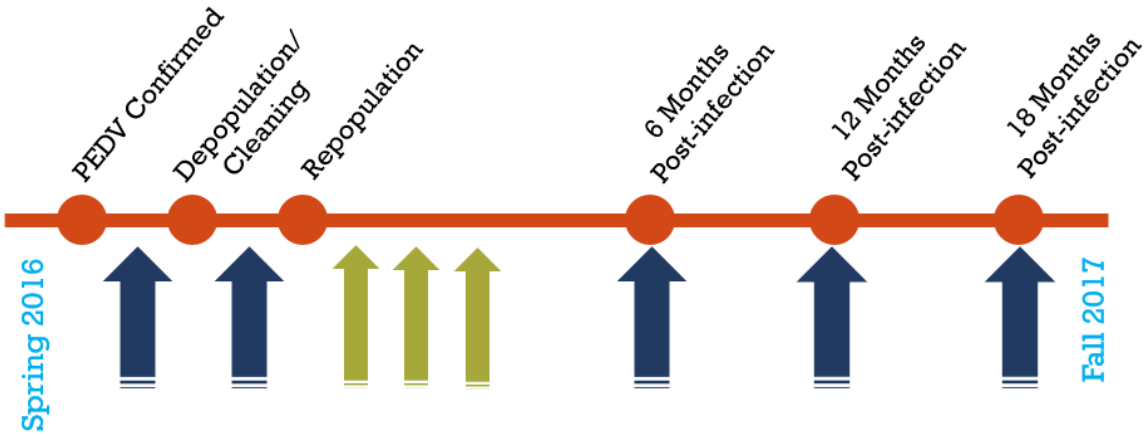

Figure 3.1. Timeline of sampling at production sites; blue arrows represent site visits for collection of samples from defined CCPs; green arrows represent rectal swab samples collected and submitted to UNL by farm personnel

Figure 3.2. Surface swab sample collections in grow-finish pig production houses (L) and breed-to-wean farm farrowing crate floor (R)

Bodily fluids from animals in each of the six monitored production areas at each site were also collected. At IL-01 and NE-02 (breed-to-wean sites), rectal swabs were collected from four sows, randomly selected but representing different parities, in each of the six monitored farrowing rooms. At NE-01 (grow-finish site), oral fluids were collected from four pens within each monitored production room using TEGO™ Swine Oral Fluids Kits (ITL BioMedical Animal, Reston, VA). Briefly, the provided rope was securely tied to the divider or gate within each pen and left in place for at least 30 min to allow pigs to chew on the rope before it was retrieved (Figure 3.3). Upon retrieval, collected fluid was wrung from each rope into a sterile plastic bag and then transferred to a sterile 50 mL conical tube for transportation back to the UNL campus.

Figure 3.3. Pigs chewing on TEGO rope

Outside the production buildings, up to three employee vehicles and all tractors/loaders were also evaluated for viral presence. For employee vehicles, surface swabs were collected from the interior floorboard and one wheel while tractors/loaders were sampled on the operator floorboard, one wheel and the bucket, using the same procedure previously described for surface sample swab collection. Fan housings were also sampled at IL-01 on the initial visit using the described surface sampling procedure.

Soil was collected from the top 5 cm of the field surface at three locations within at least one field at each site that received manure application following the initial confirmation of PEDV. Lagoon effluent and pit slurry samples (500 mL) were obtained using a long-handled pole with a cup affixed to the end where manure storages were easily and safely accessible and transferred to sterile Nalgene bottles. Grab samples of mortality compost pile material and leachate from the piles were collected at IL-01, while a surface swab of the inside surface of a bin that held mortalities intended for rendering was collected at NE-02. At NE-01, surface swabs were collected from the mortality holding area or "dead chute". All samples were transported on ice in coolers to the UNL campus and processed within 24 hours.

Sample Processing and Analysis

A PCR approach was used to detect PEDv in environmental swab samples, animal fluid samples, manure slurry/rectal swab samples, carcass compost solids and liquids, and soil samples collected at the three production sites. For every site, individual samples collected from the alleyway, pen floor and pen rail of each room were pooled by room when tested for PEDv. Samples collected from the break room and office area were

all processed individually. Samples collected from employee vehicles and tractors were pooled by individual vehicle for analysis. The RNA in each composite and break room sample was extracted using TRIzol reagent following manufacturer's suggested protocol for biological liquids and hard-to-lyse samples (Life Technologies, Carlsbad, California). Positive PEDv control for qPCR was generated using RNA extracted from reference PEDv (CO/13) using primers and conditions as described by Stevens et al. (2018). Each RT-PCR reaction mix consisted of 5 μL 5× reaction buffer, 1 μL nucleotide triphosphates, 2 μL of 25 μM-mL^{-1} $MgCl_2$, 5 μL nuclease-free water, 2 μL PEDv forward and reverse primers (10 μM each), 1 μL PEDv HEX-labeled probe (5 μM), 1 μL One Step RT-PCR Enzyme mix, and 8 μL extracted RNA. Each RT-PCR sample was analyzed on a Cepheid Smart Cycler Detection System (Cepheid, Sunnyvale, CA, U.S.) under the following conditions: 50°C for 30 min; 95°C for 15 min; and 45 cycles of 94°C for 30 s, 60°C for 60 s with optics on, and 72°C for 30 s. Validated PCR positive controls consisting of PEDv RNA and negative extraction controls were included in each run. Samples were considered positive if the mean fluorescence exceeded 30 fluorescent units prior to 40 cycles. Negative and positive PCR controls were properly classified.

For soil samples, a similar RNA extraction and qPCR approach was used but with one key exception: bead mill homogenization. For soil samples, bead mill homogenization using 0.1 mm glass beads in an Omni Bead Ruptor (Omni International, Kennesaw, Georgia) at 4.5 m s^{-1} for 45 seconds was included in the protocol to aid in cell lysis. An Applied Biosystems StepOnePlus thermal cycler (ThermoFisher Scientific, Waltham, Massachusetts) was used to amplify PEDv from soil samples; primers, probes,

and amplification conditions are as previously described (Stevens et al., 2018), with the exception that the internal PCR probe contained both 3' Iowa Black fluorescence quencher (Integrated DNA Technologies, Coralville, Iowa) located nine bases from the 5' end. Briefly, one step RT-qPCR was carried out in a 20 µL reaction containing 1 µL of RNA extract or RNA standard, 0.1 µL of both PEDv forward and reverse primer, 0.25 µL of PEDv internal PCR probe, 12.5 µL of QIAGEN QuantiTech Probe reverse transcriptase mix and 5.8 µL of water. Thermal cycler conditions: initial reverse transcription at 50°C for 30 min, followed by initial denaturation at 94°C for 15 min 40 cycles of denaturation at 94°C for 15 s, annealing at 60°C for 1 min, and extension at 72°C for 30 s. All RT-qPCR runs had reported efficiencies > 80% and R > 0.997.

To confirm that a negative PEDv PCR detection in soil was not due to poor viral RNA recovery from soil or the result of co-extraction of soil PCR inhibitors, a spiked soil experiment was conducted where 10-fold dilutions of PEDv cell culture was introduced into the soils collected at the three sites and then RNA was extracted and quantified. For each of the soils, 0.25 g of each soil was transferred into multiple extraction tubes. A stock culture of PEDv was propagated using Vero cells maintained in minimal essential media (MEM) and 10% fetal bovine serum and 100 µg mL^{-1}. For infection, two-day-old confluent monolayers of Vero cells in 150 cm2 flasks were washed two times with MEM containing 2 µg mL^{-1} L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin prior to inoculation. Monolayers were infected at approximately 0.01 multiplicity of infection (MOI) of PEDV (USA/Colorado/2013, GenBank accession no. KF272920) in MEM containing 2 µg mL^{-1} TPCK-treated trypsin, and incubated at 37°C until

maximum cytopathic effect (CPE) (48 to 96 h). Flasks were cycled through two brief freeze-thaw cycles and stored at −80°C until further processing. For purification, frozen flasks were thawed and the contents centrifuged at 2000 x g for 10 min. The media supernatant was collected, pooled, mixed, and divided into aliquots that were stored at −80°C until needed. The stock culture of PEDV was estimated to contain 1 x 10^6 TCID50 mL^{-1} based on a known qRT-PCR value of the culture material and known qRT-PCR values of log dilution of virus standard propagated similarly. The stock culture of PEDV was diluted in a 10-fold dilution series.

The stock culture was serially diluted to create culture concentrations of 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 and 10^0 PEDV genomes mL^{-1}. Each soil tube then received an addition of 600 µl of TRIzol (Life Technologies, Carlsbad, California), was mixed, and then 200 µl of one of the dilutions of PEDv stock was spiked into the RNA extraction mixture. In addition to unspiked control soils, the PEDv culture dilution series was also subjected to RNA extraction (200 µl volumes) and amplification conditions identical to the soil samples as described above.

RESULTS AND DISCUSSION

Prevalence of PEDv in Field Samples

Multiple samples collected from three swine production sites showed that the PED virus was present throughout the production buildings at each of the sites immediately after the PEDv outbreak and prior to decontamination. Prevalence of the

virus on surfaces and in biological samples outside the production buildings were lower, but not absent.

Results for the surface samples collected immediately following the PEDv outbreak at the Illinois farm (IL-01) are shown in Table 3.3. The alleyway, pen floor and pen rail were sampled in four random locations throughout each room and then combined into a single composite sample for analysis. Five of the six room composite samples (83%) were positive for PEDv, as determined by qPCR. Blood draws were performed during the initial visit to IL-01 on four sows randomly selected in each of the six farrowing rooms being monitored. Results from PCR analysis of blood samples taken from sows in each of the monitored rooms on the initial site visit at IL-01 are displayed in Table 3.4. Of the 23 sow blood samples collected, all but two (91%) were positive for PEDv.

In the employee break room at IL-01, surface swab samples were collected from the tabletop, floor and refrigerator door handle. All three samples were analyzed individually and all produced positive results for the PED virus via PCR. Because this was the first farm sampled by the project team, and the office area was not initially included in the sampling protocol, samples were not collected from the office at IL-01 on this initial visit. However, given the prevalence of the virus on surfaces in the breakroom, which was immediately adjacent to the office, it is quite likely that office area surfaces were also contaminated either directly from worker contact with animals or by transfer of infectious material between the breakroom and office.

Outside the production facility, all surface samples were negative for the virus including the samples representing employees' vehicles, farm loaders, and dust accumulated on the cowlings outside ventilation fans. Given published research that suggests the potential for airborne transmission of the PED virus on exhausted particulate matter (Alonso et al., 2014), it was surprising that no virus was detected in the samples collected from fan components at this site while an active disease outbreak was still being addressed.

Manure slurry was collected from two the deep pit storages containing manure from gestating sows and an effluent sample was collected from the anaerobic lagoon receiving flushed manure from the farrowing facilities. Despite the PEDv outbreak being active at the time of our visit, none of the manure samples produced positive results for the virus. This contradicts published research by Tun et al. (2016) and unpublished data from a research report by Tousignant (2016) that both suggest persistence of the virus in stored manure for days or weeks beyond the disease outbreak. However, these results are supported by published research from Hofman and (1989) demonstrating that PED virus persists for less than a day in virus media between pH 6 and 8, which should support viral longevity. While lagoon effluent falls within this narrow pH range, it is surprising that published studies have noted lengthy survival of viral RNA in manure storages as the conditions in stored manure do not seem to be ideal for the PED virus.

Mortalities at IL-01 were composted onsite in a structure having a monoslope roof and open front. Compost pile material was collected by grab sample from each of the bins of the compost structure – each at various stages of tissue decomposition – and

composited for analysis. Likewise, a sample was taken from a substantial volume of compost pile leachate that was pooled outside the structure. Both the compost material and the leachate tested negative for PEDv as determined by qPCR. Despite the compost process being in a relatively early stage for all bins within the structure, it is likely that viral RNA was destroyed within a matter of days or weeks following pile construction. Research investigating the survivability of the PED virus in carcass compost piles (Vitosh-Sillman et al., 2017) reported that no virus could be isolated from compost material following a 36-day primary composting cycle despite the presence of soft tissue in the material after this first heating cycle. While the compost piles at IL-01 had been operating for only a couple of weeks upon our visit to the site, it is likely that any virus-infected material in the pile was exposed to sufficient heat and unfavorable environmental conditions to inactivate the virus in a relatively short period of time.

Additional rectal swab samples were collected by farm personnel at 2 and 8 weeks post-decontamination from a random subset of sows occupying the rooms being monitored at the site. Results of PCR analysis on these samples are displayed in Table 3.5. While 30 samples were submitted for analysis, specimens were not labeled according to production room number; therefore, composites do not represent pooled samples from individual rooms. Rather, they represent six composites of five samples each taken from the 30-sample pool. Only one composite sample tested positive for PEDV (C_q = 40.0) at two weeks post-decontamination and no additional positive tests were produced at eight weeks post-decontamination.

Results for samples taken at the Nebraska finisher site (NE-01) are presented in Table 3.6. For the initial sample event, which followed the confirmed outbreak with PEDv but occurred prior to de-contamination activities, composite surface samples from all six of the monitored rooms were positive for the virus by RT-PCR. Oral fluid samples collected from four pens in each of the six monitored rooms were pooled by room and analyzed via RT-PCR. Three of the six oral fluid composite samples tested positive for the virus. While the oral fluids test results did not match the results from pooled surface samples, oral fluids were not necessarily collected from the same production pens from which surface swabs were collected. Therefore, the results cannot be directly compared. However, positive results in surface swabs and oral fluids indicate that infected biological material (i.e. manure, contaminated feed, etc.) was likely dispersed throughout the building on floor, pen wall, and/or alleyway surfaces, though not all pigs had ingested contaminated material such that the virus was present in their saliva.

In the NE-01 break room, surface swab samples collected from the tabletop, floor and refrigerator handle were analyzed individually; only the refrigerator door handle sample produced a positive result. Samples collected in the office area from the desk surface, computer keyboard and mouse, and the floor were also analyzed individually. While the desk surface did not test positive for PEDv, the computer keyboard and mouse did. One possible explanation for this is that table, floor and desktop surfaces are smooth and lack crevices and other small areas where the virus could persist while the surfaces of the computer keyboard and mouse are more complex. Swabs from these objects were

taken not just from the outermost surface, but also between keyboard keys and in the spaces between the mouse buttons.

All of the samples collected from the exterior of the production building, including employees' vehicles and facility loaders, were pooled by unit for analysis; all composite samples produced negative results for the virus on the initial visit and continued to test negative for the remaining 18-month assessment period.

At NE-02 (breed-to-wean farm) (Table 3.7), samples collected from three surfaces in four random locations within each of six farrowing rooms were pooled by room for analysis. Composites of each of the rooms were all positive for the virus. In the breakroom, samples were analyzed individually; the tabletop sample produced a positive result for PEDv while the floor and refrigerator handle were both negative. In the office area, the desk surface, computer keyboard and mouse, and the floor were analyzed individually. As was found at NE-01, only the keyboard and mouse were positive for PEDv. At this facility, a dead chute was used to facilitate disposal of expired fetal pigs to the exterior of the production facility. A surface swab sample from the interior of the chute tested positive for the virus. Samples were composited by room and, upon analysis, revealed that at least one pig in each of five of the six rooms were excreting the virus in their feces. Blood samples that were also collected from four sows randomly selected within each of the six rooms being monitored were pooled for analysis. However, the composites were not performed such that results could be applied to individual rooms. However, of the eight composites formed from these 24 samples, all but one (88%) produced positive results for PEDv.

Samples collected outside of the production facility from employees' vehicles and a loader were pooled by individual unit and none produced positive results for PEDv. However, a lagoon effluent sample collected by personnel from the swine production company operating the site within the first month following the outbreak tested positive for PEDv. This was surprising as a lagoon effluent sample from NE-01 was negative immediately following their outbreak. However, recent research published by Stevens et al. (2017) demonstrated that a positive PCR results for PED viral genome does not necessarily equate to the presence of infective viral material. Because the samples from this survey study were not tested via a live swine bioassay, it is not possible to determine whether the viral RNA detected in the lagoon sample was infectious or not.

At the two breed-to-wean sites, PEDv could still be detected two weeks post-decontamination but was no longer detected by week four. This trend may be explained by the sow sites do not depopulate and get new pigs if there is an outbreak of a virus like PEDv, the older animals are able to survive the virus. Although, piglets in-utero and pre-weaned will not likely survive the virus. The finisher production sites feed pigs to market weight and then leave to go to slaughter at which time new pigs will repopulate the site causing the virus to be limited when new healthy pigs repopulate the facility.

Soil Samples

Soil collected from fields that had received application of manure from the surveyed facilities (all within one-half mile of each facility) after the initial PEDv outbreaks were analyzed and yielded negative results by RT-PCR. To confirm that the analysis method used was efficient for detecting the PED virus in soil samples, an

additional experiment was performed to evaluate the RNA extraction method. For soils receiving both high and low treatment concentrations of PEDv culture, the RNA extraction method yielded an extract containing PEDv target sequences (i.e. a positive PCR reaction results) (Figures 3.4 and 3.5). An approximately a one-log reduction in extracted PEDv genomes per gram of soil was observed when spiked soils were compared to equivalent PEDv culture-only extracts.

At both breed-to-wean sites, PEDv was detected throughout the interiors of the production facilities, both in production and non-production areas. Rectal swabs collected at NE-02 two weeks after decontamination yielded one composite sample out of 5 composite that was positive. For IL-01, there was still PEDv detected in rectal swabs at four weeks post-decontamination sampling. However, at eight weeks, PEDV was not detected in sow rectal swab samples.

At NE-01 (grow-finish farm), PEDv was detected in samples taken from throughout the facility immediately following confirmation of PEDv infection and prior to commencement of decontamination activities. At 6, 12, and 18 months post-decontamination, no virus was detected in any of the surface or oral fluids samples collected. Because grow-finish facilities house groups of animals for only 115 to 120 d before marketing the grown animals and replacing them with a new group of pigs weighing only about 50 to 60 lbs, it is plausible to speculate that this “all-in, all-out” practice of pig movement supports more complete and effective cleaning and disinfection between groups of animals. Likewise, PEDv is more detrimental to the health of very young piglets who lack a well-developed immune system, so the larger and more

physiologically mature pigs within the grow-finish farm may have been much less susceptible to infection with PEDV.

CONCLUSIONS

Immediately after the PEDV outbreak, virus could be detected via PCR throughout the production environment at all sites, including from areas where swine were excluded (i.e. office, breakroom). However, no PEDV was detected on equipment outside the structures (i.e. employee vehicles and tractors/loaders) or in stored manure slurry or one of two lagoons tested. Mortality compost samples collected shortly after compost pile establishment and leachate from these piles also tested negative. Based upon PCR detection method, standard cleaning and decontamination practices appeared to have effectively eliminated the PED virus from swine production units and composting of infected carcasses appears to be a biosecure disposal method.

Although PED virus was easily detected in PEDv-spiked soils during a laboratory experiment to assess the efficiency of the soil testing method, the virus was not detected in manure-amended soils collected from farms. Based on PCR analysis of soils spiked with low and high concentrations of PEDV culture, even low concentrations of PEDV could be detected in soil samples. Therefore, we could conclude that the method of detection for PEDV in soil that was used in this project is an efficient way to detect the virus and we are confident that soils collected at the farms were truly negative for the PED virus. More research needs to be conducted on how the virus could potentially persist in soil amended with infected manure.

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Table 3.1. Summary of study site characteristics

	IL-01	NE-01	NE-02
Operation Type	Breed-to-Wean	Grow-Finish	Breed-to-Wean
Animal Capacity	4,000 sows	20,000 pigs	2,000 sows
Manure Storage(s)	Slurry Pit & Lagoon	Lagoon; Fresh water flush	Slurry Pit & Lagoon
Mortality Management	Compost, on-site	Rendering, off-site	Compost, on-site
Ventilation Type	Mechanical	Natural	Mechanical

Table 3.2. Sample types and quantities collected by site

Sample Type	IL-01	NE-01	NE-02
Farrowing Rooms (n=6) Pen floor (n=4) Pen rail (n=4) Alleyway (n=4) Sow rectal swab (n=4) Control (n=1)	**X**		**X**
Grow-Finish Spaces (n=6) Pen floor (n=4) Pen rail (n=4) Alleyway (n=4) Oral fluids (n=4) Control (n=1)		**X**	
Office Desk (n=1) Keyboard/Mouse (n=1) Floor (n=1)		**X**	**X**
Employee Breakroom Tabletop (n=1) Floor (n=1) Refrigerator door handle (n=1)	**X**	**X**	**X**
Employee Vehicles (n=3) Interior floorboard (n=1) Wheel (n=1)	**X**	**X**	**X**
Tractors/Loaders Interior floorboard (n=1) Wheel (n=1) Bucket interior (n=1)	**X**	**X**	**X**
Mortality Compost Unit Bins (n=4) Leachate (n=1)	**X**		
Mortality Storage for Rendering Interior surface (n=1)			**X**
Manure Storage(s)	**X**		**X**
Manure-amended Field Soil	**X**	**X**	**X**

Table 3.3. Summary of sample analysis results for IL-01

Sample Location		Pre-decontamination[1]	Post-decontamination		
			6 Months	12 Months	18 Months
Farrowing Rooms					
Room 5	Surfaces[2]	Positive (C_q = 34.45)	----	Negative	Negative
	Rectal Swabs[3]	----	----	Negative	Negative
	Control	Negative	----	Negative	Negative
Room 7	Surfaces[2]	Positive (C_q = 35.76)	----	Negative	Negative
	Rectal Swabs[3]	----	----	Negative	Negative
	Control	Negative	----	Negative	Negative
Room 8	Surfaces[2]	Positive (C_q = 37.69)	----	Negative	Negative
	Rectal Swabs[3]	----	----	Negative	Negative
	Control	Negative	----	Negative	Negative
Room 12	Surfaces[2]	Positive (C_q = 34.66)	----	Negative	Negative
	Rectal Swabs[3]	----	----	Negative	Negative
	Control	Negative	----	Negative	Negative
Room 17	Surfaces[2]	Positive (C_q = 32.68)	----	Negative	Negative
	Rectal Swabs[3]	----	----	Negative	Negative
	Control	Negative	----	Negative	Negative
Room 24	Surfaces[2]	Negative	----	Negative	Negative
	Rectal Swabs[3]	----	----	Negative	Negative
	Control	Negative	----	Negative	Negative
Office			----		
Desk		----	----	Negative	Negative
Keyboard/Mouse		----	----	Negative	Negative
Control		----	----	Negative	Negative
Employee Breakroom					
Tabletop		Positive (C_q = 37.33)	----	Negative	Negative
Floor		Positive (C_q = 39.33)	----	Negative	Negative
Refrigerator handle		Positive (C_q = 38.28)	----	Negative	Negative
Control		Negative	----	Negative	Negative
Employee Vehicles[4]		Negative	----	Negative	Negative
Tractors/Loaders[5]		Negative	----	Negative	Negative
Exhaust Fan Housing		Negative	----	Negative	----
Mortality Composter		Negative	----	----	----
Manure Storage(s)		Negative	----	----	----
Manure-amended Soil		Negative	----	----	----

[1]Positive results include PCR quantification cycle (C_q) value
[2]Composite of pen floor, pen rail and alleyway samples by room (n=12)
[3]Composite of sow rectal swab samples by room (n=4)
---- Represents "no sample collected"

Table 3.4. Results of RT-qPCR analysis on farrowing sow blood samples taken pre-decontamination at IL-01

Sample[1]	RT-qPCR Result
1	Negative
2	Positive (C_q = 37.26)
3	Positive (C_q = 35.14)
4	Positive (C_q = 35.39)
5	Negative
6	Positive (C_q = 39.68)
7	Positive (C_q = 35.80)
8	Positive (C_q = 32.67)
9	Positive (C_q = 35.96)
10	Positive (C_q = 33.66)
11	Positive (C_q = 33.94)
12	Positive (C_q = 32.86)
13	Positive (C_q = 35.96)
14	Positive (C_q = 35.01)
15	Positive (C_q = 35.64)
16	Positive (C_q = 30.05)
17	Positive (C_q = 34.14)
18	Positive (C_q = 35.39)
19	Positive (C_q = 33.86)
20	Positive (C_q = 35.31)
21	Positive (C_q = 35.26)
22	Positive (C_q = 33.25)
23	Positive (C_q = 28.55)

[1]Each sample represents a single sow; all sows randomly selected from monitored rooms at site, but samples not labeled by room

Table 3.5. Results of RT-qPCR analysis on farrowing sow rectal swab samples taken post-decontamination at IL-01 by farm personnel

Sample[1]	Week 2	Week 4	Week 8
Composite 1	Negative	Negative	Negative
Composite 2	Negative	Negative	Negative
Composite 3	Positive (C_q = 40.0)	Negative	Negative
Composite 4	Negative	Negative	Negative
Composite 5	Negative	Negative	Negative
Composite 6	Negative	Negative	Negative

[1]Samples were not taken from the same animals and were not accurately labeled with room numbers; therefore, they were not pooled by room

Table 3.6. Summary of pre- and post-contamination sample analysis results for samples collected at NE-01 by project members

Sample Location		Pre-decontamination[1]	Post-decontamination		
			6 Months	12 Months	18 Months
Grow-Finish Spaces					
Room 1	Surfaces[2]	Positive (C_q = 36.94)	Negative	Negative	Negative
	Oral Fluids[3]	Positive (C_q = 35.43)	Negative	Negative	Negative
	Control	Negative	Negative	Negative	Negative
Room 5	Surfaces[2]	Positive (C_q = 34.07)	Negative	Negative	Negative
	Oral Fluids[3]	Positive (C_q = 36.42)	Negative	Negative	Negative
	Control	Negative	Negative	Negative	Negative
Room 8	Surfaces[2]	Positive (C_q = 36.06)	Negative	Negative	Negative
	Oral Fluids[3]	Positive (C_q = 39.40)	Negative	Negative	Negative
	Control	Negative	Negative	Negative	Negative
Room 13	Surfaces[2]	Positive (C_q = 36.96)	Negative	Negative	Negative
	Oral Fluids[3]	Negative	Negative	Negative	Negative
	Control	Negative	Negative	Negative	Negative
Room 14	Surfaces[2]	Positive (C_q = 39.25)	Negative	Negative	Negative
	Oral Fluids[3]	Negative	Negative	Negative	Negative
	Control	Negative	Negative	Negative	Negative
Room 17	Surfaces[2]	Positive (C_q = 40.00)	Negative	Negative	Negative
	Oral Fluids[3]	Negative	Negative	Negative	Negative
	Control	Negative	Negative	Negative	Negative
Office					
Desk		Positive (C_q = 40.0)	Negative	Negative	Negative
Keyboard/Mouse		Positive (C_q = 40.0)	Negative	Negative	Negative
Control		N/A	Negative	Negative	Negative
Employee Breakroom					
Tabletop		Negative	Negative	Negative	Negative
Floor		Negative	Negative	Negative	Negative
Refrigerator handle		Negative	Negative	Negative	Negative
Control		Negative	Negative	Negative	Negative
Employee Vehicles[4]		Negative	Negative	Negative	Negative
Tractors/Loaders[5]		Negative	Negative	Negative	Negative
Mortality Storage		----	----	----	----
Manure Storage(s)		----	----	----	----
Manure-amended Soil		----	Negative	Negative	Negative

[1]Positive results include PCR quantification cycle (C_q) value
[2]Composite of pen floor, pen rail and alleyway samples by room (n=12)
[3]Composite of oral fluid samples by room (n=4)
---- Represents "no sample collected"

Table 3.7. Summary of pre- and post-contamination sample analysis results for samples collected at NE-02 by project members

Sample Location		Pre-decontamination[1]	Post-decontamination		
			6 Months	12 Months	18 Months
Farrowing Rooms					
Room 3	Surfaces[2]	Positive (C_q = 33.61)	Negative	Negative	Negative
	Rectal Swabs[3]	N/A	Negative	Negative	Negative
	Control	Negative	Negative	Negative	Negative
Room 4	Surfaces[2]	Positive (C_q = 35.75)	Negative	Negative	Negative
	Rectal Swabs[3]	N/A	Negative	Negative	Negative
	Control	Negative	Negative	Negative	Negative
Room 5	Surfaces[2]	Positive (C_q = 32.68)	Negative	Negative	Negative
	Rectal Swabs[3]	N/A	Negative	Negative	Negative
	Control	Negative	Negative	Negative	Negative
Room 5b	Surfaces[2]	Positive (C_q = 30.26)	Negative	Negative	Negative
	Rectal Swabs[3]	N/A	Negative	Negative	Negative
	Control	Negative	Negative	Negative	Negative
Room 6	Surfaces[2]	Positive (C_q = 33.21)	Negative	Negative	Negative
	Rectal Swabs[3]	N/A	Negative	Negative	Negative
	Control	Negative	Negative	Negative	Negative
Room 18	Surfaces[2]	Positive (C_q = 30.59)	Negative	Negative	Negative
	Rectal Swabs[3]	N/A	Negative	Negative	Negative
	Control	Negative	Negative	Negative	Negative
Office					
Desk		Negative	Negative	Negative	Negative
Keyboard/Mouse		Positive (C_q = 37.09)	Negative	Negative	Negative
Control		Negative	Negative	Negative	Negative
Employee Breakroom					
Tabletop		Positive (Cq =36.11)	Negative	Negative	Negative
Floor		Negative	Negative	Negative	Negative
Refrigerator handle		Negative	Negative	Negative	Negative
Control		N/A	Negative	Negative	Negative
Employee Boot Room		Positive (C_q = 36.60)			
Employee Vehicles[4]		Negative	Negative	Negative	Negative
Tractors/Loaders[5]		Negative	Negative	Negative	Negative
Dead Chute		Positive (C_q = 35.71)	----	----	----
Lagoon		Positive (C_q = 36.11)	----	----	----
Manure-amended Soil		----	----	Negative	Negative

[1]Positive results include PCR quantification cycle (C_q) value
[2]Composite of pen floor, pen rail and alleyway samples by room (n=12)
[3]Composite of rectal swab samples by room (n=4); analysis results for blood samples collected on initial site visit (pre-decontamination) summarized in Table 3.X
[4]Composite of samples from surfaces (n=2) of employee vehicles (n=3)
[5]Composite of samples from surfaces (n=3) of tractors/loaders at site (n=1)
---- Represents "no sample collected"

Table 3.8. Results of RT-qPCR analysis on farrowing sow blood samples taken pre-decontamination at NE-02 by project members

Sample	RT-qPCR Result
Composite 1	Positive 31.82
Composite 2	Positive 37.22
Composite 3	Positive 32.79
Composite 4	Positive 34.10
Composite 5	Positive 33.82
Composite 6	Positive 36.89
Composite 7	Positive 37.94
Composite 8	Negative

[1]Samples (n=24) were not accurately labeled with room numbers; therefore, they were not pooled by room

Table 3.9. Results of RT-qPCR analysis on farrowing sow rectal swab samples taken post-decontamination at NE-02 by farm personnel

Sample[1]	Week 2	Week 4	Week 8
Composite 1	Positive (C_q = 36.43)	Negative	Negative
Composite 2	Negative	Negative	Negative
Composite 3	Negative	Negative	Negative
Composite 4	Negative	Negative	Negative
Composite 5	Negative	Negative	Negative
Composite 6	----	Negative	Negative

[1]Samples were not taken from the same animals and were not accurately labeled with room numbers; therefore, they were not pooled by room

---- Represents "no sample collected"

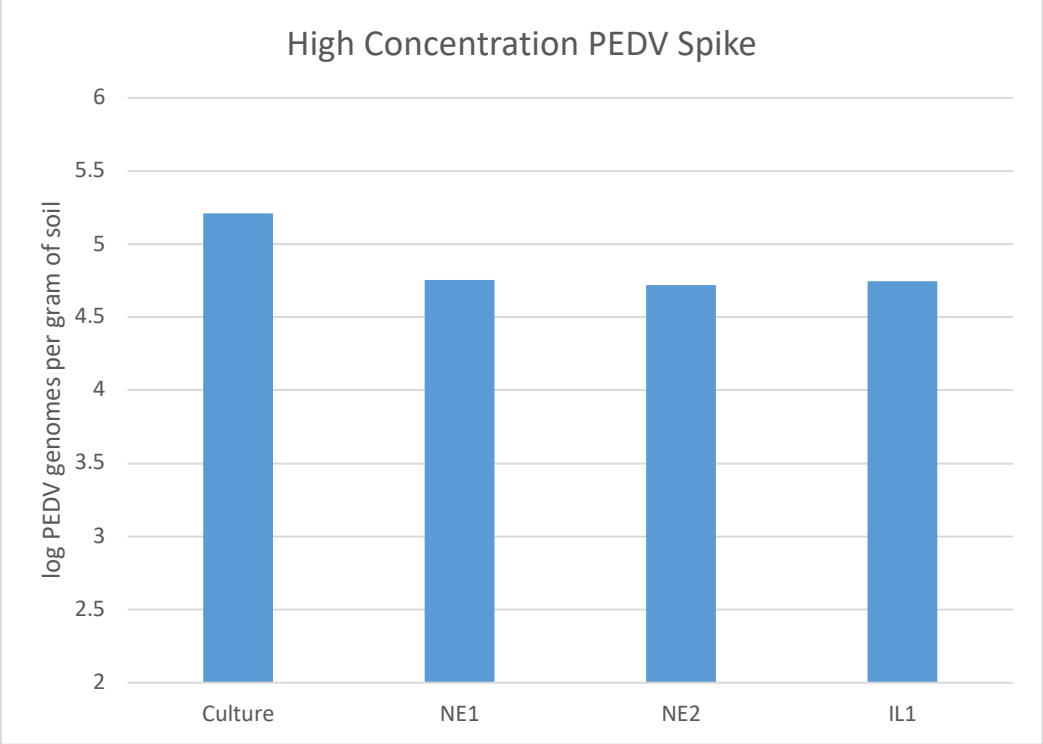

Figure 3.4. PED viral genome concentrations of culture added to soils at a "high" dose and subsequently recovered from soils

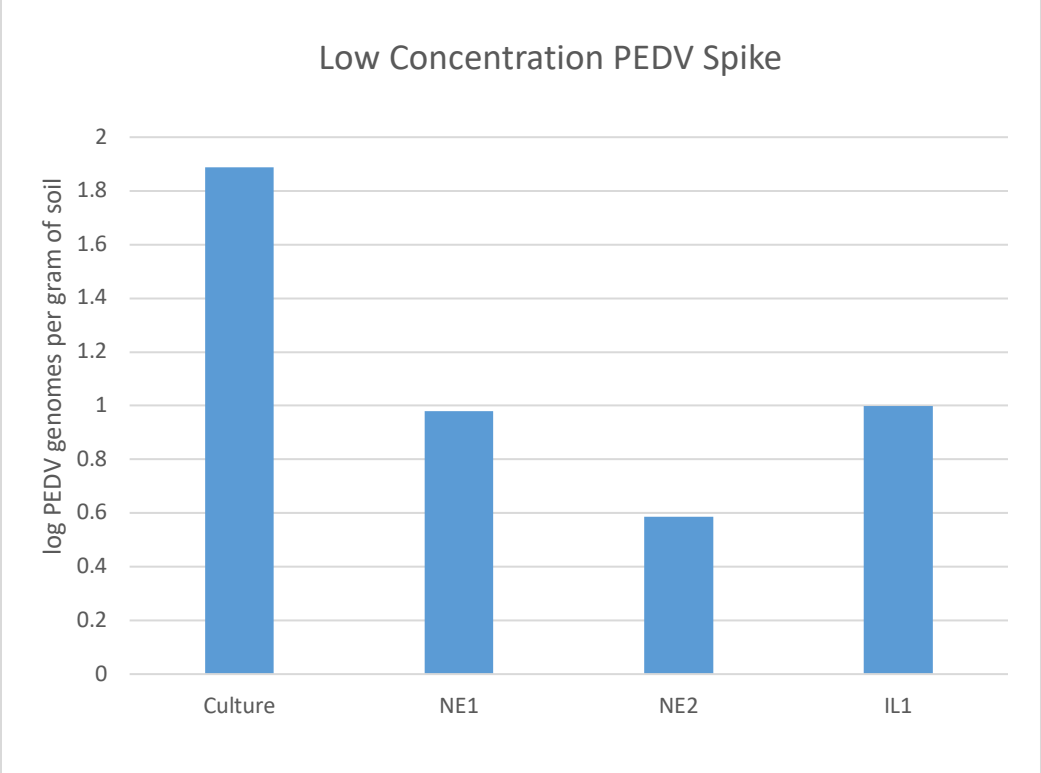

Figure 3.5. PED viral genome concentrations of culture added to soils at a "low" dose and subsequently recovered from soils

CHAPTER IV.

GENERAL CONCLUSIONS

With the emergence of the porcine epidemic diarrhea virus (PEDv) in the United States in 2013, the U.S. pork industry suffered billions of dollars in losses through animal mortalities, loss of productivity, and trade limitations. Previous research had not evaluated how the virus was spreading between swine production sites and whether it could re-emerge after industry-recommended decontamination protocols were employed within farm boundaries.

Multiple critical control points (CCPs) have been identified for preventing or mitigating an outbreak of PEDv and other swine enteric coronavirus diseases (SECDs). To minimize the potential for any disease outbreak, strict biosecurity planning and implementation of practices for equipment, workers, and livestock entering the farm is always the first line of defense. Other carriers of PEDv could be vermin or wildlife, infected feed ingredients and domestic pets. Ingestion of PEDv-infected manure by healthy pigs is the primary transmission route of the virus (Li, 2018). Thus, after an outbreak, inactivating or destroying the virus in stored manure and on equipment, supplies and other surfaces having contact with manure is critical.

Because manure is commonly utilized as a soil amendment with little to no pre-treatment, the survivability of PEDV in manure-amended soil was identified as potential transmission risk. As such, a manure treatment practice to inactivate the virus prior to land application of infected manure was considered. Likewise, the effectiveness of

decontamination practices at swine production sites following an outbreak is of paramount importance to limit the risk for reinfection of naive pigs introduced to a production system following the initial herd infection.

A series of laboratory studies and field surveys were conducted to:

i) evaluate a treatment option to inactivate PEDv in fresh manure slurry, and
ii) assess the distribution and persistence of PEDv in and around three swine production facilities immediately after a PEDv outbreak and over an 18-month period following the decontamination.

Alkaline stabilization of manure with hydrated lime, a treatment method approved for pathogen control in land-applied human septage (40 CFR Part 503), was proven an effective treatment to render PEDV-positive swine manure slurry non-infective. Pig bioassays demonstrated that the virus was not infectious when manure slurry was treated to a pH 10 or greater for at least one hour (Stevens, 2018). This treatment goal can be achieved with a dosing rate of 22.7 kg (50 lbs) of lime per 3,785 L (1,000 gal) of swine manure.

While treatment to pH 10 for one hour is an effective way to limit infectivity, alkaline treatment of manure to a pH less than 10 for a longer period of time than was assessed in these studies may be just as effective. Similarly, a pH greater than 10 for less time may be a useful treatment. One important observation made during these experiments was that, when lime-amended slurry below pH 10 was evaluated in the pig bioassay, some pigs did not become infected (Reference your published paper). One

possible explanation is that the pigs utilized for the bioassay were weaned, making them slightly larger and older than the pre-weaned piglets known to be most impacted by exposure to the PED virus. These weaned pigs may have been less susceptible to PEDv and, therefore, less likely to become clinically ill from manure treated at a lower pH. Additional research is needed to answer this question.

The pH of manure also plays an important role in determining the predominant forms of nitrogen (N) in the manure. As a nutrient input to cropland, the concentration of nitrogen in manure greatly impacts the fertilizer value of the product. As manure pH increases beyond 9.25, the amount of nutrient loss via ammonia (NH_3) volatilization also increases as ammonium (NH_4^+) shifts to NH_3. This volatilization loss results in manure having less N available as a fertilizer when manure slurry is applied to cropland. In our studies, we measured the concentration NH_3 in treated manure samples at time points representing simulated treatment overnight in a slurry pit and during transport in a slurry tank to a land application site. The ammonia loss from samples in the pit simulation and tanker simulation were approximately 30% and 15%, respectively. The greater NH_3 loss from the pit simulation was likely due to the manure slurry being exposed to air, allowing for free gas exchange, and treatment lasting 12 h while the tanker simulation was performed over a two-hour period and slurry samples were loosely capped. While both treatment simulations produced economically significant nitrogen losses from the manure, when alkaline stabilization of manure is necessary to inactive the PED virus in manure, treatment in a tanker is preferable to slurry pit treatment. Of greatest concern

with treatment in a slurry storage is the risk of ammonia asphyxiation to workers and animals; an additional concern is the significant odor risk to neighboring residents.

Live swine bioassays (LSBs) are considered one of the most sensitive and conclusive methods to determine whether live virus is present in a source. However, LSBs are laborious, expensive, and take several days to evaluate effectiveness. The qPCR assay is a quicker method that is relatively inexpensive and yields a result within 24 hours. Comparing bioassay results to the qPCR results, the qPCR method provided semi-quantitative information on virus genome abundance in a sample, but it did not provide information about infectivity of the virus. PEDv RNA could still be detected using PCR, though lime treatment eliminated the capacity of the virus to cause infection. The envelope of the virus is critical for infection and was likely disrupted during the alkaline treatment with lime. So, while naked viral RNA genomes were still detected in slurry, they were not infectious. Therefore, our research supports the assertion that PCR is a reliable source for detection of PEDv presence, but is not a reliable indication of potential to cause infection.

While “lime” is a term that is broadly used in this thesis, it is an imprecise term. Multiple forms of lime exist: agricultural lime, quick lime, and hydrated lime, among others. Agricultural lime is essentially crushed limestone (calcium carbonate), composed of fairly course particles. It is commonly broadcast across crop fields, is very slow to react (dissolve), and quite safe to handle. Initial tests conducted as part of this thesis research using ag lime produced little change in manure slurry pH over several days, and it was quickly abandoned as a potential liming agent for manure slurry. Quick lime

(calcium oxide) is highly reactive, and when mixed into water is very exothermic (i.e. produces heat). As a fine powder, it can be easily inhaled and is very harmful to human and animal health. Proper personal protection equipment (PPE) should be worn when handling lime to prevent contact with skin. Hydrated lime (calcium hydroxide) is much less reactive and safer to handle; therefore, it was used throughout the course of the laboratory studies.

The results from the survey of field sites provide a unique insight into the effectiveness of decontamination practices after a PEDv outbreak. Many CCPs exist within the boundary of a swine farm. In the field survey reported in this thesis, sampling was performed throughout three commercial swine production farms to assess the prevalence and persistence of the PED virus over time following initial infection on the farm. Surface samples were collected at spots where manure would be in contact with surfaces through-out the facilities and where personnel and vectors could transmit manure to other surfaces such as the office and breakroom. Field samples including surface swabs, oral fluid samples, blood samples, and rectal swabs were collected from inside swine production areas at the time of a PEDv outbreak at three different swine facilities in March and April of 2016. These tested positive for PEDV (qPCR method) throughout each of the facilities.

At the NE-01 finisher swine site 100% of the production room pooled surface samples tested positive for PEDv. Oral fluids were positive for three of the six (50%) rooms sampled and did not match with pooled room surface results. This indicates that saliva is a good target for monitoring PEDv at the room or pen scale; fecal-impacted

surfaces likely have higher concentrations PEDv compared to oral samples. In non-swine areas, such as on breakroom surfaces, PEDv was not indicated, but positive samples were detected in the office area. Outside of the production facility employee vehicles and equipment at this site were negative for PEDv.

At the NE-02 sow site all six (100%) of the swine production rooms were positive for PEDv. Consistent with methods used at NE-01, these results represent pooled surface samples for each room. Five of the six rooms (83%) produced positive results for fecal samples. This difference may be because fecal swabs were not collected from the same pens where wipe samples were collected. The four sows in this one (surface wipe) positive room may not have become sick with PEDV. At NE-02, PEDv was not isolated from swabs of the breakroom surfaces, but PEDv was detected in the office. Inside the production facility, the boot room and dead chute from this site also produced positive results. Outside of the production area, employee vehicles and loader tested did not have PEDv detected.

At the Illinois sow farm, six rooms were monitored using the same protocol described for the Nebraska farms. Five of the six production rooms (83%) tested positive for PEDv, and fecal samples from sows produced positive results for three of the six rooms (50%). PEDv was isolated from swabs of the breakroom surfaces (the office area was not initially sampled). Employee vehicles and equipment were both negative, as were the fans on pig housings and compost facility samples. Here and at the other two Nebraska operations, control samples were collected in all areas ensuring personnel

collecting samples did not cross contaminate samples. All control samples were negative for the virus.

After the initial out-break of PEDv, samples collected 6, 12 and 18 months post-disinfection at all sites in the three operations were all negative for PEDv. At these three sites, we conclude that biosecurity procedures, such as shower-in, shower-out was effective since no PEDv was detected outside the buildings immediately after the outbreak. The on-farm survey study indicate that industry-recommended biosecurity practices appear to be effective for mitigating PEDv. Indeed, some of the room decontamination practices involved white wash which uses alkaline agents to kill the virus. This would not only affect PEDv on surfaces but should also eliminate PEDv in the fresh manure when washed into the manure collection areas. PEDv reemergence was not observed over 18 months; decontamination of the production site was thorough and effective.

Soil samples at the three production sites were all PEDv negative even though these soils received manure slurry that was potentially infected with PEDv. It is possible that the RNA extraction procedure which worked well with manure slurry samples was ineffective at recovering PEDv RNA from soil. To ensure that the RNA extraction method could extract PEDv from a contaminated soil, a laboratory study was conducted using soil samples collected from each of the swine operations spiked with a culture of PEDv. Results of this soil extraction study confirm that the extraction method is effective, though spiked PEDv recovery was not 100% efficient. However, the results of

soil extractions from the surveyed sites appear to be reliable—no PEDv was present in the soil.

In conclusion to the first study, using lime as a treatment for infectious manure can be a practice used for limiting the pathogenicity of the virus. We found that raising the manure pH to at least 10 for an hour did not cause infection to animals. Although, after obtaining a pH of 9.25, ammonia emissions via volatilization is a concern for personnel and animals that could be exposed to the gas emissions. Exposure to the emissions could potentially cause asphyxiation to personnel and animals if air ventilation is not adequate. Since manure is typically applied to fields as fertilizer, there is concern that lime treatment will cause a loss in N source via ammonia volatilization. More studies need to be conducted on whether raising the pH for a less amount of time or a lower pH for more time can be as effective as pH 10 for an hour.

In conclusion of the last study conducted, recommended biosecurity practices for swine production impacted with PEDv proved to be effective at controlling the virus. At the initial sampling time when the production facilities were infected with PEDv, the virus could be found in almost all places including where pigs were housed and employee quarters. After 4 weeks post-decontamination, the virus could not be detected at any of the production facilities at any sampling point (6, 12, and 18 months). Soil samples were collected from fields that had been applied with manure from production facilities that were contaminated with the virus were tested via PCR for PEDv. No soil samples collected at the initial sample collection period were positive for PEDv. To test our RNA extraction method used for PCR, soil samples were spiked with different dilutions of a

stock strain of PEDv in a laboratory setting at high and low dilutions. PEDv was detected via PCR at high and low concentration concluding that our method used to extract viral RNA is a reliable source for PEDv detection. Soil samples collected from the production sites truly did not have detectable PEDv in them. More studies are being conducted currently on the persistence of PEDv in manure amended soil.