Strategies to impact swine feed biosecurity

by

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Abstract

Heightened concern surrounding pathogen spread within animal food manufacturing and farm facilities has led to increased interest in monitoring techniques. A series of two experiments were conducted to determine the impact of four different environmental swab types on the detection of Porcine Epidemic Diarrhea Virus (PEDV) on two different surfaces (stainless steel and woven polyethylene totes), and then determine the differences in detection of PEDV in soybean meal (SBM) between environmental swabbing, individual probe sampling, and composite probe sampling. The first experiment demonstrated a dose × surface type × swab type interaction ($P<0.0001$; SEM=0.96), with Dacron-tip and cotton gauze swabs yielding the most detectable PEDV from the surfaces. The second experiment utilized miniature totes of SBM inoculated with a small amount of PEDV to compare detection rates between environmental sampling, individual probe samples, and composite probe samples. It was determined that 37% of individual probe samples, 33% of environmental swabs, and 100% of composite probe samples found to contain viral RNA, demonstrating the inability for individual samples to dependably detect the presence of viral contamination. Subsequently, the environmental monitoring techniques identified in Experiment 1 were used to monitor and both a multiple-stage swine operation and the feed mill supplying it during an outbreak of PEDV. Data were collected throughout the duration of the outbreak and was used as an informational tool for employees and to monitor efficacy of cleanup efforts. The changes in viral presence as detected by PCR throughout the duration of the outbreak illustrate how differences in biosecurity procedures and employee behavior can impact clean-up efforts, and the ability of environmental monitoring to be used as a tool during a disease outbreak. A study conducted during this specific outbreak evaluated the presence of Enterobacteriaceae when compared to PEDV and rotavirus utilizing

environmental sampling, as well as a comparison of two different testing methods for Enterobacteriaceae (a traditional laboratory culture analysis and a “rapid” on-site detection method). This study noted differences in mean reported PEDV Ct values throughout different areas on-farm, with lower values noted in areas with pig contact, non-pig contact, and within the main office area of the farm, while the feed mill had no environmental samples show PEDV presence throughout the duration of the study. There was no evidence of correlation noted ($r \leq 0.20$, $P > 0.05$) between the presence of PEDV or rotavirus and the presence of Enterobacteriaceae, however he “rapid” Enterobacteriaceae test had a significantly strong correlation ($r = 0.65$, $P < 0.0001$) with the cultured testing results, indicating that it could be used to monitor levels on-farm as an alternative to laboratory culturing methods. A second study was conducted in Brazil to identify Enterobacteriaceae presence within the feed manufacturing facilities of a multi-farm system experience a viral outbreak. Zone 5 had the lowest growth outside of feed and ingredient samples collected. Similar growth was noted in zones 1,2,3,4, 6 as well as the highest levels of growth in groups 2,3,4, 6 and 7. There was a moderate correlation found between different zoned areas of the feed manufacturing facilities and level of Enterobacteriaceae growth, suggesting that there is potential for the use of Enterobacteriaceae monitoring to help facilities determine areas of concern for facility hygiene or biosecurity practices.

Table of Contents

List of Figures

List of Tables

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Dedication

I would like to dedicate this thesis to my incredible family due to the endless support and encouragement, and for putting up with my constant rambling about environmental swabs over the last two years.

Chapter 1 - A review of strategies to impact swine feed biosecurity

ABSTRACT: Global pork production has largely adopted on-farm biosecurity to minimize vectors of disease transmission and protect swine health. Feed and ingredients were not originally thought to be substantial vectors, but recent incidents have demonstrated their ability to harbor disease. The objective of this paper is to review the potential role of swine feed as a disease vector and describe biosecurity measures that have been evaluated as a way of maintaining swine health. Recent research has demonstrated that viruses such as porcine epidemic diarrhea virus and African Swine Fever Virus can survive conditions of transboundary shipment in soybean meal, lysine, and complete feed, and contaminated feed can cause animal illness. Recent research has focused on potential methods of preventing feed-based pathogens from infecting pigs, including prevention of entry to the feed system, mitigation by thermal processing, or decontamination by chemical additives. Strategies have been designed to understand the spread of pathogens throughout the feed manufacturing environment, including potential batch-to-batch carryover, thus reducing transmission risk. In summary, the focus on feed biosecurity in recent years is warranted, but additional research is needed to further understand the risk and identify cost-effective approaches to maintain feed biosecurity as a way of protecting swine health.

Keywords: biosecurity, feed, pathogen, PEDV, virus, swine

Pathogenic bacteria in swine feed and ingredients

Biological hazards that may be pathogenic to swine health include bacteria, such as *Salmonella* spp. and *Escherichia coli*, and viruses, such as PEDV, ASFV, SVA, Classical Swine Fever Virus (CSFV), Pseudorabies Virus (PRV) and Foot and Mouth Disease (FMD). These hazards differ in chemical and molecular structure, and therefore their prevalence may differ in feedstuffs. However, fecal contamination may lead to entry of many of these pathogens into ingredients, and the type of feedstuff and manner of contamination event may impact their survivability in feed and infectivity in swine.

Of the potential biological hazards in feed, *Salmonella* spp. is the most researched and understood. Feed-based transmission of *Salmonella* has been demonstrated to impact swine health, including a feed-based outbreak of *Salmonella enterica* subsp. enterica serovar Cubana in Sweden (Östererg et al., 2006). Furthermore, commercial feed was reported to have a high significance as a potential vehicle for *Salmonella* transmission in the United States by Molla et al. (2010). The researchers found 3.6% of feed samples and 17.2% of fecal samples positive for *Salmonella enterica* subsp. *enterica* 36 barns and more than 6,500 pigs. Of the *Salmonella* isolates, more than half were genotypically related with similar phenotypes and patterns of antimicrobial resistance. Currently, the United States Food and Drug Administration (FDA) considers *Salmonella enterica* serotype Choleraesuisas an adulterant in swine feed, but adulteration by other serotypes is evaluated on a case-by-case basis (FDA, 2013). While *Salmonella* spp. has been reported by the FDA to be present in approximately 8% of animal feeds, neither *Salmonella* Cubana nor Choleraesuis are in the top 25 most prevalent serotypes found by the agency during routine surveillance (Li et al., 2012).

One of the emerging serotypes of concern for swine feed is *Salmonella enterica* serovar 4,5,12:i:−, a monophasic variant of *Salmonella enterica* serovar Typhimurium. This serotype was responsible for a recall of whole roaster hogs in the United States in 2016, and has been associated with resistance to many common antimicrobials (Moreno Switt et al., 2009, Centers for Disease Control, 2016). In 2012, Li et al. reported the serotype was the 6th most prevalent serotype found in animal feeds, and the 7th most common serotype in human infections. In a recent survey of 11 United States feed mills, *Salmonella enterica* serovar 4,5,12:i:− was found in the manufacturing environment of two different mills (Magossi et al., 2018). Contaminated surfaces included the ingredient pit grating, floor dust in the ingredient receiving area, and floor dust in the control room (Magossi et al., 2018). Due to its multidrug resistance and links to both pork safety and prevalence in feed mills, *Salmonella enterica* serovar 4,5,12:i:− is likely the key *Salmonella* serotype to control through future feed biosecurity.

The presence of other pathogenic bacteria in swine feed is less established. Tulayakul et al. (2012) reported 17 of 24 nursery, finishing, and sow feed samples collected in central Thailand were positive for *E. coli*, but only one sample had > 100 colony forming units/mL. Doane et al. (2007) reported two of 24 United States swine feed samples contained *E. coli* O157:H7, both of which were obtained in the state of Washington. The recent survey of 11 United States feed mills described above also identified *E. coli* in one sample of finished swine feed (Magossi et al., 2018).

Both *Salmonella* and *E. coli* belong to a family of bacteria called Enterobacteriaceae. Active surveillance of this bacteria family may act as an indicator of biosecurity compliance and even predict future outbreaks. Enterobacteriaceae and *Salmonella* spp. in the 11 feed mills by Magossi et al. (2018) are shown in Figure 1.1. Most Enterobacteriaceae identified in feed or the

manufacturing environment were generally non-pathogenic in nature, such as *Enterobacter* and *Citrobacter*. However, areas with high levels of Enterobacteriaceae also had high levels of *Salmonella* spp. (association $P = 0.05$). Analysis of retained samples showed that worker shoes also carried Senecavirus A in one feed mill. When another mill that was part of the surveillance was associated with an outbreak of Porcine Deltacoronavirus, the virus was found in the load-out auger, cooler air intake, ingredient pit grating, all locations of floor dust, broom, and worker shoes. Enterobacteriaceae is commonly used to indicate hygiene and/or biosecurity compliance in human food, rendering, and poultry feed manufacturing facilities (Jones and Richardson, 2004; Van Schothorst and Oosterom, 1984; Nestle, 2014). The proactive monitoring of Enterobacteriaceae should be further evaluated and considered as a method to better identify and control the highest risk points of entry into the swine feed supply chain.

Pathogenic viruses in swine feed and ingredients

Research has demonstrated that viruses, such as PEDV, ASFV, SVA, CSFV, PRV, and FMD, are able to survive in at least some commonly imported feed ingredients (Dee et al., 2018). Modeling done to simulate the environmental conditions during transport of ingredients from China to the United States has shown that a viable PEDV sample is able to survive in certain ingredients, including soybean meal (both conventional and organic), vitamin D, lysine hydrochloride, and choline chloride (Dee et al., 2016). In addition to PEDV, 11 other pathogens have been subjected to a similar modeling procedure in a variety of different ingredients (Dee et al., 2018). The survivability of a pathogen varied depending on the genetic and physicochemical properties of the virus, and differed between pathogens and the feed ingredients tested. Certain feed ingredients or feed products presented a better matrix for virus survival than the others and

select ingredient matrices seemed to enhance the survival of multiple viruses. For example, conventional soybean meal had a higher level of virus survival in comparison to organic soybean meal. The exact reason for this difference in survivability in sources of soybean meal is unknown, but could be attributed to the higher levels of fat present in the organic variety used in the trial, as there has been some evidence that medium chain fatty acid blends have viricidal effects (Cochrane, 2018). It has also been hypothesized that higher protein ingredients have greater capability of retaining viral infectivity, but the mechanism is not yet understood. Overall, laboratory simulations have indicated that certain feed ingredients exhibit a higher risk of transporting viral pathogens (Dee et al., 2018). Additional research is needed to better understand what ingredient attributes are associated with enhanced survivability.

Infectivity of biological hazards in swine feed and ingredients

Once it has been established that biological hazards can survive in feed and ingredients, it is important to understand their infectivity at a dose that may cause infection. Infectivity frequently relies on ensuring the viral capsids or bacteria lipid membranes are intact as they protect the pathogen from deterioration during storage. Sufficient numbers of intact particles are needed to cause infection in otherwise healthy animals, and this is known as the minimum infectious dose. Loynachan and Harris (2005) first published the minimum infectious dose of *Salmonella enterica* serovar Typhimurium in pigs as $>10^3$ colony forming units (CFU)/g of feed. Cornick and Heldgerson (2004) reported the infectious dose of *Escherichia coli* O157:H7 is 6×10^3 CFU/g in 3-month old pigs. As Österberg et al. (2006) reported, infectious dose is difficult to determine, especially in bacteria, because challenge doses are strongly associated with fecal shedding, but weakly associated with infection.

Schumacher et al. (2016) reported the minimum infectious dose for PEDV-inoculated feed is 5.6×10^1 $TCID_{50}$, equivalent to aqRT-PCR Ct of 37.1. Notably, this was above the threshold of many PEDV PCR assays in diagnostic laboratories. This research helped demonstrate why PEDV was so easily spread through a feed matrix, as 1 g of feces from an acutely infected pig could infect 500 tonnes of feed, with all the feed being infected at a dose capable of causing illness.

Ongoing research focuses on determining the median infectious dose of African Swine Fever Virus in both feed and water (Niederwerder, 2018). Additional research is needed to determine the minimum or median infectious dose for a number of bacteria and viruses, including Enterotoxigenic *Escherichia coli*, SVA, CSF, and PRV. These doses are necessary as they become targets for mitigation measures. While ideally there is no detectable pathogen in feed or ingredients, it must at least be prevented or reduced to levels below an infectious dose to sustain animal health.

Once biological hazards that are considered a risk have been identified, procedures should be created that prevent entry of the hazard into the mill, as well as procedures for mitigation and decontamination in case hazard entry cannot be prevented. Cochrane et al. (2016) published an overview of a feed mill biosecurity plan that can easily serve as the foundation for developing a mill-specific biosecurity plan. Some of their recommendations are highlighted below.

Preventing biological hazards in swine feed and ingredients

The most effective component of a feed mill biosecurity plan is prevention of hazard entry. There is incentive to prevent a hazard's entry into a facility altogether because it has been

shown that the introduction of a contaminated material into a feed mill can lead to the mill being contaminated for an extended period (EFSA, 2008). Controlling the entry of biological hazards into a facility should begin with evaluation of the ingredient suppliers. The development of a supplier verification program that includes specific requirements for ingredients being purchased, as well as communicating safety expectations to the supplier of an inbound ingredient is an important step in preventing the entry of a biological hazard. This may also include verification of ingredient-supplier protocols and on-site manufacturing facility reviews and assessments. As mentioned in the previous section, some ingredients have the potential to maintain bacteria or virus survivability and infectivity more than others. As a result, the best way to prevent hazard entry into the mill is to eliminate high risk ingredients from diet formulations. Thus, coordinated efforts between nutritionists, formulators, purchasers, and the rest of the integrated feed supply team is essential to maintaining an effective feed mill biosecurity plan.

While having a supplier control program is an important step when controlling the entry of a biological hazard into a facility, routine sampling and analysis of bagged, bulk, or liquid ingredients that are considered high-risk for certain pathogens is a valuable tool. All samples collected should be done using an aseptic method, as cross-contamination of samples during the collection process needs to be prevented. If an ingredient is considered high risk, every lot should be analyzed separately. If it is lower risk, it may be more practical to collect samples and pool them for more intermittent analysis as a way to reduce analytical cost. Determining and setting a schedule for sampling of ingredients that are considered higher risk, as well as defining an inventory holding procedure until analytical results are obtained can help lower the potential of a biological hazard being introduced into the mill. Traceability of ingredients is essential, and

maintaining records that indicate information such as the receiving date, time, lot number during unloading, and prior haul data that is connected to specific batches of finished feed allows for a quick response if a biological hazard is suspected.

Movement of people or vehicles in or out of a facility also has the potential to introduce biological hazards. Employees in the feed mill and visitors, such as guests, truck drivers, and subcontractors have the ability to introduce contaminants into a feed manufacturing facility. People may unknowingly carry fecal, dirt, or dust particles contaminated with undesirable microorganisms on the bottoms of their shoes or on clothing and are at a particularly higher risk if they are coming from another farm or feed mill where the hazard is present. The risk of people introducing biological hazards is easily illustrated in Figure 1.1 (Magossi et al. 2018), as 91% of samples collected from worker boots were contaminated with Enterobacteriaceae. Controlling and minimizing foot traffic across receiving pit grates or around hand-add port grates is a logical, low-cost method to reduce the risk of a biological pathogen being introduced into the manufacturing system, and can easily be accomplished by covering the grates when not in use. No-walk zones or even hygienic zoning may be appropriate to include in biosecurity plans in feed mills that have a higher risk of biological pathogen introduction. Procedures requiring that all visitors must be accompanied at all times by a trained employee can to help prevent biosecurity breaches. Visitors should be provided clean footwear, plastic boots, or boot cover-ups to limit the entry of outside hazards. This includes the drivers of inbound trucks. Ideally, drivers should stay inside their trucks at all times to minimize foot traffic, especially over the receiving grates. If the driver must exit the vehicle, wearing disposable plastic boots or covers will limit the potential of hazards being introduced from their shoes. Trucks entering the feed mill should have mud and sludge removed from the trailer opening before the vehicle reaches the

receiving pit, and the pit should remain covered until the truck is ready to unload. Ingredients may be contaminated prior to unloading, but they may also be contaminated during the unloading process due to mud or floor sweepings intermingling with ingredients at the point of entry. Ensuring the receiving pit remains covered while trucks are being moved reduces the risk of contamination during unloading, which is important considering the impracticality of thoroughly cleaning conveying equipment such as the central pit or, bucket elevators. Use of cones and funneling devices can also be used to limit the quantity of material that spills during unloading and prevents mill employees from sweeping spilled ingredients into the pit.

Floor sweepings, including those from the unloading process, should be disposed of and not swept into the pit. In addition, many feed manufacturing facilities have grain cleaners and dust collection equipment in place, and it has been well established that dust and other screened particles can act as a carrier for biological hazards including PEDV (Gebhardt, et al., 2016) or mycotoxins (Yoder et al., 2018), among others. Many feed manufacturers have the mentality that adding back the dust or screened material to the finished feed is acceptable because it will reduce ingredient shrink. However, the cost associated with reduced animal performance and/or increased mortality is much greater than the loss of mill efficiency, and therefore all dust and screened materials should always be disposed of compared to being added back into the feed.

Reducing biological hazards in swine feed and ingredients

Once a biological hazard is introduced into a facility, it can be almost impossible to control because most feed manufacturing facilities were not hygienically designed. Furthermore, mitigation strategies that may be possible in some systems may not work in others because of differences in facility design and equipment, manufacturing operations, and other associated risk

factors among feed mills. For instance, Muckey (2016) reported that the surface type (concrete, plastic, rubber, stainless steel, etc.) impacts pathogen survivability in the presence of different decontamination procedures. Stainless steel and smooth plastic surfaces, while easier to clean than tires, rubber belts, or polyethylene totes, are more difficult to sanitize due to the formation of biofilms that protect the bacteria or virus from a chemical sanitizer. Therefore, both cleaning and sanitizing is often necessary, and nearly impossible based on current equipment design constraints.

Physical prevention of hazard spread via cross-contamination is especially difficult due to the highly infective nature of contaminated dust and the impracticality of physical clean-out in most mills (Figure 1.2). In Schumacher et al. (2017), the role of PEDV cross-contamination in feed mills was evaluated. Initially, a PEDV-negative corn- and soybean meal-based nursery pig diet was mixed, conveyed, and discharged using pilot scale feed manufacturing equipment. Next, a diet was manufactured, including an ingredient that had been spiked with infectious PEDV. Subsequently, four separate PEDV-negative diets were mixed, conveyed, discharged to test how many negative diets were necessary to 'flush' contamination from the manufacturing surface. Environmental swabs were collected prior to and after each batch of feed by swabbing direct feed contact surfaces, adjacent surfaces located within 1 m of manufacturing equipment, and other surfaces located at least 1 m away from manufacturing equipment. The presence of PEDV RNA was reported in cycle threshold (Ct) of qRT-PCR using PROC GLIMMIX (SAS Institute, Inc., Cary, NC). The statistical model evaluated the effect of manufacturing sequence (negative, positive, flush 1, flush 2, flush 3 and flush 4) and location (direct feed contact, adjacent, or other surface) and the associated interaction. The LSMEANS procedure compared surface type among treatments within animal food-contact surfaces by pairwise comparison. Both main effects and

the interaction were significant ($P < 0.05$). Subsequently, Gebhardt et al. (2018) demonstrated that dust collected from feed manufacturing surfaces can cause infectivity in a swine bioassay. Therefore, limiting and controlling dust created during manufacturing should be a priority, as it can serve as a vector in viral disease transmission such as PEDV. Sequencing procedures in order to minimize risk to the most sensitive phases of production should be utilized. Furthermore, flushing protocols should be established to help minimize cross contamination risk. Gebhardt et al., (2016) showed in a PEDV model that rice hull flushes can be a cost-effective strategy to reduce cross-contamination risk.

For RNA viruses in particular mitigation techniques depend on disrupting the viral capsid which removes the protective shell around the virus (Cliver, 2009). Three main categories of mitigation strategies have been identified and include biological, physical, and chemical. Deng and Cliver (1995), reported that biological inactivation typically occurs with the use of specific enzymes or other products of microbial origin that attack viruses or bacteria, but research is lacking to determine if this is a feasible mitigation strategy for the feed manufacturing industry. Physical inactivation in feed manufacturing is most commonly achieved thermally, but should be considered a point-in-time mitigation strategy, because it would not prevent post-processing contamination risk. The use of chemical agents, such as formaldehyde or medium-chain fatty acids as feed additives have been shown to have excellent potential to inhibit virus and bacterial hazards in feed. The benefit of these chemical agents is that they have the potential to have immediate as well as residual efficacy which could help with mitigation from the point of application until the time the feed is consumed. Specific research identifying mitigation strategies that can be used in the feed manufacturing process are reviewed below.

Thermal Processing: In a benchtop model, Goyal (2013) confirmed that PEDV is a heat-sensitive virus and that a temperature x time relationship could be used as a guide for PEDV inactivation. Based on this information, two studies were conducted to determine if passing feed through a pellet mill would be sufficient to apply thermal insult to a great enough extent to prevent PEDV infectivity. Cochrane et al. (2017) showed in the first trial that when a low or high dose of PEDV was used to inoculate feed, with the resulting feed subsequently processed at 1 of 9 combinations of conditioning temperature (68, 79, or 90 °C) or conditioner retention time (45, 90, or 180 s) all processed batches of feed were unable to generate infectivity in a pig bioassay model, even though the unprocessed feed did lead to PEDV infectivity. In a subsequent trial, the same researchers processed feed through a conditioner utilizing a 30 s retention time and 1 of 5 condition temperatures (38, 46, 54, 63, or 71°C) and observed that feed processed at or above 54 °C was able to prevent PEDV infectivity, while feed that was processed at the two low temperatures did lead to PEDV infection when fed to pigs. This series of trials demonstrated that thermal mitigation is a possible means of minimizing PEDV-associated risk, and more importantly demonstrated that equipment commonly found in commercial feed mills was effective at applying the thermal stress. However, it is important to remember that even though the feed mill may target a specific processing temperature adequate to inactivate PEDV, there are times during the feed manufacturing process (such as at equipment startup, or if steam flow is turned off to ameliorate a plugged die) that the feed may not be processed at a high enough temperature to effectively eliminate all virus transmission risk. Furthermore, the research demonstrates that the pellet mill is an effective point-in-time mitigation strategy, but it cannot prevent post-processing recontamination risk.

Residual Control Measures

The use of chemical feed additives as strategies to reduce biological hazards in feed is appealing because they allow for efficacy throughout the remainder of the feed supply chain, with the potential to also influence animal performance once consumed. As a result, a number of different products have been tested as chemical-based feed hazard mitigants. Some compounds that have shown mixed efficacy at reducing or eliminating virus or bacterial risk include organic acids (Eklund et al., 1985), essential oils (Orhan et al., 2012), sodium bisulfate (Knueven, 1998), or sodium chlorate (Smith et al., 2012); however, the cumulative data suggests that the effectiveness of any chemical-based feed mitigant is not only target specific but also feed ingredient/matrix specific (Cochrane, 2018). Of all the potential chemical mitigants available, the two that have garnered the most commercial interest are formaldehyde and medium chain fatty acids.

Formaldehyde has been shown to be effective at preventing risk associated with PEDV (Dee et al., 2014; Dee et al., 2015; Cochrane, 2018) as well as *Salmonella* (Cochrane et al., 2016). However, regulatory restrictions can limit some applications as the product is only approved for use to prevent contamination with *Salmonella*. Additionally, specialized equipment must be used for accurate application, and there are worker health concerns as well as negative perception by some consumers, which can lead to formaldehyde being limited in its commercial application. Furthermore, the use of formaldehyde in feed may lead to detrimental bacterial shifts in the pig gut (Williams et al., 2018).

The use of medium chain fatty acids (MCFA) as chemical-based feed mitigants was reviewed by Cochrane et al. (2018). They observed that MCFA are effective at preventing risk associated with feed contaminated with PEDV in addition to their effectiveness against

Salmonella (Cochrane et al., 2016). Through a series of trials this group of researchers has shown that combinations of caproic, caprylic and capric acid are the most effective with little efficacy of lauric acid against PEDV. Interestingly, the same group of researchers also showed that increasing concentrations of a 1:1:1 blend of caproic, caprylic and capric acid also resulted in a linear increase in growth performance with a 1.50% inclusion resulting in an almost 2 kg BW advantage compared to a diet with no MCFA after feeding nursery pigs for 35 d (Thomson et al., 2018). Furthermore, Gebhardt et al. (2018) showed that feed used in this trial that was collected 40 d after MCFA application was still successful at reducing PEDV risk which demonstrates the residual mitigation potential of MCFA.

Addressing feed mills contaminated with biological hazards

Due to the high quantity of airborne particulates in animal food manufacturing facilities, dust contamination is a widespread mechanism for both viral and bacterial hazard transmission (Figure 1.2). This can be specifically challenging because of the difficulties associated with physical cleaning (Muckey, 2016). Highly aggressive procedures, such as use of liquid chemical sanitizers and heat have been shown to be necessary when reducing bacteria on environmental surfaces to completely decontaminate manufacturing surfaces (Figure 1.2; Huss et al., 2017; Schumacher et al., 2017). Effective cleaning, which may require both physical cleaning and the use of cleaning solutions, removes biofilm formations that will allow for subsequent penetration and removal of vegetative bacteria by a sanitizer. Both steps are necessary, but can prove to be difficult in many feed manufacturing systems due to a lack of access or ability to thoroughly clean out or safely sanitize dry bulk manufacturing systems. Cleaning of non-animal food-contact surfaces should not be overlooked as biological hazards can efficiently spread throughout

a facility through dust and other airborne particulates. This contamination is not mitigated during flushing procedures, and can contaminate subsequent feed batches (Schumacher et al., 2017).

Because complete physical clean-out of feed manufacturing systems can prove to be difficult, flushing procedures including the use of added substances such as formaldehyde, MCFA, and dry essential oil blends may be used to help reduce the presence of biological hazards on feed-contact surfaces. Data suggests that biological hazard risk can be reduced after a third flush, or after the use of a chemically enhanced flush (Gebhardt et al., 2016, Muckey, 2016, Schumacher et al., 2017). Formaldehyde-based products and an MCFA blend have been shown to reduce the presence of PEDV on these surfaces when used in conjunction with a rice hull flush. Similarly, MCFA blends have been found to be effective at reducing *Salmonella* enterica serovar Typhimurium on stainless steel surfaces, in addition to reducing the quantity of post-processing *Salmonella* enterica serovar Typhimurium contamination if 2% is applied to swine feed prior to its inoculation with bacteria (Cochrane, 2016).

Future directions for swine feed safety

Clearly, additional research is necessary to better understand both the risk and prevention of biological hazards in swine feed and ingredients. Our knowledge of survivability, infectivity, mitigation, and decontamination strategies all must be improved to maintain the safety of swine feed in the future. Additional research is warranted to evaluate the role of beneficial bacteria to competitively exclude pathogens in feed manufacturing environments, such as those described by Zhao et al. (2013) for controlling *Listeria monocytogenes* and *Listeria* in poultry processing plants. The concept of competitive bacteria for inhibitory exclusion is being tested for controlling

Salmonella and other pathogenic bacteria in meat processing and rendering facilities, and may be viable to consider for feedstuff production facilities. Furthermore, it will be important to understand hygienic design for retrofits and new construction of feed mills in the future.

One of the items that limits the ability to make faster progress on feed safety is that few molecular diagnostics methods have been appropriately validated for feedstuffs. Our team has consistently witnessed lower recovery rates of viral nucleic acids when moving from inoculant (virus stock) into dry feed or ingredients. We reported this challenge in Schumacher et al. (2016), where we established the infectious dose of PEDV via feed. Increasing levels of virus were associated with lower qRT-PCR cycle threshold (CT) in the inoculum. However, the CT value of the same virus dose in feed was, on average, 9.5 CT higher than that in the inoculum after correcting for the dilution. The loss in viral RNA and diagnostic sensitivity did not appear to be dose-related, since a similar CT was detected despite different amounts of PEDV were spiked into the feed. This was later confirmed by Cochrane et al. (2017), where we reported a consistent loss in PEDV detectability by RT-PCR when feed was inoculated with a low (10^3 $TCID_{50}$/g) or high (10^5 $TCID_{50}$/g) dose of PEDV. Tissue culture medium inoculum was 20 or 13 CT, while the detectable PEDV in feed was 30.7 or 23.9 CT, leading to a 10.7 or 10.9 CT loss in sensitivity for both the low and high PEDV doses, respectively. This loss in viral RNA and diagnostic sensitivity is not isolated to our laboratories. A similar loss in sensitivity was found by Iowa State University, when our samples were tested in their diagnostic laboratory. In our collaboration with Pipestone Systems and SDSU, we (Dee et al., 2016) reported an up to 11.4 CT loss when moving from stock virus to feed or ingredients using diagnostic assays developed at the SDSU Veterinary Diagnostic Laboratory. The challenge in loss of sensitivity is not confined to PEDV. We recently observed a similar CT sensitivity loss between liquid inoculum and dry

feed with Senecavirus A (SVA) (Sardella et al., 2019). Furthermore, Dee et al. (2018) reported a loss in sensitivity between stock virus and various ingredients or complete feed for SVA, BVDV (surrogate for CSFV), PRRSV, and ASFV. Finally, the loss in diagnostic sensitivity appears to vary from one feed or ingredient matrix to another. Cochrane et al. (2016) demonstrated that complete swine diet, blood meal, meat and bone meal, and spray-dried porcine plasma inoculated with the same quantity of PEDV inoculum resulted in CTs ranging from 26 to 31 using identical sample preparation, extraction and RT-PCR conditions. Furthermore, Dee et al. (2016) reported a loss of sensitivity ranging from 1.4 to 11.4 CT for PEDV, depending upon ingredient matrix.

We initially accepted this loss in sensitivity was inherently part of the assay, and that it posed a problem for veterinary diagnostic laboratories but had limited biological relevance. However, we have recognized that the poor recovery of nucleic acids using the current methods in ingredients has substantial ramifications: it leads to false negative results via a type II error. Natural fecal contamination in ingredients or from cross-contamination due to poor biosecurity is likely to have low levels of virus, and a 10-CT reduction in sensitivity may lead to the determination that a contaminated product is actually safe. For example, we reported in Schumacher et al. (2018) that a pig gavaged with a PEDV-spiked feed sample that was qRT-PCR-negative (> 40 CT) became infected and symptomatic, i.e. presence of infectious PEDV was confirmed in the bioassay.

Currently, the current methods for sample preparation, extraction, and detection of nucleic acids in feedstuffs are too variable and pose too high of a risk for a false negative, which negates the value of the test. Urgent research is needed to validate molecular detection methods in feedstuffs, which can then be used to create an appropriate sampling method and point-of-use diagnostic devices. Until then, environmental monitoring and product testing is not a viable

option for ensuring feed safety; instead efforts must be more preventative than reactive. The swine feed industry must embrace feed biosecurity as regulators and consumers shift their thinking of our product as swine feed to swine food.

Recommendations to maximize swine feed biosecurity

In conclusion, biosecurity is a well-known topic at the farm level, but only recently has begun to gain importance in the feed manufacturing process. Evidence demonstrating the ability of feed and feed ingredients to support virus infectivity and bacterial survivability has been collected which points to the fact that feed and ingredients can be a vector for biological hazard transmission. Consequently, a series of steps should be taken to help maximize feed biosecurity:

1. Assess biological hazard risk: Feed manufacturing facilities must take a proactive approach to understanding biological hazards for their own operations and the security of their customers. The biosecurity procedures employed by a specific mill may not be the same as other mills depending on the customers they serve and the associated risk tolerance vs. price for mitigation strategies that are employed.
2. Define protocols to prevent entry of hazard into the mill: The most important part of a feed mill biosecurity plan is to prevent hazards from entering the mill. Identifying and eliminating high risk ingredients, minimizing entry via people and equipment, covering all open points of entry when not being used, and other strategies can be used to prevent hazard entry into the mill.
3. Utilize mitigation strategies to prevent risk: Not all hazards can be prevented from entering the mill and consequently mitigation strategies should be utilized. The best option is to identify the mitigation strategies that are effective against the specific hazards of concern

and utilize a combination of point-in-time mitigants as well as those that have residual effectiveness for continue protection through the remainder of the feed supply chain. Some mitigation strategies have multiple benefits. As an example, dust collection and elimination not only create a safer and better environment for the workers, but also can eliminate a major point of contamination.

4. Feed mill decontamination: While it is extremely difficult to completely accomplish, a feed mill decontamination strategy must be developed and should include a combination of physical cleaning, chemical cleaning, and if applicable the use of high heat as the final step.

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Figures and Tables

Figure 1.1 Presence of Enterobacteriaceae in 11 US feed mills

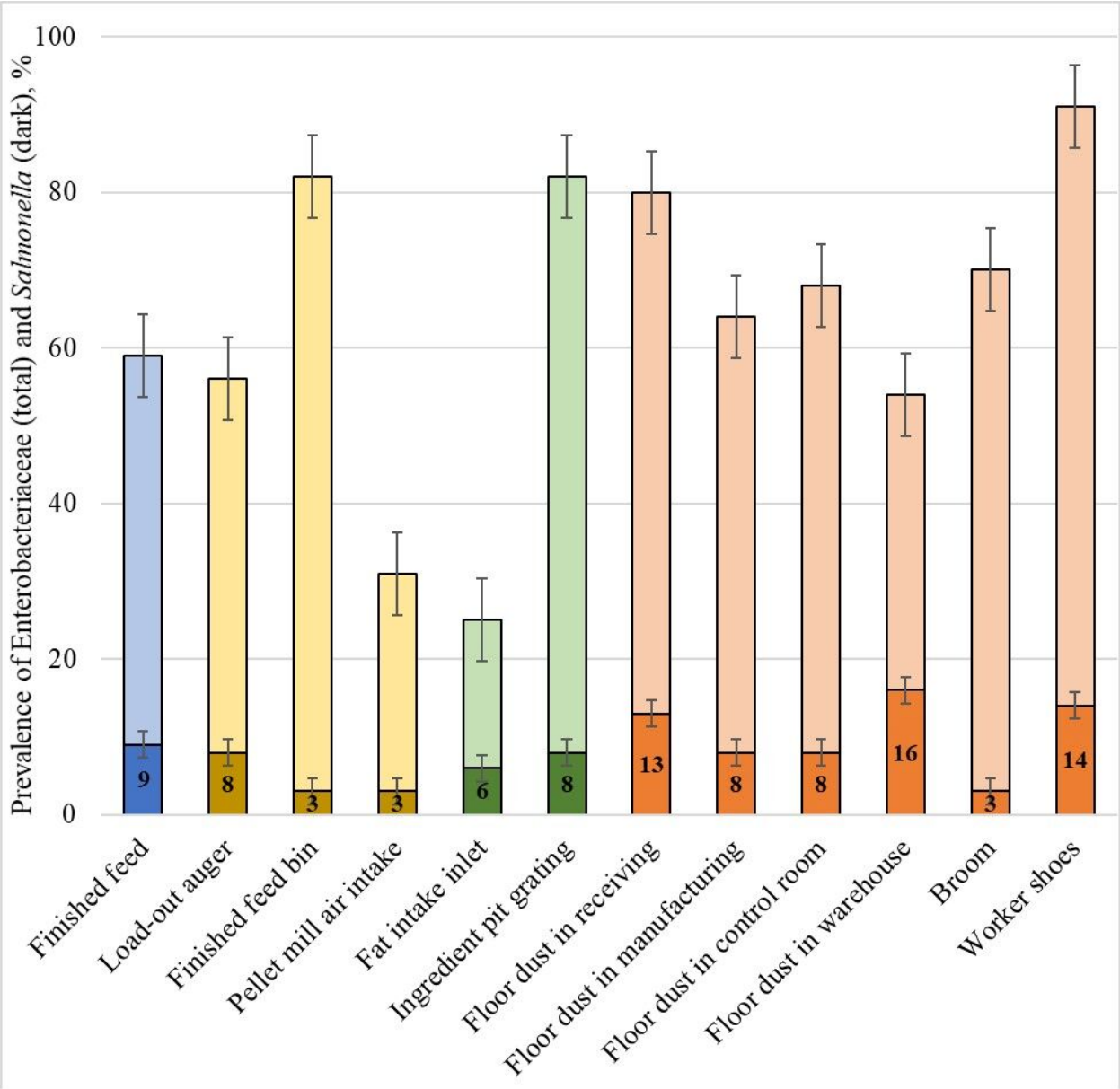
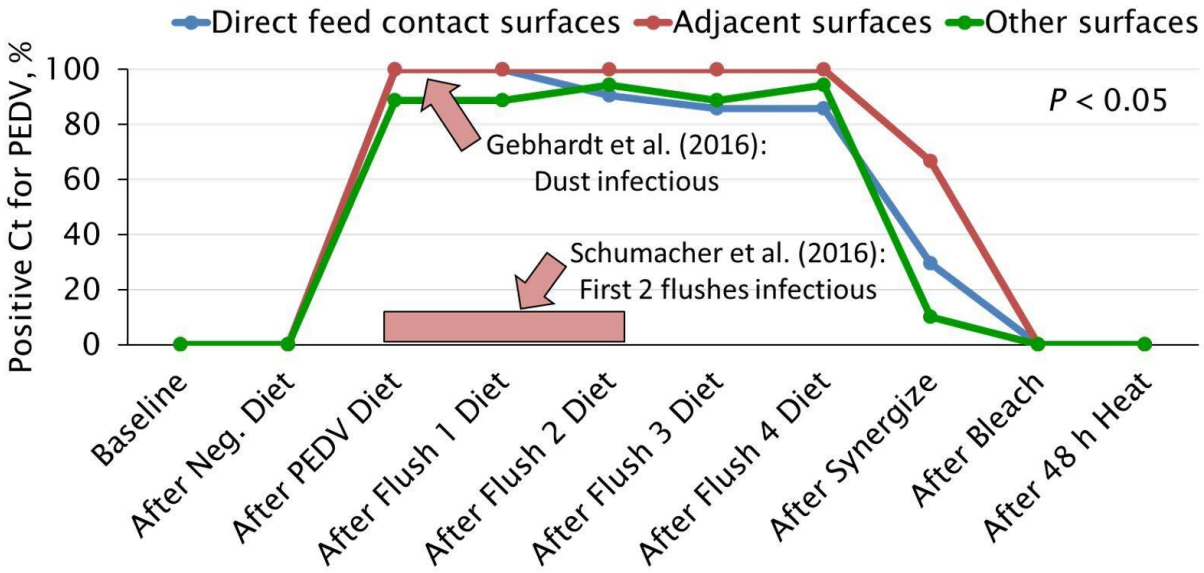


Figure 1.2 PEDV surface contamination of feed manufacturing environment throughout an experimental manufacturing and cleaning process



Chapter 2 - Methods for detecting porcine epidemic diarrhea virus within feed and ingredients

Introduction

Feed and ingredients have been demonstrated vectors of biological hazards, such as Salmonella and porcine epidemic diarrhea virus.[1, 2] Other biological hazards categorized as foreign animal diseases (or their surrogates) have been demonstrated to survive transboundary shipment, demonstrating the viability for ingredients to be a source of introduction of African Swine Fever Virus, Classical Swine Fever Virus, Pseudorabies Virus, and Foot and Mouth Disease.[3] Currently, there are limited options to monitor or analyze feed or ingredients for these hazards, but the technology is growing rapidly and already exists for pathogens like PEDV. As our diagnostic capabilities improve, we must have a validated method to sample ingredients for biological hazards in order to truly understand ingredient risk.

Obtaining a representative sample from bulk feed and ingredients can be challenging. The current methodologies typically involve collecting 10 subsamples from evenly distributed locations, but at varying material depths. The 10 samples are then mixed to create a composite sample that is representative of the larger quantity of product.[4] While this method is readily used for precise sampling of drug[5] or nutrients,[6] it is more difficult to collect a representative sample for hazards that are not evenly distributed. For example, the AAFCO Feed Inspector's Manual[4] reports that "the true aflatoxin concentration in a lot cannot be determined with 100% certainty" because the hazard exists in 'hot spots' within a bulk ingredient instead of being evenly distributed. Similarly, biological hazards in bulk ingredients are likely to exist in 'hot spots' of fecal contamination, tick infestation, or other pockets of contamination that concentrate the hazard into

small areas instead of being evenly distributed. This makes it more challenging to collect a representative sample.

If an ingredient were to be contaminated with fecal material carrying PEDV and then loaded into a 1-tonne polyethylene tote bag commonly used to transport ingredients, the virus is not likely to be evenly distributed throughout the material. Instead, it is more likely that the ingredient particles nearest the source feces have the greatest contamination. In most situations, facilities will collect one sample from the top of the tote when testing for ingredient quality. If tested for the PEDV, the PCR results from this sample may be negative. Even in scenarios where thorough product testing involving collection of 10 probes of product is conducted, it is plausible that the ingredient may test PCR negative because the 'hot spot' of the ingredient is not breached by one of the probes.

This is a similar challenge to what human food and pet food manufacturers face with biological hazard contamination in finished product. Pet food manufacturers struggle to design sampling methodologies to effectively "test for a negative" when confirming finished kibble is free of Salmonella. To address this challenge, these industries frequently rely on environmental swabbing during the manufacturing run to indicate risk of biological hazards instead of product sampling in finished product. This in-production environmental sampling is not yet a realistic expectation of foreign ingredient suppliers. However, we can extend the principles to sampling methodology. It has been demonstrated that once virus is introduced to the manufacturing process, nearly all manufacturing surfaces become contaminated and retain the contamination.[7, 8] The surfaces may be better representatives than the diet itself. In PEDV, this surface contamination contained a lower concentration of viral particles (higher Ct) than the contaminated feed, but still caused infectivity.[9] This is a notable difference. Environmental sampling is less likely to detect the

exact level of contamination (i.e. number of viral particles per gram), but has the advantage of being more consistent at detecting the presence of contamination. Thus, it is likely that the surface of the tote may have residual viral contamination due to the loading process that is otherwise undetected in the product. Swabbing the interior surface of the tote is therefore a better indicator of viral presence than sampling the product itself. Theoretically, these findings should extend to assessing risk in bulk ingredients, but no data exists to confirm its extrapolation. Likewise, no methodology has been published or recognized as appropriate for sampling bulk products for assessing pathogen contamination. It is appropriate to confirm the appropriate methods for environmental sampling in both enveloped and non-enveloped viruses. Therefore, the objective of this proposal is to develop a validated method to test bulk feed products and apply it to monitoring shipments for pathogen contamination.

The objective of the following experiments is twofold; first, determine the impact of different swab and surface types on the detection of a RNA virus (PEDV), and second, determine the differences in detection of PEDV in soybean meal (SBM) between environmental swabbing, individual probe sampling, and composite probe sampling.

Materials and Methods

Experiment 1

Experiment 1 was arranged as a 2 × 2 × 4 factorial with two doses of PEDV (low: 10^3 or high: 10^5 TCID50), two surface types (stainless steel or polyethylene tote), and four swab types (1: sponge-tip swab packaged with neutralizing buffer; 2: Dacron-tip swab packaged in Dey-Engley neutralizing broth; 3: dry dust-mops (Swiffer® Sweeper Dry Unscented Sweeping Cloth, Proctor and Gamble Company, Cincinnati, OH, USA) ; or 4: 5 cm x 5 cm cotton gauze squares

soaked in sterile phosphate-buffered saline with three replicates per combination of swab type, viral level, and surface type.

Dry dust-mop swabs were prepared prior to inoculation by being cut in half and then placed into plastic sample bags (Whirl-pak®, Nasco, Fort Atkinson, WI, USA). Cotton gauze square swabs were prepared by placing gauze into a 50 ml conical tube, then adding 2.5 ml of sterile 7.2 pH phosphate-buffered saline (PBS). Both the sponge-tip and Dacron-tip swabs did not require any preparation prior to use.

All initial inoculation and swabbing work were performed in a Class II Biological Safety Cabinet (BSC) located at the Kansas State University College of Veterinary Medicine.

For each replicate, 15 g of soybean meal (SBM) was inoculated with one PEDV dose at a 1:10 ratio. The virus was added into the SBM, which was then agitated to allow for even distribution throughout the sample. The individual portions of SBM sat at room temperature for 5 minutes after virus addition prior to being used; replicates were randomly assigned an order within a grouping of PEDV dose, with dose groups run in order from lowest to highest assumed viral load.

After the viral addition process, SBM portions were placed onto 10 cm × 10 cm stainless steel coupons, which were then covered and allowed to set at room temperature for 15 min. After the 15 minutes, each portion of SBM was conveyed onto a 10 cm × 10 cm square of polyethylene tote material, which were then covered at room temperature for 15 minutes. After removal of the SBM from the stainless steel, each coupon was swabbed using a randomly assigned swab type. Following the 15-minute rest period, the SBM was removed from each polyethylene tote square; each square was swabbed with a randomly assigned swab type, and a 1 g sample of the inoculated

SBM was retained for analysis. Gloves were changed between handling and collection of each sample.

Experiment 2

Experiment 2 was set up as a 2 × 3 factorial plus control, utilizing 2 different inoculation levels of PEDV and three sample types (environmental, individual probe, and composite probe). For this experiment, 168 samples, consisting of environmental samples, individual probe samples, and combined composite probe samples were obtained from polyethylene tote bags containing 1 kg of SBM and then analyzed for PEDV. Two different levels of viral contamination were used in addition to a negative control; a "low" virus level of 10^3 $TCID_{50}$/g and a "high" virus level of 10^5 $TCID_{50}$/g. A total of 13 miniature totes were used for this experiment, with an environmental sample and 10 individual probe samples taken from each.

All initial inoculation and sample collection work were performed in a Class II Biological Safety Cabinet (BSC) located at the Kansas State University College of Veterinary Medicine.

To simulate real-world ingredient handling situations for each tote, 997.5 g of SBM were placed into a shallow aluminum tray. 2.5 g of SBM was inoculated at a 10:1 ratio; 0.5 g was retained for analysis and the remaining 2 g were dropped into the tray. The contents of the tray were then conveyed into a tote. The totes were tied closed and transferred to a separate container. After 15 minutes, totes were moved again, and the tops opened. Environmental samples were collected along the ingredient contact points of the top flap of the totes using Dacron-tip swabs. After environmental sampling, 10 individual probe samples were collected at varying depths and locations within the tote. 1 g of SBM was collected from each individual sample, and an additional 1 g from each location was taken and combined to produce a composite sample. Probes were

single-use plastic replicates of traditionally used grain probes. Gloves were changed between the handling and collection of each sample, and care was taken to prevent potential environmental contamination between samples and replicates.

Sample preparation and analysis

Experiment 1

Controls of virus were analyzed for both doses of PEDV to establish baselines, in addition to a negative control, resulting in a total of 87 samples. After collection, samples and swabs were refrigerated and transported to a BSL-2 laboratory located at the Kansas State University Research Park. 20ml of PBS were added to dust-mop swabs, 10 ml of PBS to the cotton gauze swabs, and 9 ml of PBS added to the SBM samples. Swabs were stored overnight at 4˚C prior to analysis. Samples of inoculum, SBM, and swabs were analyzed for PEDV via qRT-PCR with an upper cycle threshold (Ct) value limit of 40.

Experiment 2

After collection, samples were refrigerated and transported to a BSL-2 laboratory located at the Kansas State University Research Park. 7.2 pH PBS was added to all ingredient samples at a 1:5 ratio. Samples were stored overnight at 4˚C prior to analysis. Samples of inoculum, SBM, and swabs were analyzed for PEDV via qRT-PCR with an upper cycle threshold (Ct) value limit of 40.

Statistical Analyses

Experiment 1 data were analyzed via the GLIMMIX procedure of SAS (Version 9.4, SAS Institute Inc., Cary, NC, USA), using a 2 × 2 × 4 factorial design with two levels of inoculation, two surface types, and four swab types). Experiment 2 data were analyzed using GLIMMIX using a 2 × 3 factorial design with two levels of inoculation and three sample types. For both experiments, results were considered significant at $P \leq 0.05$.

Results

Experiment 1

There was a dose × surface type × swab type interaction ($P<0.0001$; SEM=0.967; Table 2.1). In stock virus and SBM, increased dose resulted in lower ($P<0.05$) detectable PEDV. However, no virus was detected in sponge-tip swabs used on stainless steel surfaces. Dacron-tip and cotton gauze swabs yielded the most detectable PEDV from surfaces but was still 1.2 to 5.1 Cycle threshold (Ct) higher than SBM. All control samples were confirmed to not contain any detectable PEDV RNA.

Experiment 2

There was a virus level × surface interaction ($P<0.05$, Table 2.2) on the prevalence and mean detection quantity of PEDV within the samples. All control samples were confirmed to not contain any detectable PEDV RNA, while the inoculum used for each tote were found to contain a mean Ct of 32.4 (low level) or 22.3 (high level).

There were no samples taken from totes inoculated with a low virus does that had detectable PEDV RNA present. When inoculated with high levels of virus, 22 individual samples

(out of 60) recovered PEDV RNA, with a mean Ct of 37.7. Composite samples from the 6 high virus totes all contained RNA, with a mean Ct of 35.7. Two of the 6 environmental samples were positive, with a mean Ct of 39.2.

Discussion

Experiment 1

Differences on detection were noted when both high- and low-level virus were present. There was a difference in overall detection levels when comparing stainless steel to polyethylene tote. This could potential be attributed to the more abrasive qualities of the woven texture of the polyethylene tote surfaces; it could have potentially abraded swab surfaces and allowed for an increase of surface area to pick up virus particles. For samples within both viral levels, PEDV Ct values in SBM samples was approximately 11 Ct higher than in stock virus/inoculant.

There are several factors that could influence the recovery within different swabbing methods, including swab moisture level, surface dryness, and the technique used to collect the samples[10]. Additionally**,** biofilms that are naturally occurring can also impact recovery for microbiological samples, and there is limited data available on biofilm presence in feed manufacturing facilities, as well as how the presence of biofilms could potentially impact virus survival or detection. Traditionally, environmental monitoring swabs have been developed for use within environments that are easily sanitized and cleaned; feed manufacturing environments are inherently dirty, which may impact the efficacy of swabs. Therefore, traditional environmental or hygiene swabbing techniques should be adapted to better handle these changes. Sponge tip applicators are used for bacterial monitoring, but as shown in these data, do not have nearly as good of viral recovery when in an environment typical of feed manufacturing. A 2007 study

comparing several types of swabs on recovery of *E. coli* from stainless steel coupons found that type of swab did impact recovery; with Dacron swabs performing better than both rayon and cotton-tipped swabs.[11] In a study that compared detection of *L. monocytogenes* on three different surfaces commonly found in food manufacturing facilities (stainless steel, high density polyethylene, and rubber), sponge stick swabs had the worst recovery when compared to both a foam-tip and a dense-tip environmental swab.[12] While the poorer performance of the sponge-tip swab is consistent to the PEDV recovery, the data in these studies demonstrated that cotton gauze squares performed similarly to the Dacron-tip swabs. This could potentially be attributed in part to the physical differences between viral and bacterial particles, or the addition of organic material to the sample collection area. While dry dust-mops have been used for environmental monitoring in feed environments, they do not perform as well as cotton-based swabs within the two surfaces tested, especially in environments where the levels of virus present are low. When higher levels of virus are present, the dry dust-mop performed similarly to both the Dacron®-tip applicator and cotton gauze swabs; this could be due to the lack of a wetting agent present when swabs were taken.[13] There was also a difference between surface areas of individual swabs; the Dacron®-tip applicator and sponge-tip swab had smaller surface exposed to the contaminated surface than the cotton gauze swab, and all 3 had smaller exposed areas when compared to the dry dust mop. The physical characteristics of each swab also differed, which may have contributed to the difference in detection. The sponge-tip swab was very porous compared to the other three swab types; the dry dust-mop exhibited characteristics that would indicate some static charge differences when used on the stainless steel and polyethylene tote coupons, and the Dacron®-tip applicator was very dense in comparison to both the dry dust-mop and cotton gauze square.

There was also a difference in performance at the two different viral levels. When low levels of virus were present, the dry dust-mop and sponge tip swabs had decreased performance when compared to the Dacron®-tip and cotton gauze swabs. The dust-mop swab did have increased detection when higher levels of virus were present, while the sponge-tip swab remained above the limit of detection (40 Ct). Both the cotton gauze and Dacron®-tip swabs performed similarly at both viral levels, with a consistent decrease in measured Ct from the SBM of approximately 11 Ct.

It should be noted that while the two swabs performed similarly, the cotton gauze swabs require material that is more readily available to producers and clinicians. While some preparatory work was necessary, it is possible to store swabs for future use. The Dacron®-tip swabs had to be purchased from a specific supplier, and require refrigeration prior to use, which could be challenging for some applications.

Experiment 2

Prevalence of PEDV varied across viral level, with no RNA detected within any sample at the lower viral inoculum level. This potentially could be attributed to the different challenges associated with recovery of nucleic acids in ingredient or environmental samples especially considering the generally accepted loss of 10-13 Ct of sensitivity when comparing an inoculum and a feed or ingredient sample. [6, 9, 14, 15] Due to the lower starting level of the virus for this set of samples (32.4 Ct) and the current upper detection limit of 40 Ct, some of the lack of detection with the low-level samples could be attributed to this difference. This potential inability to detect virus present at a level below the minimum infectious dose within a feed matrix is cause for concern, as

cases of samples without PCR detected RNA have been documented to cause infection within swine bioassays[14].

The samples taken from totes with higher levels of virus present (22.3 Ct contaminate) did have a higher level of recovery across all sample types; 37% of the individual probe samples, 100% of composite samples, and 33% of environmental samples were found to contain viral RNA. This further supports that the limitations of qt-PCR for viral RNA detection could have impacted the results of the samples taken from totes contaminated with the lower level of PEDV. It also helps support previous data showing that bulk sampling methods can detect contamination that is not evenly distributed within an ingredient.[16, 17] The creation of a composite sample from 10 individual samples was able to detect contamination in all of the high virus totes and is more sensitive for viral detection than either individual probes or environmental swabs, it is important to point out that composite sample collection takes more manhours due to the collection of 10 samples instead of one. This has the potential to create logistical strains on facilities if utilized instead of individual samples or environmental swabbing.

Only 33% of the environmental samples collected from high PEDV contaminated totes had PEDV RNA detected, contrary to previous research which would suggest that the presence of PEDV RNA is more consistent in environmental samples than product samples.[8] While every attempt was made to accurately scale-down this project for a benchtop model, there were visual differences noted in the amount of dust accumulation on the miniature totes when compared to typical industry environments, potentially due to the air handling system within the BSC where the experiment was formed.

In addition to the noted loss of analytical precision when moving from inoculum to feed matrix or product to environmental swabs, rapid detection PCR methods have been developed for

specific RNA viruses for clinical sample use[18] and measure presence, not viability of virus within samples. Viral samples may contain viable and non-viable genetic material, which is currently not distinguished between by PCR. PCR results from environmental swabs for viral pathogens should be interpreted as an indicator of presence, not communicability or viability.

Further research is needed to develop and determine how best to utilize sampling methods within a feed manufacturing environment for disease monitoring. Current technology and lack of ability to extract and detect nucleic acids from feed and ingredients creates limitations on the use of PCR to detect virus in such samples, especially when low levels of virus are present. Environmental monitoring may provide a more comprehensive analysis of pathogen presence in feed manufacturing facilities when compared to more traditional raw material or finished product sampling, but only in environments where virus levels are high.

Conclusion

In summary, environmental swabbing probe sampling can detect the presence of RNA viruses such as PEDV, but with limitations in environments with lower levels of virus present, and at less precision than product samples. Accuracy has been demonstrated to be dependent upon swab type for detection of PEDV; of the swabs tested, Dacron®-tip and cotton gauze are superior (more accurate) for environmental analysis than dry dust-mops. Sponge-tip swabs should not be used for environmental monitoring of PEDV. Analysis of a single probe sample from a bulk ingredient is not a sensitive or reliable method for detecting viral presence, but composite sampling consisting of at least 10 individual samples can accurately determine presence of PEDV. Further research, development, and validation of both sample collection tools as well as laboratory techniques is necessary to fully utilize environmental monitoring moving forward.

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Figures and Tables

Table 2.1 Impact of porcine epidemic diarrhea virus (PEDV) dose, manufacturing surface type, or swab type on PEDV detectability as determine by qRT-PCR[1]

	PEDV dose, $TCID_{50}$		
Item;	None	10^3	10^5
Virus	40.0[a]	21.4[h]	14.3[i]
Soybean meal[1]	40.0[a]	32.6[c]	25.8[g]
Stainless steel			
Sponge-tip	40.0[a]	40.0[a]	40.0[a]
Dry mop	40.0[a]	39.0[a]	32.6[c]
Dacron-tip	40.0[a]	35.7[b]	29.5[de]
Cotton gauze	40.0[a]	35.6[b]	30.9[d]
Polyethylene Tote			
Sponge-tip	40.0[a]	40.0[a]	39.4[a]
Dry mop	40.0[a]	36.0[b]	29.6[d]
Dacron-tip	40.0[a]	34.1[c]	28.1[ef]
Cotton gauze	40.0[a]	34.1[c]	27.9[f]

[1]Soybean meal was inoculated with PEDV, then placed on stainless steel followed by polyethylene tote coupons. After removing the soybean meal, surfaces were swabbed with one of four types. Samples were analyzed for PEDV via qRT-PCR, and results reported as cycle threshold (Ct). There were three replicates per treatment. There was a dose×surface type×swab type interaction ($P<0.0001$; SEM=0.967).

[abcdefghi]Means within the table without a common subscript differ $P<0.05$.

Table 2.2 Impact of sample type and Porcine epidemic diarrhea virus (PEDV) dose on PEDV detectability as determined by qRT-PCR[1]

PEDV Dose	Contaminant	Individual Probes	Composite Sample	Swab	SEM
Prevalence of samples containing PEDV, %					
Control	-	0 (0/10)	0 (0/1)	0 (0/1)	-
Low (10^3 $TCID_{50}$/g)	100[a] (6/6)	0[c] (0/60)	0[c] (0/6)	0[c] (0/6)	12.7
High (10^5 $TCID_{50}$/g)	100[a] (6/6)	37[b] (22/60)	100[a] (6/6)	33[b] (2/6)	12.6
Mean quantity of PEDV, Ct					
Control	> 40	> 40	> 40	> 40	-
Low (10^3 $TCID_{50}$/g)	32.4[d]	> 40[a]	> 40[a]	> 40[a]	0.85
High (10^5 $TCID_{50}$/g)	22.3[e]	37.7[b]	35.7[c]	39.2[d]	0.92

[1] Soybean meal totes were contaminated with PEDV inoculated soybean meal, sampled by three different methods. Samples were analyzed for PEDV via qRT-PCR, and results reported as cycle threshold (Ct). There were six replicates per treatment, consisting of either one environmental swab, 10 individual probes, or one composite probe sample.

[abcde] Means within the table without a common subscript differ $P<0.05$.

Chapter 3 - The application of environmental monitoring of PEDV within a swine system during a disease outbreak

Abstract

The use of environmental swabs as a monitoring tool was implemented during a 2019 porcine epidemic diarrhea virus (PEDV) outbreak that occurred at the Kansas State Swine Teaching and Research Center (FARM), a farrow to finish facility located in Manhattan, KS. A series of environmental samples were collected to be used as an evaluation tool for biosecurity practice improvements over the course of 16 weeks after initial infection. Environmental swabs were analyzed for both PEDV and rotavirus using quantitative real-time PCR. In addition to the environmental monitoring on-farm, the feed manufacturing facility was swabbed, as well as two close-proximity sites containing pigs. Throughout the duration of the sample collection, the only site with a positive result was the FARM. Viral testing results were communicated to farm staff after collection, and changes in biosecurity practices were implemented by the facility veterinarian based on results as well as employee suggestion. Separation of on-farm areas into six different zones determined by animal and feed ingredient contact and proximity (vehicles, direct pig contact surfaces, non-pig contact surfaces within individual barns, surfaces in the main office, direct feed contact surfaces, and non-feed contact surfaces) allowed for a targeted approach to clean-up efforts, as well as evaluation of the efficacy of control efforts. Additionally, the zone monitoring allowed for rapid feedback to employees during clean-up, and changes in positive samples between collection timepoints were noted.

Keywords: environmental monitoring; PEDV; feed

Introduction

Environmental monitoring has been commonly used in food and other facilities manufacturing end-consumer products for years[1,2]. Recently, it has begun to gain traction as a method to determine the presence of pathogens that typically indicate fecal presence (fecal indicators)[3]. In addition to facilities producing direct-to-consumer goods, some healthcare systems have used environmental monitoring of both virus and bacteria to determine hygiene and biosecurity risk, including bacteria strains known to be resistant to antibiotics[4]. The potential use of environmental monitoring of viral pathogens within a farm environment has seen an increase in popularity with the growing pressure placed on production systems from diseases like porcine epidemic diarrhea virus (PEDV) and rotavirus. Environmental swabs have been shown to be an effective method to detect viruses within feed manufacturing environments[5] and with on-farm use for swine operations. The ability for PEDV to be transmitted via contaminated feed ingredients and for contaminated feed to produce animal illness within research settings[6,7], as well as the epidemiological evidence to support historical animal feed transmission within North America[8,9] has brought increased levels of scrutiny on mills supplying feed to swine operations.

Despite the documented use within several industries, there is also a lack of information available on the impact that environmental monitoring results can have on employee behavior and engagement within a facility.

The present investigation aimed the presence of PEDV within a system currently experiencing a PEDV outbreak, as well as the attempt to minimize PEDV transmission through the combined use of biosecurity practices and environmental monitoring. It also attempted to evaluate the efficacy and cost-efficiency of using different environmental monitoring indicators as tools during a disease outbreak.

Case summary and timeline of changes

The opportunity to evaluate the impact of environmental monitoring arose when the Kansas State Swine Teaching and Research Center (FARM) experienced an outbreak of PEDV in spring 2019. The facility includes sow, nursery, and finisher housing, separated into different barns based on phase (Figure 3.1) and maintains a 160-head batch farrow sow herd, with additional group housing for nursery, growing, and finishing pigs. On March 8, 2019, a group of weaned pigs were noted with scours; over the course of the next two days diarrhea was noted within the gestation barn. Fecal samples submitted to the Kansas State University Veterinary Diagnostic Laboratory (KSUVDL) and analyzed using PCR confirmed the presence of PEDV at the facility.

Due to the teaching and research mission of the FARM, there is typically a considerably higher level of foot traffic from students and researchers than would be found on a typical swine operation of this size. However, the herd has historically maintained a high health status and was naïve to PEDV at the time of infection in March 2019, having avoided the disease entirely until that time. Being a farm with high health, there had been limited environmental monitoring done at the site prior to the outbreak.

Biosecurity prior to outbreak

Pre-outbreak biosecurity procedures included a fenced perimeter buffer zone with limited vehicle and personnel access, off-site quarantine and PRRS testing of new gilts for 8 weeks prior to farm entry, and requirements that supplies delivered are from pig-free areas of origin. Personnel and visitor entry were restricted, with visitor policies posted and a visitor log used to document all visitor access to the facility. Initial requirements for entry consisted of the use of a Danish bench system to establish a clear line between the farm perimeter and changing area. Outside footwear

was not permitted to cross the bench, with all entrants required to don provided coveralls and boots once through the shower. Showering into the facility was only required in situations where prior exposure to pigs, livestock facilities, processing plants, or laboratories handling known pathogens or diagnostic samples had occurred earlier on the day of the visit (less than 1 night of downtime). The area prior to crossing the Danish bench was considered dirty, with the showers and change rooms acting as an intermediary between it and the transition zone within the main office area (Figure 3.2). The office area itself contained two different access points to the outside paths leading to different barns; the only requirement for moving between areas or barns was to rinse visible debris from boots using water and change gloves.

Day 0

Upon the confirmation of PEDV within the facility, changes in biosecurity procedures were implemented, with the use of environmental monitoring of the facility to help determine efficacy of the changes and help determine when or where additional changes in procedure were necessary. The first changes in biosecurity procedures at the facility (Phase 1) were implemented immediately after fecal samples tested positive for PEDV. Specific changes included: restricting all non-essential access, designating employees to specific areas when possible, requiring employees to wear boot covers and gloves between the farm entry and their vehicles, implementing a barn-specific captive boot system, and instituting a feed transfer zone consisting of both a feed truck to deliver feed from the mill as well as a truck that remained on-farm (Figure 3.3).

Day 14

Environmental swabs were taken throughout the facility 14d after the initial diagnosis and biosecurity process changes. The samples indicated that there was viral RNA present throughout the facility (Figure 3.4), including in areas that were expected to not have RNA present, such as transition zones surrounding the main office as well as the main entrance to the farm. As expected, the areas where animals were still present did have the highest level of viral RNA present. The evidence of viral spread throughout the facility triggered additional changes to the biosecurity procedures. Phase 2 biosecurity procedure changes included requiring employees change coveralls when moving between barns, a considerable change to prior protocol. Requiring that entrants don a set of scrubs upon initial entry into the farm enabled workers to don and doff coveralls upon entering and exiting individual housing units, and to return to the main office without bringing coveralls or boots back. Additional transition zones and Danish bench entry procedures were added to individual barns as well as when returning to the main office. The main office area was swabbed again 4 days later, and a reduction in viral RNA presence within transition zones was reduced or eliminated (Figure 3.5).

Day 28

Additional changes were implemented after environmental monitoring done 28 days after diagnosis indicated that there was still some viral RNA transfer between the barns and main office, specifically in transition zones and near the laundry facilities (Figure 3.6). The areas with pigs, both animal-contact as well as within the housing buildings were still contaminated, and despite the changes implemented, there was still viral RNA present within the main office. After this timepoint, an additional laundry area was set up to allow for the separate laundering of outer

clothing being worn in barns and the clothing being worn under coveralls when in the main office or walking between barns.

Continued evaluation (day 42-115)

Environmental monitoring continued for four additional timepoints. After 115 days post-diagnosis, all transition zones to the main office had no detectable viral RNA present, despite some presence still being noted within individual barns (Figure 3.7). Throughout the monitoring period, results of the environmental monitoring were communicated with on-farm employees to help communicate efficacy of the changes.

Materials and methods

Swabbing method and locations

Samples were collected at 7 different timepoints by swabbing a surface area of approximately 20 cm × 20 cm with a 10 cm × 10 cm cotton gauze square soaked in 5 ml of phosphate buffered saline (PBS) with a pH of 7.2, as described by Griener[10] and were analyzed for PEDV and rotavirus. Throughout the duration of this disease outbreak, environmental swabs were taken biweekly. Samples were analyzed for PEDV and rotavirus. Additional analysis was run on separate samples to evaluate for presence of Enterobacteriaceae.

After the extensive initial environmental swabbing of the feed manufacturing facility did not obtain any positive results, 10 collection areas deemed to be high-risk for potential fecal contamination were selected for continued monitoring. The sample locations were split into two separate zones; surfaces that come into direct contact with finished feed or feed ingredients (feed

contact surfaces), and surfaces that do not come into direct contact with feed or feed ingredients (non-contact surfaces).

On-farm swabbing locations had more variation between collection timepoints, but sample points were selected within one of four zones. The zones included on and off-farm vehicles, including feed delivery trucks, tractors, and employee vehicles (vehicles), direct pig contact surfaces including pen flooring, pen walls, feeders, and waterers (pig contact), non-pig contact surfaces within one of the barn areas including employee walkways, work areas, feed storage, and in-barn transition zones (non-pig contact), and surfaces in the main office building including laundry areas, change rooms and shower areas, and transition zones upon entering and exiting the building (main office).

Biosecurity updates were reported biweekly to farm employees, with adjustments made to biosecurity protocols accordingly between swabbing timepoints.

Sample preparation and analysis

Environmental swabs submitted for viral testing were initially prepared by adding 5 ml of PBS to a cotton gauze square in a conical tube. After samples were collected, swabs were transported to a laboratory where 20 ml of additional PBS were added. Swabs were stored overnight at 4˚C the submitted to KSUVDL for quantitative real-time PCR, with an upper Ct limit of 45 for PEDV and 40 for rotavirus.

Statistical Analysis

PCR results for viral analysis were analyzed and reported in both Ct level and prevalence of positive PCR test within combination of location and sampling week. Prevalence of PCR

positive samples was fit using a binary distribution with each sample either being PCR positive or negative. All data were analyzed using the GLIMMIX procedure of SAS (Version 9.4, SAS Institute Inc., Cary, NC) with the Tukey-Kramer adjustment using the assigned location zones as the levels with the response variables of PEDV Ct and rotavirus Ct. Treatment means were separated using pairwise comparisons of means performed using the DIFFS option from the LSMEANS statement of SAS. For the analysis for prevalence of PCR positive test results, degrees of freedom were approximated using the Kenward-Roger approach and the LSMEANS procedure was used to output interactive means. The SLICEBY option was used to slice interactive means by both factors and pairwise means separation within simple effects was performed using the LINES option for interpretation of interactive effects using a Bonferroni multiple comparison adjustment to control type I error rate. Results were considered significant at $P \leq 0.05$ and marginally significant if $0.05 < P \leq 0.10$.

Results

Zoning and containment boundary areas

Utilizing environmental monitoring during the disease outbreak helped illustrate the ever-changing situation. A reduction in positive samples across multiple zones was seen, specifically within transition areas and areas outside of barns, as shown in Table 3.1. Upon initial sample collection at 2 weeks post-infection, 44% of samples obtained from exterior areas adjacent to the facility perimeter (worker's vehicles, on-site student housing, and near the entry bench) tested PCR positive for PEDV. At that same timepoint, 81% of the transition zone areas (including the shower/changing area and main office) as well as 66% of samples from non-pig contact areas within the barns were PCR positive.

Reduction in the number of PCR positive results was seen beginning at week 4; there was a 29% reduction in the positive results outside of the perimeter boundary, a 60% reduction in positive samples seen in transition zones, a 16% reduction in non-pig contact areas outside of barns, and a 20% reduction in non-pic contact areas within barns. At this timepoint, environmental monitoring of pig-contact areas was also initiated; this remained 100% PCR positive until the final collection (16 weeks). These reductions were not necessarily steady throughout the entire data collection period, but upon the final collection at 16 weeks post infection, samples collected outside the perimeter, within transition zones, and in non-pig contact areas outside of barns had remained negative for 4 weeks.

There were differences noticed across the 6 identified zones with similar PEDV Ct.. Mean PEDV Ct were impacted by zone; zones 1, 5, and 6 were all negative for the duration of the monitoring, while the mean of zone 4 was lower ($P \leq 0.05$). Comparing the zones with PEDV RNA detected by PCR observed, the mean Ct of zone 4 (the main office area) and zone 3 (non-pig contact surfaces within barns) was higher, while zone 3 and zone 2 (pig contact surfaces) shared similar mean Ct.

There was no evidence of a sampling location × sampling week interaction ($P = 1.000$). In addition to the test of interactive effects, interactive effects were sliced by both factors to evaluate the simple effects due to missing combinations of sampling location and sampling time. There was no evidence that the prevalence of PCR positive test results differed over time within a sampling location ($P < 0.05$) or between locations within a sampling week ($P < 0.05$). Within the transition zones, there was marginally significant evidence ($P = 0.056$) that the prevalence of PCR positive test results differed among the weeks evaluated, and further characterization demonstrates that the prevalence of PCR positive results for PEDV for week 4 was less than week 2 ($P = 0.043$). There

was no evidence that the prevalence of PCR positive test results differed among other sampling timepoints within the transition zone ($P \geq 0.104$).

Discussion

The results of this study were evaluated by separating samples out into those that had PEDV RNA detected by PCR and those that did not. While the PCR results were reported using quantifiable Ct, due to the nature of the environmental samples collected in the dynamic environments of a feed mill and swine farm, forgoing the numerical values and instead running analysis on the number of samples with or without detectable PEDV RNA helps account for the variety of surface types and locations. Feed mills contain a wide variety of equipment shapes, sizes, and surface types, so using the reported Ct as a comparison between sample locations may not be the best indicator of viral presence. Focusing on the number of PCR positive samples is a better approach when faced with data that includes samples from a wide variety of surfaces and equipment.

Additionally, these data illustrate how difficult it can be to eliminate a viral contaminant like PEDV from a premise once infected. All pig-contact surfaces swabbed throughout the 16-week sample collection period had PEDV RNA detected by PCR until week 16, when there was a 25% reduction in the number of samples with PEDV RNA detected by PCR observed. Extensive disinfection of the facility, including the areas swabbed was done throughout the timeframe.

Implementation of biosecurity changes

After receiving a positive PEDV diagnosis, there were initial changes that were made to the biosecurity plan. This included requiring new coveralls to be put on when entering a new area

or room and halting all non-essential entry into the farm; students and faculty who would potentially be visiting the facility for research or class were not allowed on farm. Essential employees were assigned to specific areas; either working exclusively in the finishing rooms, farrowing and nursery areas, or the breeding and gestation barns.

Since a small particle of contaminant has the potential to infect large quantities of a feed or feed ingredient, there was concern surrounding the feed delivery protocol that was standard practice when the outbreak first occurred. In order to help minimize the risk of transmission to the feed mill and other off farm areas, a change was made in the feed delivery protocol; instead of the feed delivery truck driving from the feed mill onto the farm to fill the bins, the driver began bringing the truck to the perimeter barrier and transferring the feed to an intermediary truck that remained within the perimeter.

Based on the environmental data gathered across the different timepoints, the spread of virus through the compound was able to be tracked to an extent. While the feed mill remained negative for the duration of the outbreak, the virus was found in areas within the farm that were not initially observed to have PEDV RNA detected by PCR, as well as within transition zones, indicating that the initial changes to the biosecurity protocols were not successful in limiting viral spread within the facility. This led to further enhancements to the protocol, including requiring gloves and boot covers to be worn by all entrants from their vehicles to the farm entrance bench, and instituting a captive boot system for each specific barn. The farm was separated out into three main areas, finishing, farrowing and nursery, and breeding and gestation, with a more strictly enforced separation of employees within them.

Lasting changes to the biosecurity protocol were implemented throughout the duration of the outbreak. Entrants to the farm now don scrubs after passing through the entrance, putting on

clean coveralls over the scrubs prior to entering a barn and then removing the coveralls and leaving them in a dirty laundry collection area prior to returning to the main office. Dirty laundry is transported in a bio-secure manner to a designated laundry area when necessary. Boot covers are worn while walking between the main office and the barns and changed prior to entering the main office through either transition zone.

The main office and shower/entrance areas are disinfected multiple times per day, and clearly visible transition zones or swing benches were placed in barns where not already present.

Biosecurity protocols were sculpted and adapted as the biweekly environmental swabbing results were reported. Problem areas were noted, especially locations that had multiple PCR detection of PEDV RNA present results across different timepoints. Specific areas of concern included on-site vehicles, including those that were being used to transport or dispose of waste or carcasses, transition zones in barns as well as those within the main office, and areas within the main office that were part of the “clean” area in the biosecurity plan.

Additionally, to minimize the risk and in an attempt to minimize risk, periodic environmental monitoring has continued after the initial data collection period described within this paper. Biosecurity procedures have continued to evolve, and now include showering in and out of the facility in addition to the prior changes. Each facility has individual needs, and these data gathered over the data collection period help illustrate the need for customized protocols and re-evaluation when situations change. Biosecurity plans should be living documents and reviewed as needed.

Employee response

Workers play a huge role in any facility's biosecurity, and employee buy-in and compliance can make or break a biosecurity plan. Being able to show tangible metrics surrounding the cleanup effort after a disease outbreak can serve as an informational and motivational tool while trying to get employee buy-in. After each timepoint within the data collection, results of different areas were reported back to employees on farm. This allowed for problem areas to be pointed out and addressed, in addition to help influence areas requiring additional manpower or attention. Sharing the data with the employees also helped with finding solutions to reoccurring issues; for example, the laundry area for the facility was originally located directly next to the showers. The suggestion of adding a laundry area within the "dirty" area of the facility resulted in the elimination of positive results within the shower area at the next data collection timepoint. While there were continuing PCR results with RNA presence indicated in some areas, there was a marked improvement in areas of high concern, and employees were very responsive, especially with the initial push for tighter biosecurity regulations. As time went on, some areas did see a relapse into higher amounts of PEDV RNA indicated, which could be attributed in part to employee complacency. Without having the environmental data collected at these timepoints, there would be no tangible way to measure or rectify the increase.

Conclusion

Environmental monitoring can be an important tool for managing a disease outbreak within a system. In this case, it was able to help indicate locations with a potential for disease spread outside of the infected perimeter, as well as identify areas within the farm with holes in the

biosecurity plan. Ensuring open communication and information transfer to on-farm staff is crucial during outbreaks and subsequent clean-up attempts.

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Figures and Tables

Figure 3.1 Map of Kansas State Swine Teaching and Research Center (FARM)

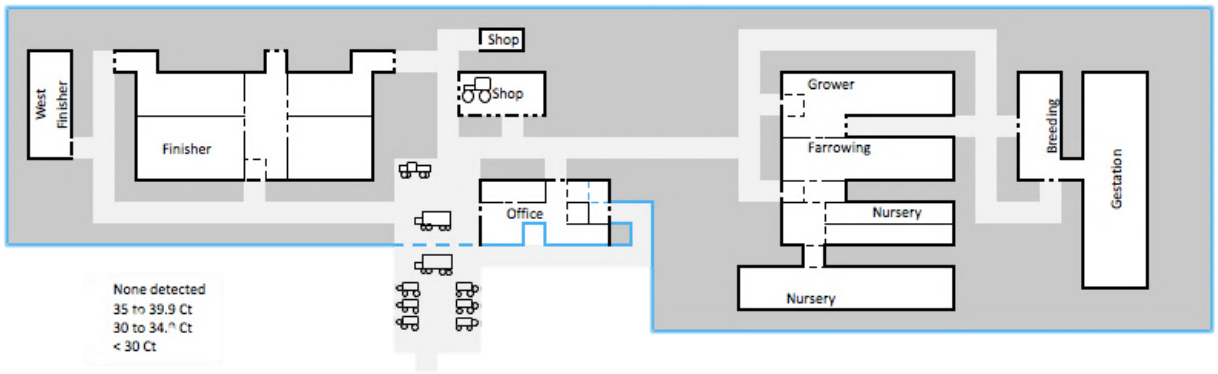

Figure 3.2 Main office space on FARM

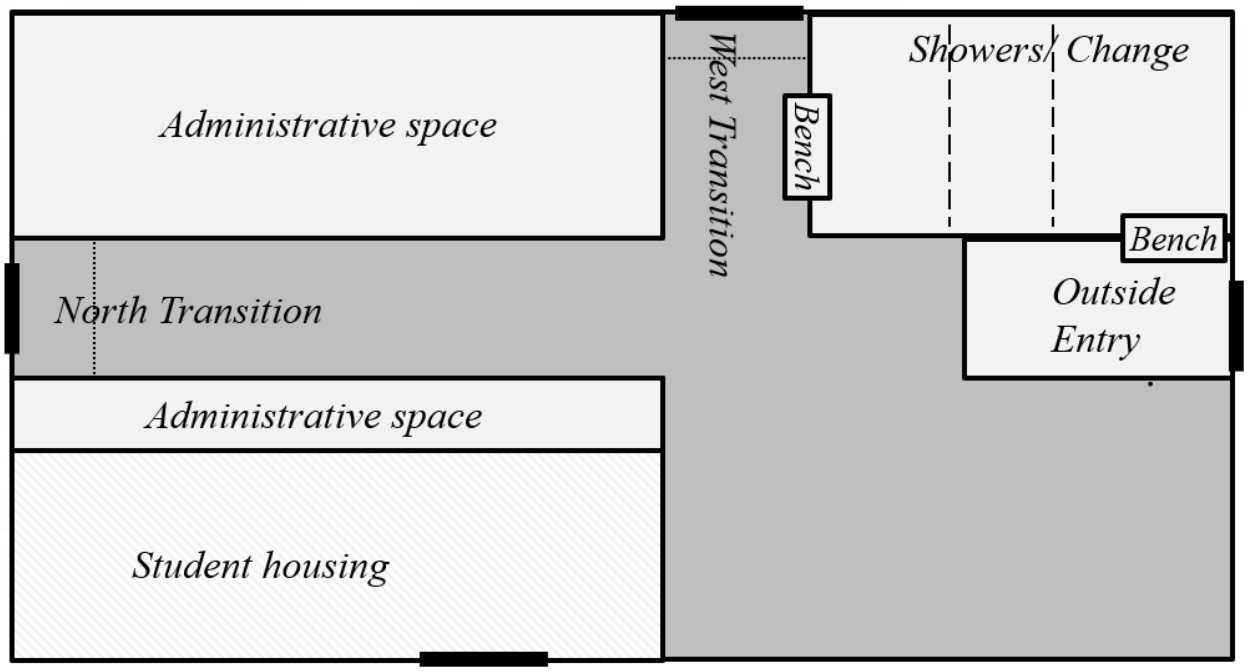

Figure 3.3 Feed transfer system implemented at FARM

Figure 3.4 Porcine epidemic diarrhea virus (PEDV) RNA levels 14 days post-diagnosis

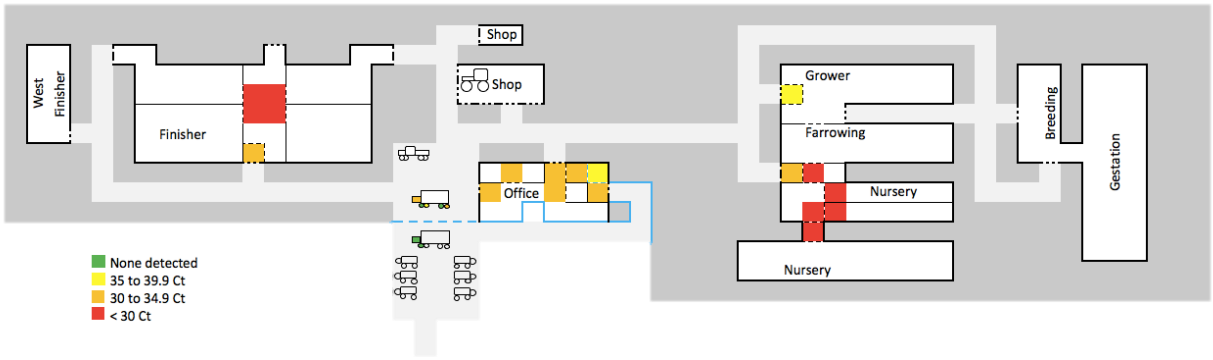

Figure 3.5 PEDV RNA levels 18 days post-diagnosis

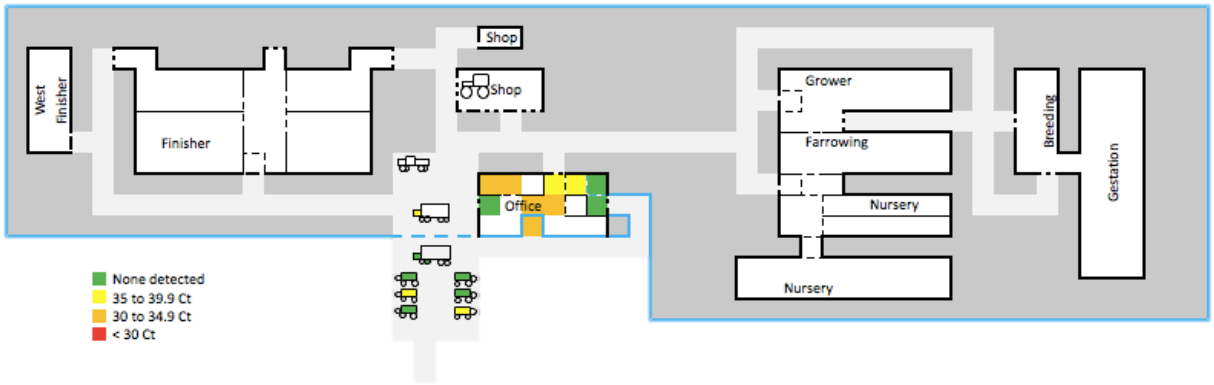

Figure 3.6 PEDV RNA levels 28 days post-diagnosis

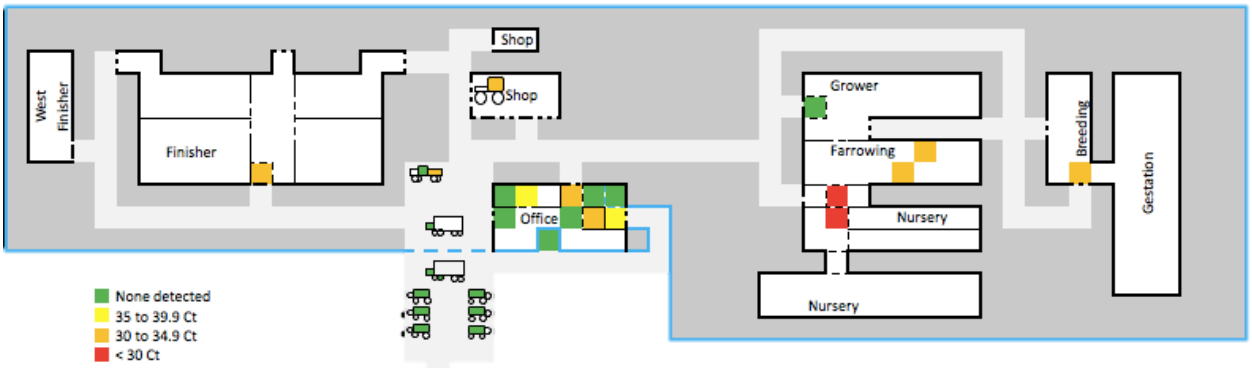

Figure 3.7 PEDV RNA levels 115 days post-diagnosis

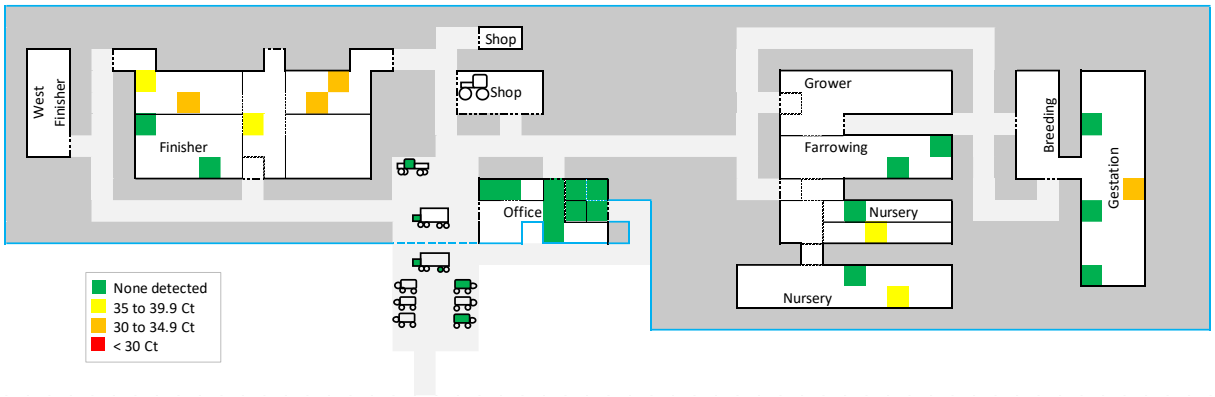

Table 3.1 Percentage of PCR + PEDV results in different farm locations across collection timepoints

	Weeks after initial diagnosis					
	2	4	6	8	12	16
Zone:						
Outside perimeter	44% (4/9)	13% (1/8)	0% (0/1)	25% (1/4)	0% (0/2)	0% (0/3)
Transition zones	81% (13/16)	21% (3/14)	29% (4/14)	44% (4/9)	0% (0/6)	0% (0/7)
Non-pig contact (outside)	66% (4/6)	50% (2/4)	25% (1/4)	NS	0% (0/4)	0% (0/5)
Non-pig contact (inside)	100% (12/12)	80% (4/5)	88% (8/9)	75% (3/4)	100% (4/4)	80% (4/5)
Pig contact	NS	100% (2/2)	100% (2/2)	100% (4/4)	100% (4/4)	75% (3/4)

NS= not sampled.

Location × sampling week interaction ($P = 1.000$).

The slice for the transition zone was marginally significant, with evidence of a difference in prevalence between week 2 and week 4, but no evidence of a difference across other pairwise comparisons within that slice.

There was no evidence that the prevalence of PCR positive test results differed over time within a sampling location ($P < 0.05$) or between locations within a sampling week ($P < 0.05$).

Chapter 4 - Indicators of biosecurity on farms and feed mills

Introduction

Environmental monitoring is used in food and other facilities manufacturing end-consumer products[2], and has gained traction as a method to determine the presence of pathogens that typically indicate fecal presence (fecal indicators)[3]. In addition to facilities producing direct-to-consumer goods, some healthcare systems have used environmental monitoring of both virus and bacteria to determine hygiene and assess biosecurity risk, including bacteria strains known to be resistant to antibiotics[4]. A correlation between the presence of *Salmonella* spp. and Enterobacteriaceae within feed mills has been demonstrated[5], but little information is available on how the presence of Enterobacteriaceae correlates with viral pathogen presence. The potential use of environmental monitoring of viral pathogens within a farm environment has seen an increase in popularity with the growing pressure placed on production systems from diseases like porcine epidemic diarrhea virus (PEDV), senecavirus A (SVA), and rotavirus. Environmental swabs have been shown to be effective when detecting viruses within feed manufacturing environments[6] and with on-farm use for swine operations. The ability for PEDV to be transmitted via contaminated feed ingredients and for contaminated feed to produce clinical disease within research settings[7,8], as well as the epidemiological evidence to support a historical animal feed transmission within North America[9,10] has brought increased levels of scrutiny on mills supplying feed to swine operations.

Two studies were conducted within different swine systems in an attempt to both evaluate the presence of Enterobacteriaceae when compared to PEDV and rotavirus within a system which experienced a PEDV outbreak, as well as compare two different testing methods for Enterobacteriaceae (traditional laboratory culture analysis and “rapid” on-site detection). An

additional study attempted to identify Enterobacteriaceae presence in the feed manufacturing facilities of a multi-farm system experiencing a viral outbreak.

Background

Study 1

The Kansas State Swine Teaching and Research Center (FARM) experienced an outbreak of PEDV in Spring 2019. The facility includes sow, nursery, and finisher housing, separated into different barns based on phase and maintains a 160-head batch farrow sow herd, with additional group housing for nursery, growing, and finishing pigs. There is typically a considerably higher level of foot traffic in and out of the FARM from students and researchers than would be found on a typical swine operation of comparable size. However, there has historically been high health at the facility and because of this, little need for environmental monitoring at the site prior to the outbreak. Throughout the duration of the outbreak and subsequent facility clean-up efforts, environmental samples were taken within barns, the office, on-farm transportation, and the feed manufacturing facility. Samples were analyzed for both viral and Enterobacteriaceae presence. Both cultured laboratory and a rapid on-site Enterobacteriaceae environmental samples were taken for comparison.

Study 2

A multi-farm swine system experienced viral disease outbreaks among several sites during 2018. In an effort to determine feed manufacturing facility hygiene and establish areas of concern, three different feed mills supplying feed to the affected sites were swabbed and tested

for Enterobacteriaceae. Three feed mills located in south-central Brazil, in addition to one multiplication site being fed by one of the mills, were sampled. Bacterial data were collected with samples across different areas (zones) of the facilities to pinpoint areas of potentially higher biosecurity risk or areas of lower hygiene.

Materials and Methods

Swabbing method and location

Study 1

Throughout the duration of this disease outbreak, environmental swabs were taken biweekly. Samples were analyzed for PEDV, rotavirus, and Enterobacteriaceae. At three of the 7 timepoints, sponge-tip stick swabs were submitted for analysis of Enterobacteriaceae. Additionally, the Enterobacteriaceae samples submitted for laboratory testing were paired with a commercially available on-site or rapid Enterobacteriaceae test utilizing a luminometer (Hygiena, Camarillo, California, USA), currently being used in food manufacturing environments.

After the extensive initial environmental swabbing of the O.H. Kruse Feed Technology Innovation Center at Kansas State University did not obtain any positive results, 10 collection areas deemed to be high-risk for potential fecal contamination within the feed manufacturing process were selected for continued monitoring, including both feed-contact surfaces (receiving pit grates and the interior surface of the corn cleaner) as well as non-contact and environmental surfaces (floor mat in employee entrance, forklift tires, feed truck steps, feed cab surfaces, feed truck tires, truck scale, and control room floor) were taken.

On-farm swabbing locations had more variation between collection timepoints, but sample points were selected within one of four zones. The zones included on and off-farm vehicles,

including feed delivery trucks, tractors, and employee vehicles (Zone 1), direct pig contact surfaces including pen flooring, pen walls, feeders, and waterers (Zone 2), non-pig contact surfaces within one of the barn areas including employee walkways, work areas, feed storage, and in-barn transition zones (Zone 3), and surfaces in the main office building including laundry areas, change rooms and shower areas, and transition zones upon entering and exiting the building (Zone 4). Within the feed mill, feed-contact surfaces were considered Zone 5, while non-feed contact surfaces were labeled as Zone 6.

Biosecurity updates were reported biweekly to farm employees, with adjustments made to biosecurity protocols accordingly between swabbing timepoints.

Study 2

A total of 573 samples were taken over the course of four days, with 381 of those samples consisting of feed ingredient or finished feed, and the remaining 192 samples environmental swabs, collected across the 4 sites.

Feed ingredient and finished feed samples were collected using single-use plastic tubs. For each separate item, 10 individual samples were collected initially. For bulk-storage products, samples were either drop-collected or grabbed at multiple timepoints while being conveyed. For bagged products, samples were obtained from each of 10 different bags onsite. Each sample was kept separate for individual analysis, with an additional blended composite sample created from the 10 samples analyzed.

For the environmental samples, two different collection methods were used. Cotton gauze swabs were utilized for areas within the mill that had easy access for swab collection. The gauze swabs were collected by swabbing a surface area of approximately 20 cm x 20 cm with a 10 cm x

10 cm cotton gauze square soaked in 5 ml of phosphate buffered saline (PBS) with a pH of 7.2. For areas without easy access, such as the interior of storage bins or truck trailers, a paint roller was utilized, as described by Dee et al., 2014[10]. Locations did vary based on each individual site, but within the 3 feed manufacturing facilities (Sites 1-3), similar locations were chosen. Each swab was assigned one of four zones, including direct feed or ingredient contact surfaces (Zone 1), close proximity (within 1m) non-contact surfaces (Zone 2), non-contact surfaces without close proximity (>1 m of separation) (Zone 3), and transient surfaces, such as moveable tools, employees, and non-feed or ingredient delivery vehicles (Zone 4). Swabs taken from the fourth facility, the multiplier farm, were assigned zones based on proximity to pigs. This included direct feed-contact surfaces (Zone 5), direct pig-contact surfaces (Zone 6) including pen flooring, pen walls, feeders, and waterers (pig contact), and non-pig contact surfaces (Zone 7) including employee walkways, work areas, feed storage, and barn ventilation fans.

Sample preparation and analysis

Study 1

Rapid Enterobacteriaceae samples were prepared and analyzed on-site per manufacturer's instructions (Hygiena, Camarillo, California, USA), including the use of a two-part snap tube test system which includes both an enrichment device for initial collection and incubation and then a detection device for use in the luminometer. Initial swabs were brought to ambient temperature prior to use, then after collection were incubated for 8 hours at 38˚C. After the incubation period, the sample was placed into the detection device. The swabs were then analyzed using the Hygiena EnSure luminometer, which uses a bio luminogenic reaction to measure the quantity of Enterobacteriaceae present within the sample. Cultured Enterobacteriaceae samples were done so

on MacConkey agar with semi-quantitative counts reported by the Kansas State University Veterinary Diagnostic Laboratory, Samples were assigned an index value based on reported growth (cultured samples) or machine readout (rapid test). The cultured samples were assigned either 0, 1, 2, or 3 based on reported growth of either none, few/low, moderate, or heavy, respectively. Rapid test results were assigned a value of 0-3 based on the numerical readout at time of analysis, with 0 representing a negative result and values 1, 2, or 3 a low, moderate, or high positive result, respectively.

Study 2

Multiple samples of each feed and ingredient were collected. For each product, a composite sample was created by dividing and blending approximately 25 g from each individual sample. All product samples were stored at 4˚C until shipped.

Cotton gauze environmental swabs submitted for testing were initially prepared by adding 5 ml of PBS to a 10 cm x 10 cm uncoated cotton gauze square (Johnson & Johnson, New Brunswick, New Jersey, USA) in a 50 ml conical tube (SPL Life Sciences, Korea) prior to collection. After samples were collected, 20 ml of additional PBS were added. Swabs were kept at 4˚C until shipped. The paint rollers used for sample collection were placed into large zipper-seal plastic bags immediately after use. To prepare them for shipment, 200 ml of 7.2 pH PBS was added to each roller. The sample was then agitated and allowed to set for 1 hour. 10 ml of the PBS was removed from each sample and stored at 4˚C until shipped. After collection, samples were store and shipped on dry ice to the Iowa State Veterinary Diagnostic Laboratory. Samples were cultured on MacConkey agar, and the three types of bacteria with largest growth for each sample were identified and reported by assigning a growth index value.

Statistical Analyses

Study 1

PCR results for viral analysis were analyzed based on reported Cycle Threshold (Ct) level. The data were analyzed using the GLIMMIX procedure of SAS (Version 9.4, SAS Institute Inc., Cary, NC) with the Tukey-Kramer adjustment using the assigned location zones as the levels with the response variables of PEDV Ct, rotavirus Ct, rapid Enterobacteriaceae results and cultured Enterobacteriaceae results. Treatment means were separated using pairwise comparisons of means performed using the DIFFS option from the LSMEANS statement of SAS. Results were considered significant at $P \leq 0.05$ and marginally significant if $0.05 < P \leq 0.10$. Data were also analyzed with the CORR procedure of SAS, with the variables including PEDV, rotavirus, rapid Enterobacteriaceae results and cultured Enterobacteriaceae results.

Study 2

Bacterial growth results were assigned an index value of either 0, 1, 2, 3, or 4 based on reported growth, with 0 representing a negative result and values 1, 2, 3, or 4 a few, low, moderate, or high positive result, respectively. Growth values were reported as individual bacteria, with each sample receiving an overall index sum. The data were analyzed using the GLIMMIX procedure of SAS (Version 9.4, SAS Institute Inc., Cary, NC) with the Tukey-Kramer adjustment using the assigned location zones as the levels with the response variables of total growth (sum of index values) and presence of bacteria typical used to indicate fecal matter is present (fecal indicators). Treatment means were separated using pairwise comparisons of means performed using the DIFFS option from the LSMEANS statement of SAS. Results were considered significant at $P \leq 0.05$ and

marginally significant if $0.05 < P \leq 0.10$. Data were also analyzed with the CORR procedure of SAS, with the variables including site, zone, and presence of fecal indicator bacteria.

Results

Study 1

There were differences noticed across the 6 identified zones with similar PEDV Ct and the numerical index values assigned to the rapid testing. Mean PEDV Ct were impacted by zone. Zones 1, 5, and 6 were all negative for the duration of the monitoring. Zones 3 and 4 had lower ($P < 0.05$; Table 4.1) Ct values compared to zones 1, 5, and 6, and zone 2 had a lower Ct value ($P < 0.05$) compared to zone 4. There was no evidence of a difference in PEDV Ct value between zones 3 and 4. There was no detected difference ($P > 0.05$) within the mean rotavirus Ct or the numerical index values for the cultured Enterobacteriaceae testing reported across the zones. The rapid Enterobacteriaceae testing results did differ depending on zone location. Zones 1, 5, and 6 had greater mean Enterobacteriaceae index indicator compared to zone 4 ($P < 0.05$), with Zones 2 and 3 being intermediate. None of the zones had mean rapid Enterobacteriaceae index values that indicated completely negative (or no presence). There was no detected difference for Enterobacteriaceae across time points or zones.

There was no evidence of correlation noted ($r \leq 0.20$, $P > 0.05$) between the presence of PEDV or rotavirus and the presence of Enterobacteriaceae, however there was evidence of a moderate correlation ($r = 0.65$, $P < 0.0001$) between the cultured Enterobacteriaceae samples and the rapid testing (Table 4.2).

Study 2

Bacterial growth comparison within facility zones

Due to the unique nature of each of the three sites visited for this study, the use of zones based on feed or pig contact and/or proximity was utilized. Table 4.3 shows the comparison of mean reported Enterobacteriaceae index values from within each zone. Zones 2, 3, 4, 6, and 7 had the similar reported mean growth values ($P \leq 0.05$) assigned, ranging from a mean index score of 18.1 for Zones 2 and 3 to a mean index value of 21.4 for Zone 7. Zones 1, 2, 3, 4, and 6 had similar mean growth values ($P \leq 0.05$). Zone 5 had the second-to-lowest demonstrated growth of all sample groups ($P > 0.05$), with the group of samples with the lowest growth index value being the raw ingredient and finished feed samples.

In addition to the differences between the reported growth between zones, there was also a wide variety of bacteria identified within the sample groups, and there was some variation between zones. It should be noted that the results reported were only of the top 3 growth species for each sample; there may have been other species present that were not identified within the samples.

Correlation

As shown in Table 4.4, there was evidence of moderate correlation noted ($r = 0.463$, $P \leq 0.0001$) between the zone and presence of Enterobacteriaceae, but no evidence of correlation ($r = 0.029$, $P > 0.05$) between zone a presence of fecal indicator bacteria. There was significant evidence of a weak correlation ($r = 0.201$, $P \leq 0.0001$) between Enterobacteriaceae presence and site.

Discussion

Study 1

Currently, Enterobacteriaceae testing is one method of hygiene monitoring done within the human food industry as an indicator of contamination within a process. Other methods, such as measuring adenosine triphosphate (ATP) or salmonella, are also done with commercially available "rapid" or on-site tests, and traditional culture tests are also utilized for monitoring, but may not be as convenient or quick as on-site testing.[12] For this experiment, Enterobacteriaceae was selected over some of the other methods in an attempt to avoid some known issues when doing environmental monitoring in areas with high levels of organic material present, as well as potential impacts from different materials present within the facilities. Despite being used widely within food manufacturing facilities, there is little scientific data available for ATP monitoring; what data is available has shown that there is considerable impact on results based on the surface type as well as heat treatment.[13] Since ATP tests quantify the amount of organic matter present, it is difficult to use in areas with high levels of dust or other material consistently present. Salmonella testing that is traditionally done in food manufacturing facilities is unable to differentiate between salmonella types and does not detect the presence of other coliforms that could be considered fecal indicators.

While the results obtained at this one facility did not indicate that there was a correlation between the presence of Enterobacteriaceae and PEDV or rotavirus, further testing at other facilities may indicate otherwise. Because the feed mill for this system did not have any viral samples detect PEDV or rotavirus with PCR throughout the outbreak, there was no way to determine if Enterobacteriaceae used in a mill setting would correlate with the presence of a viral pathogen like rotavirus or PEDV.

There was however, a strong correlation observed between the cultured Enterobacteriaceae results and the rapid "on-site" Enterobacteriaceae testing, indicating that the rapid test could offer a relatively quick alternative for facilities wishing to monitor cleanliness in a more quantifiable way. Other novel environmental monitoring methods have also been shown to perform comparably to more traditional laboratory testing.[14] Submission of samples for culture at a diagnostic laboratory typically costs $20-35 per sample and can take anywhere from 2-7 days to receive results once received by the laboratory. In contrast, sample cost for the rapid Enterobacteriaceae test used in this experiment is under $6, excluding the initial purchasing cost of the readout equipment, and the results are available after 8 hours. For this study, the cultured Enterobacteriaceae samples were submitted for general culture growth data without a request for specific colony identification. Laboratory testing can provide more in-depth identification of specific bacteria present depending on the type of analysis performed, so if the intention is to obtain detailed bacteria population data and/or determine if certain indicator bacteria such as coliforms are present, the additional price and wait time for results is necessary. It should be noted that the rapid Enterobacteriaceae test provides general count levels, and there are no industry-established guidelines for acceptable thresholds for the count results within feed manufacturing facilities or farms at this time. Human food manufacturers establish a set of guidelines when considering common hygiene markers like Enterobacteriaceae or ATP levels to remain compliant with Good Manufacturing Practices (GMPs) within specific industries, but those values are not necessarily standardized across different facilities, and are not easily applicable to an inherently dusty environment such as a feed mill or swine farm.

Study 2

Three separate feed manufacturing facilities (Sites 1-3) were evaluated and sampled for this study, with a brief biosecurity evaluation and audit performed during each visit. Each mill offered its own biosecurity challenges, either with the normal operating procedures or required tasks to be performed within facility limitations.

Overall, a higher presence of Enterobacteriaceae as indicated by the growth index value was noted in areas that did not have direct feed contact. Zone 5 (feed contact surfaces on-farm) had lower mean index values than non-feed and non-pig contact surfaces. This could indicate that there should be more concern placed on the spread of potentially harmful bacteria by environmental factors, or that areas that are not constantly in contact with physically abrasive feedstuffs may act as a better representative sample than feed-contact surfaces themselves. The feed and ingredient samples that were analyzed had significantly lower mean index levels, which could indicate that the material itself is not necessarily as hospitable to the bacteria as other environments. A variety of ingredients, including animal byproducts and cereal grains, in addition to finished feed products, were used for this study. Each ingredient and feed has specific physical characteristics, which could impact bacteria survival rate.

The use of zoned sample groups allows for the comparison of how different areas within the facility compare to the actual raw ingredient or finished feed when cultured. The use of an index assigned based on the laboratory reported values of “none, few, low, moderate, or high” allowed for a quantification of a qualitative value. The overall score for each sample was calculated based on the sum of the three top growth bacteria that were reported. It did not account for how many other bacteria were present in each sample, which could have varied, or variability between

reporting structures. While these index scores can be compared across samples and allow for comparison across zones or sites, it does not account for the whole microbiome of a sample.

Additionally, these samples were shipped internationally, which may have also had an impact on bacteria survivability. While the utmost care was taken while preparing the samples for shipment, there were still more stress placed on them than a typical domestic shipment would receive. Repeating this study within an integrated system then having the samples analyzed domestically could potentially offer additional insight or differing results.

It is also important to note that each facility and system is unique and faces challenges based on required use and even facility design. For example, one feed production site in this system shared a truck scale with vehicles hauling live pigs. This creates some biosecurity concerns that may not be present at other sites within the system or separate facilities. There is no "one size fits all" solution for feed mill or on-farm environmental monitoring plans or biosecurity practices.

Conclusion

Enterobacteriaceae testing may prove to be a useful tool in on-farm and feed manufacturing settings, but further research in a variety of settings is needed to fully determine the extent of use, and each facility may encounter unique challenges when attempting to use environmental monitoring. Commercially-available "on-site" Enterobacteriaceae testing proved to have results that were highly correlated with Enterobacteriaceae testing done within a laboratory, even in settings that are inherently dirty. This may provide an appealing alternative for feed manufacturing or swine facilities that would like to use Enterobacteriaceae as a monitoring tool. There is evidence that Enterobacteriaceae populations and growth differ based on location within integrated swine systems. While this study did not demonstrate a strong correlation between different zones and

Enterobacteriaceae presence, a case could be made that areas with higher bacterial growth present when sampled should be focused on when putting in efforts to update or change facility hygiene or biosecurity policies, based on the moderate correlation found with these data and data available within the human food and pet food industries.

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Tables and Figures

Table 4.1 Mean Porcine Epidemic Diarrhea Virus (PEDV), rotavirus, and Enterobacteriaceae environmental sample results within different zones in a US swine production facility and feed mill

Zone	PEDV Ct	Rotavirus Ct	Cultured Enterobacteriaceae	Rapid* Enterobacteriaceae
1. Vehicles†	45.0[a]	39.8	0.8	1.2[a]
2. Pig contact surfaces	32.9[c]	38.5	0.3	0.9[ab]
3. Non-pig contact surfaces	34.3b[c]	39.6	0.9	0.7[ab]
4. Main office on farm	38.5[b]	39.6	0.2	0.2[b]
5. Feed contact surfaces	45.0[a]	40.0	1.0	1.2[a]
6. Non-feed contact surfaces	45.0[a]	39.1	0.9	1.5[a]

Note: The threshold for QT-PCR for PEDV and Rotavirus was 45 and 40, respectively. Any result over that threshold was considered negative. Laboratory samples were analyzed at the Kansas State University Veterinary Diagnostic Laboratory, rapid Enterobacteriaceae samples were analyzed following manufacturer's guidelines.
Enterobacteriaceae samples were assigned an index value ranging between 0-4, based on the amount of growth or value reported.
[abc] Means within a column with different superscripts differ $P < 0.05$.

† On- and off-farm, including select locations on feed delivery trucks, tractors, and employee vehicles.

*Hygiena EnSURE MicroSnap™ EB

Table 4.2 Correlation between PEDV, rotavirus, cultured Enterobacteriaceae, and rapid* Enterobacteriaceae testing

	r	*P*-value	*n*
PEDV			
Rotavirus	0.179	0.183	57
Cultured Enterobacteriaceae	-0.082	0.553	55
Rapid Enterobacteriaceae	0.109	0.429	55
Rotavirus			
Cultured Enterobacteriaceae	-0.084	0.542	55
Rapid Enterobacteriaceae	-0.064	0.644	55
Rapid Enterobacteriaceae			
Cultured Enterobacteriaceae	0.646	<0.0001	51

*Hygiena EnSURE MicroSnap™ EB test kit.

r= Pearson correlation coefficient.

n= number of samples considered.

Correlation considered significant when $P < 0.05$.

Table 4.3 Mean bacterial growth index scores within different zones in an integrated swine system in Brazil

Zone	*Growth index value*
Feed/Ingredients	1.2[d]
Feed mill Zones	
1. Direct feed or indirect contact surfaces	16.5[b]
2. Close proximity (< 1m) non-feed contact surfaces	18.1[ab]
3. Non-feed contact surfaces (> 1m of separation)	18.1[ab]
4. Transient surfaces (employees, tools, vehicles)	18.7[ab]
On-farm Zones	
5. Direct feed contact surfaces	10.6[c]
6. Pig contact surfaces	20.0[ab]
7. Non-pig contact surfaces	21.4[a]

Note: The growth index value represents the cumulative mean of the top three identified bacteria after plating. Samples were assigned values based on amount of growth present, with higher numbers indicating higher levels of bacterial growth. Samples were analyzed at Iowa State University.

[abcd] Means within a column with different superscripts differ $P < 0.05$.

Table 4.4 Correlation between zone, Enterobacteriaceae presence, and fecal indicator bacteria presence

	r	*P*-value	*n*
Zone			
Enterobacteriaceae growth index value	0.463	<0.0001	414
Fecal indicator present	0.029	0.6170	309
Enterobacteriaceae growth index value			
Fecal indicator present	0.212	0.0002	309
Enterobacteriaceae growth index value			
Site	0.201	<0.0001	414

r= Pearson correlation coefficient.

n= number of samples considered.

Correlation considered significant when $P < 0.05$.