Understanding the transmission of monophasic *Salmonella enterica* 4,[5],12:i:- through alternate exposure methods in swine and demonstrating the importance of common biosecurity measures on swine farms

by

Olivia Lynn Harrison

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Approved by:

Co-Major Professor
Dr. Valentina Trinetta

Approved by:

Co-Major Professor
Dr. Cassandra Jones

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Abstract

Salmonella is a major foodborne pathogen in the United States, causing approximately 31% of foodborne illnesses in the United States. Swine are capable of replicating *Salmonella* serotypes without showing clinical signs of infection and can produce contaminated pork products. The first experiment surveilled swine finishing operations to determine the distribution of *Salmonella* within farms and their environments, 186 samples from five finishing farms were collected in the fall of 2020. 100 (53%) of the samples were culture positive for *Salmonella* and 14 (7.5%) were confirmed *Salmonella* by PCR. Of the PCR positive samples, 12/14 were from either feces or locations in contact with feces such as the pen floor, gate, and fence. *Salmonella* was rarely observed in locations not typically associated with feces such as the feeders and waterers. 13/14 PCR positive samples originated from a single farm. These results suggest that *Salmonella* prevalence on surfaces and in feces is impacted by the farm and its current group of pigs, disease challenges, and the environment. The second experiment focused on *Salmonella enterica* 4,[5],12:i:- which is an emerging serotype commonly linked to contaminated pork products with a high degree of antimicrobial resistance. Transmission of this serotype to weaned pigs was conducted via contaminated feed, water, and aerosolized particles. *S. enterica* 4,[5],12:i:- was intermittently shed in the feces, rectal and nasal swabs, and in the environment of all inoculated pigs. Furthermore, *S. enterica* 4,[5],12:i:- was observed in the respiratory, lymphatic, and digestive systems of the infected pigs. Contaminated fomites such as feed and water are capable of *S. enterica* 4,[5],12:i- transmission and can introduce this serotype into the pork supply chain.

The final experiment focused on the importance of biosecurity to prevent pathogen entry. Glo Germ is a fluorescent powder which can be used to track contamination in a simulated

situation without risking the health of the farm. Glo Germ was spread prior to common biosecurity measures on a swine operation. Before and after photographs were used to evaluate whether Glo Germ had spread into the area following the biosecurity measure. Common biosecurity protocols are able to limit the spread of Glo Germ throughout a swine operation. Implementing similar visual techniques can help demonstrate to employees the importance of biosecurity on swine farms.

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Dedication

To my dad, Curt Harrison, who passed before I could complete this chapter of my life. He was and always will be my biggest supporter and favorite person to people watch with.

Chapter 1 - Potential causes of dilated cardiomyopathy phenotype in canines[1]

Abstract

The intent of this paper is to review the current literature associated with dilated cardiomyopathy in canines regarding the current diet preferences of consumers. Dilated cardiomyopathy (DCM) in canines has been linked to low whole blood and plasma taurine concentrations. Previous research has shown similar symptoms in felines caused by a taurine deficiency in the diet. The diagnosis of DCM decreased in felines once supplemental taurine was added and is now considered an essential amino acid in feline diets. While certain breeds of canines have always had a predisposition to DCM, reported cases of taurine deficient DCM have increased in recent years. In 2018, the FDA linked the rise in cases to the increased popularity of grain-free diets. However, few studies have been able to implicate the pulse ingredients which replace grains as the factor causing dilated cardiomyopathy. Follow-up research has instead shown disparities between the taurine pre-cursors (sulfuric amino acids) needed to maintain body condition score and the amount needed to maintain its sulfuric amino acid intake per molecular body weight. This was particularly true for larger breeds of dogs. When larger dogs were fed to maintain their sulfuric amino acid intake, they were not able to synthesize the same proportion of taurine in their liver as smaller bred canines. In conclusion, grain-free diets are most likely not causing DCM because they replace the more common carbohydrate sources with pulses. Further research

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should be conducted to better understand the bioavailability of the sulfuric amino acids as consumer preferences change.

Introduction

In July of 2018, the FDA released a report monitoring the increase of DCM phenotype in canines (FDA, 2019). Dilated cardiomyopathy phenotype causes the heart, usually the left ventricle, to enlarge due to weakening of the myocardium (ACVIM, 2014). The concern of the FDA report is not the rise in cases, which is likely due to better and more consistent reporting, but the increased number of cases in small breed dogs not typically predisposed to genetic DCM. The report suggests that this increase in incidence in small breed dogs is due to the increased popularity of BEG diets (FDA, 2019). The FDA specifically refers to grain-free diets, or those diets where corn, soy, and other grains are replaced with legumes, or pulse ingredients, such as peas and lentils or varying forms of potatoes, as a potential cause of DCM phenotype (Mansilla et al., 2019). Alternate protein sources like duck, bison, and venison, could also pose health concerns due to their less defined amino acid profiles compared to more traditional proteins sources like chicken or beef. Research has yet to understand the interactions these alternate protein and carbohydrate sources play in terms of amino acid bioavailability and nutrient digestibility (FDA, 2019).

Dilated cardiomyopathy phenotype is most common in larger breeds of dogs such as Doberman pinschers, Great Danes, Irish wolfhounds, Newfoundlands, and golden retrievers (Sanderson et al., 2001; Ko et al., 2007; Adin et al., 2019). While the cause is largely unknown, the prevalence suggests a genetic link between these larger breeds and development of the disease (Broschk and Distl, 2005; Simpson et al., 2015; Simpson et al., 2016). One of the most

prevalent theories is DCM phenotype caused by a taurine deficiency similar to what occurred in feline diets during the 1980's (Pion et al., 1987; Pion et al, 1998). Cats, like dogs, utilize taurine for bile acid synthesis which requires a greater taurine synthesis rate than other mammals. Taurine is synthesized from cysteine and methionine via the CSA decarboxylase enzyme. However, the CSA decarboxylase activity in cats is not great enough to produce sufficient taurine from dietary precursors (Pion et al., 1998). Supplemental taurine was added to all feline diets and is now considered an essential amino acid to reduce risk of DCM phenotype and other heart related diseases (Pion et al., 1987). As dogs' CSA decarboxylase activity is typically greater than cats, there has been little evidence for supplemental taurine in their diets (Pion et al., 1987; Pion et al., 1998). However, the results of the FDA's investigation suggest that there may be a link between grain-free diets and DCM phenotype induced by a taurine deficiency.

Grain-free diets

Previous clinical data have shown that dogs fed a grain-free diet have reduced body weight compared to those fed a grain-based diet at the time of DCM phenotype diagnosis (Adin et al., 2019). Dogs that were switched from a grain-free to a grain-based diet and provided supplemental taurine showed significant improvement in their LVIDdN and LVIDsN. Given the improved health with diet change and supplemental taurine, grain-free diet induced DCM phenotype may be reversed through diet change +/- supplemental taurine (Adin et al., 2019). A similar case study with golden retrievers reported decreased LVIDd and LVIDs through diet change and supplemental taurine (Kaplan et al., 2018).

Conversely, Donadelli et al. (2020) reported no change in the overall health of Labrador retrievers when fed a commercially available grain-free diet over 26 weeks. Dogs were provided

a grain-based diet for 26 weeks prior to the start of the experiment to establish a baseline. Blood was collected at weeks 0, 13, and 26 and analyzed for amino acid content. Taurine concentration increased and met or exceeded common reference values at all time points. This increase in blood taurine concentrations may have been due to the greater taurine concentration in the grain-free diet compared to the grain-based (0.14 vs 0.07% DMB of diet, respectively; Donadelli et al. 2020). Similarly, beagles fed either a grain-based or a grain-free diet for 28 d had similar blood taurine concentrations, which increased from baseline. Both experimental diets had greater taurine concentrations (grain-based: 0.33; grain-free: 0.35% DMB of diet) compared to the basal diet (0.25% DMB of diet). These data suggest that carbohydrate source is likely not the cause of DCM phenotype, provided the diet is sufficient in taurine; however, none of these studies evaluated amino acid intake and the diet's ability for adequate taurine synthesis (Pezzali et al., 2020).

Diets with differing fat and carnitine levels

Bile acids, including components such as taurine, aid in the digestion of fat and can be recycled throughout the digestive tract reducing the need for supplemental taurine. In diets with high levels of fat or alternate protein sources, bile acid components may remain bound and be excreted in the feces; therefore, increasing the need for supplemental taurine in the diet (Pion et al., 1987; Adin et al, 2019). This need could potentially be decreased with additional carnitine in the diet. Like taurine, carnitine is synthesized from cysteine and methionine in addition to lysine (Sanderson et al., 2001). Consequently, research was conducted to evaluate taurine concentrations and echocardiographic data when healthy dogs were fed either a low fat, a high fat diet, or a high fat diet supplemented with carnitine and protein was restricted. Cysteine and

methionine concentrations met or exceeded AAFCO recommendation. After 48 months on test, there was no difference in blood taurine concentrations between the low, high fat, or high fat + carnitine diets. Echocardiographic data showed no difference between diets, and no dogs in either diet were diagnosed with DCM phenotype. There was an overall decrease in whole blood taurine concentrations from month 0 to 48 for all diets. While this decrease did not result in DCM phenotype, these data suggest that blood taurine concentrations can still decrease below normal levels even if cysteine and methionine concentrations meet AAFCO recommendations (Sanderson et al., 2001).

Kittleson et al. (1997) supplemented American cocker spaniels with DCM phenotype with taurine and carnitine or a sugar and cornstarch placebo over a 2-month period. Dogs supplemented with taurine and carnitine had improved echocardiographic data after 2 months of supplementation when compared to their beginning baseline. Similar improvement from the baseline data was seen between 4 to 6 months when the placebo dogs were switched to taurine and carnitine supplementation (Kittleson et al., 1997; Mansilla et al., 2019). The results of these trials suggest DCM phenotype caused by a taurine deficiency is likely related to the amino acid profile of the diet rather than ingredient composition, as reported by the FDA investigation.

Cysteine and Methionine requirements

The current cysteine and methionine requirements for canines are 0.085g/kg $BW^{0.75}$ (methionine) and 0.17g/kg $BW^{0.75}$ (cysteine and methionine); however, there is little research on whether those requirements should increase at a larger proportion as body weight increases (NRC, 2006; Mansilla et al., 2019). To evaluate the differences in cysteine and methionine requirements between small and large dogs, six beagles (12.8 ± 0.4 kg) and six mixed breeds

(37.9±2.1 kg) were fed the same diet in a series of two trials during which they were fed to either maintain an ideal BCS of 5/9 (Exp. 1) or fed to maintain similar amounts of SAA (cysteine and methionine) intake per kg of MBW (Exp. 2). In order to maintain the same SAA intake per kg of MBW as the small dogs, large dogs had to eat more feed in Exp. 2 than they were eating to maintain BCS in Exp. 1. Even when larger dogs ate to maintain a similar SAA intake per unit of MBW, smaller dogs maintained higher plasma taurine concentrations and whole blood taurine concentrations. The increased blood taurine concentrations in small dogs correlates to the increased taurine synthesis rates observed in the small dogs' livers. When liver weights, the main area of taurine synthesis, were normalized between the large and small dogs, small dogs were able to synthesize more taurine in both Exp. 1 and 2 (Mansilla et al., 2019). Beagles and Newfoundlands were given the same diet, and both taurine and cysteine blood concentrations were evaluated. Dogs were fed to maintain their estimated ME requirements based off body weight. Similar to the previous study, the Newfoundlands were seen gaining weight during the trial due to increased feed intake needed to maintain ME versus eating to maintain BCS. Although these dogs had greater feed intake, the Newfoundlands had lower blood taurine, plasma taurine, and plasma cysteine, than the beagles on trial (Backus et al., 2006). Both trials suggest a disparity between the SAA intake a large dog needs to synthesize adequate taurine and the SAA levels currently recommended by AAFCO to maintain body condition.

Conclusions

As the FDA continues to monitor the increase of DCM phenotype cases in dogs of all sizes, further emphasis must be placed on the nutrient profile rather than ingredient composition (Kittleson et al., 1997). Preliminary epidemiological studies do support a causal relationship

between grain-free diets and DCM phenotype (Morris et al., 1994; Ko et al., 2007); however, further investigations have not reported changes in cardiac health when dogs were given a grain free diet (Kaplan et al., 2018; Donadelli et al., 2020). Furthermore, when additional taurine was added to diets (between 0.14-0.35% DMB of diet) blood taurine concentrations and echocardiographic data showed no difference between grain-free and grain-based diets (Kaplan et al., 2018; Donadelli et al., 2020). Future research should focus on both cysteine and methionine concentrations and the possibility of supplementing taurine in canine diets. Emphasis should be placed on large breed dogs, especially those predisposed to genetic DCM, and their ability or inability to synthesize adequate taurine from the current SAA levels recommended by AAFCO. As consumer preferences lean more towards BEG diets, a better understanding of diet composition, digestibility, and amino acid bioavailability will be integral in reducing DCM phenotype in canines.

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Chapter 2 - Understanding the environmental presence of *Salmonella* spp. in finishing pigs at commercial swine farms in Kansas

Abstract

Salmonellosis remains a major foodborne disease threat to public health worldwide. Swine are considered a reservoir for many *Salmonella* serotypes that can infect humans; however, not all serotypes of concern in food animal products cause clinical signs of infection in swine. Therefore, the objective was to evaluate the presence and distribution of *Salmonella* spp. in finishing pigs at commercial swine farms across Kansas. Five commercial farms were selected and sampled within four weeks of the first load out for marketing. A total of 37 samples per farm were collected: 5 swabs from each of 6 individual pens on the front gate, floor, back wall, feeder, waterer, feces (~ 20g) from each pen and one ventilation exhaust fan. Samples were collected using a pre-moistened sponge and transported at refrigerated conditions to the lab for processing following USDA-FSIS guidelines. Presumptive positive samples were confirmed as *Salmonella* by PCR. A total of 186 samples were collected among the five farms. A total of 100 (53%) samples were culture positive, and 14 (7.5%) were confirmed *Salmonella* positive by PCR with 3 of 5 farms having no PCR-positive samples. No difference for proportion of culture positive samples was observed between farm, between sampling location within pen, or their interaction ($P > 0.05$). Feces or locations prone to fecal contact tested positive more often at one farm than other locations on any other farms. These results suggest that *Salmonella* prevalence on surfaces and in feces is impacted by the farm which might be related to its current group and age of pigs, disease challenges, or other environmental factors.

Introduction

Bacterial contamination of meat products continues to be a concern for public health. Salmonellosis remains a major foodborne disease worldwide. It is estimated that every year over 31% of foodborne illnesses are linked to *Salmonella* in the United States (CDC, 2011). Swine are considered a reservoir for many *Salmonella* serotypes and contaminated pork products can cause foodborne infection in humans (Boyen et al., 2008). Therefore, the surveillance of *Salmonella* in farms could be a tool to better understand contamination risks and identify areas of concern within farms.

In 2006, the United States Department of Agriculture (USDA) observed *Salmonella* prevalence in 7.2% of collected feces with 52.6% of the farms (n=135) having at least one *Salmonella* positive sample (USDA-APHIS, 2009). Bjork et al. (2018) reported a similar percent of positive farms (52.9%) with a slightly higher prevalence of individual *Salmonella* positive feces (14.2%). As pigs are transported to the processing facility, the individual prevalence of *Salmonella* is thought to increase as pigs are introduced to new cohorts with differing health status. In fact, finishing pigs are capable of shedding *Salmonella* two hours after initial exposure to the pathogen (Hurd et al., 2001; Gebreyes et al., 2004; Boughton et al., 2007). *Salmonella* transmission during transport and holding at the processing facility is primarily thought to occur via fecal-oral route (Gopinath et al., 2012). However, alternate transmission routes, such as nose-to-nose contact and airborne have also been found to be viable pathways (Oliveira et al., 2006 and 2007).

Contaminated environments have been found to increase the prevalence of *Salmonella* positive pigs (Stärk et al., 2002; Beloeil et al., 2004; Andres and Davies 2015). The increased prevalence can be linked to inappropriate cleaning and disinfection practices (Funk and

Gebreyes, 2004; Argüello et al., 2011; Pires et al., 2013). Furthermore, few studies linked contaminated environment and equipment to increased *Salmonella* shedding of finishing pigs. The purpose of our research was to evaluate the presence of *Salmonella* spp. in finishing pigs and their environment at commercial swine farms across Kansas to better understand the role a contaminated environment may play in *Salmonella* infection as pigs enter the food processing chain.

Materials and Methods

Farm sampling

Five farms were selected across Kansas and sampled between September and November 2020. Different genetic flows and marketing within four weeks of the sampling date were the criteria used for farm selection. A total of 37 samples per farm were collected: 5 swabs from 6 individual pens on the front gate, floor, back wall, feeder, waterer and a fecal grab (~ 20g) from the floor of each pen. Additionally, one sample per site from the ventilation exhaust fan was collected. Overall, the number of samples collected were 186 (one additional waterer was sampled at farm 1). Procedures for sample collection followed the Food Safety and Inspection Service manual (FSIS-USDA, 2019). Briefly, peptone water pre-moistened sponges (3M, St. Paul, MN) were used to swab an approximate 10×10 cm^2 area of the gate, floor, fence and fan; feeders and waterers. Approximately 20 g of feces was aseptically collected into a Whirlpak bag (Nasco, Inc., Fort Atkinson, WI). All samples were placed on ice for transportation to the laboratory within 24 hours and processed immediately upon arrival.

Salmonella Isolation from environmental samples

Environmental samples were transferred into 60 mL buffered peptone water (BPW; BD Difco, Sparks, MD) and incubated for 20-24 hours at 37°C (USDA-FSIS, 2019). After incubation, samples were homogenized for 30 s. A 500 µL aliquot was transferred into 10 mL of tetrathionate broth (TTB; HiMedia, West Chester, PA) and an additional 100 µL was added into 10 mL of Rappaport-Vassiliadis (RV; BD Difco, Sparks, MD). Samples were incubated at 42°C for 24 hours. Subsequently, a 10 µL aliquot was taken from each enrichment broth and plated on both Brilliant Green Sulfa (BGS; HiMedia, West Chester, PA) and Xylose Lysine Tergitol 4 (XLT4; Criterion, Santa Maria, CA). Plates were incubated at 37°C for 24 hours. Between 3-5 colonies that appeared presumptive positive for *Salmonella* (pink-white to red colonies (BGS) and yellow to red with black centers (XLT4) were streaked onto Tryptic Soy Agar (TSA; Criterion, Santa Maria, CA) and incubated for 24 hours. Plates were stored at refrigerated temperatures until further confirmation.

Salmonella Isolation from fecal samples

A subsample of fecal matter (10 g) was combined with 90 mL of TTB and incubated at 37°C for 24 hours for enrichment. After incubation, samples were homogenized for 30 s. A 100 µL aliquot of TTB was transferred into 10 ml of RV at 42°C for 24 hours before 10 µL was streaked onto Hektoen enteric (HE; HiMedia, West Chester, PA) agar. Plates were incubated at 37°C for 24 hours (Smith et al., 2016) and presumptive positive colonies (greenish-blue with black centers) were streaked onto Xylose Lysine Deoxycholate (XLD, Criterion, Santa Maria, CA) containing 4.6 mL/liter Tergitol (Remel, Lenexa, KS), 10 mg/L novobiocin (BD Difco, Sparks, MD), and 10 mg/L cefesulodin (Biosynth Carbosynth, San Diego, CA) (XLD_{tnc})

(Brichta-Harhay et al., 2012). After the second selective streak between 3-5 presumptive positive colonies were streaked onto TSA and stored at refrigerated temperatures until further confirmation.

Salmonella Confirmation

Presumptive positive colonies were analyzed via real-time PCR. A single colony from each TSA plate was transferred to the PCR mixture following the protocol developed by Bai et al. (2018) which targets the *invA* gene present in all *Salmonella enterica*. In each experiment, a non-template control (nuclease-free distilled water), a negative control (*Escherichia coli*, ATCC 29425), and a positive control (*Salmonella enterica* Infantis, ATCC 57141) were included. Samples were considered PCR positive if the Ct value was lower than 40.

Statistical Analysis

Power analysis was conducted to determine sample size necessary to estimate the prevalence of *Salmonella* detection in the environment with alpha set at 0.05 and a precision value of d = 0.05 (Naing et al., 2006; Pourhoseingholi et al., 2013). The prevalence was based off previous research of farm environmental samples and feed processing samples from feed mills across eight states (Keelara et al., 2013; Magossi et al., 2019a). The estimated sample size was 162 samples between the five selected farms.

Presumptive positive samples were denoted as culture positive and those found to contain *invA* were PCR positive and were considered *Salmonella* positive. Associations between explanatory variables (sampling site and location) with the prevalence of positive samples were analyzed using generalized linear mixed models using the GLIMMIX procedure in SAS 9.4

(SAS Institute Inc., Cary, NC). Models were fit using a binary distribution, logit link, Laplace approximation and ridge-stabilized Newton-Raphson algorithm were used when fitting statistical models similar to Magossi et al. (2019a and 2019b). The outcome was the presence or absence of *Salmonella* spp. in the environmental and fecal samples determined by culture analysis and PCR. The independent variables were farm and sampling location in pen. Fixed effects were evaluated as both univariable and multivariable models; however, due to hierarchical structure of the study, a random intercept of sampling location in pen nested within farm was included (except when either was evaluated as a fixed effect). If more than one fixed effect was found significant (indicated if $P < 0.05$) within the univariable model it was followed by the multivariable model. Mean probabilities and their 95% confidence intervals were also computed.

Results and Discussion

Kansas is home to almost 1,000 hog farms; however, more than 99% of the state's pork production comes from 150 sites (Kansas Pork Association, 2019). In this study, 5 of these larger farms were sampled in order to evaluate *Salmonella* presence at finishing swine operations. Samples were collected from 6 different pens within each farm. The number of head per barn ranged from 300-1,000 pigs with Farm 1 and 4 being wean-to-finish, and Farms 2, 3, and 5 being grow-finish. A short interview via email over herd health and farm history were conducted before sampling. Farms 1, 4, and 5 had no reported clinical signs or confirmed laboratory results of *Salmonella* or other *Enterobacteriaceae*. Farms 2 and 3 observed positive *E. coli* and rotavirus cases in the grower stage and occasional *Salmonella* positive cases were reported during late finisher in previous groups of pigs. No farms used additional antibiotics or antimicrobials to combat bacterial pathogens during the finishing stages.

Of the total 186 samples collected, 100 resulted culture positive. All farms had at least one culture positive sample at each sampling site except the exhaust fan (Table 1). However, there was not a significant difference ($P > 0.05$) between any of the farms or sampling sites. The 100 culture positive samples resulted in 325 isolates for PCR analysis, since up to 5 colonies were picked from each positive sampling site for confirmation. PCR detected the *invA* gene in 108 of the isolates originating from 14 samples (Figure 1) of which 12 were from feces and areas more prone to fecal contact (pen gate, floor, and fence). No fan samples were *Salmonella* PCR positive. There was also no difference ($P > 0.05$) between farm or sampling location for the PCR positive samples.

Similar to our study, Rodriguez et al., (2006) found a higher *Salmonella* prevalence in fecal material and rectal swabs (29/67 and 16/67, respectively) than environmental samples from fresh feed, trough feed, and soil (4/67, 7/67, and 11/67, respectively). However, Keelara et al. (2013) reported a higher prevalence of *Salmonella* in environmental samples than fecal samples (11.7% vs 4.0%, respectively). The higher prevalence in the environment could be explained by the categorization of barn floors as environmental while in our study they were considered areas in contact with feces.

After PCR analysis, two of the five farms (40%) were confirmed as *Salmonella* positive, primarily from fecal samples and the pen itself (floor, wall, and gate). Haley et al. (2012) found that 42 out of 126 farms in 2000 and 71 out of 135 farms in 2006 were *Salmonella* positive via fecal samples. Similarly, in a study conducted by Rajic et al. (2005), 60/90 (66.7%) of farms had at least one *Salmonella* positive sample during the 3 visits where both environmental (empty pen, boots, dust, and main drain) and fecal samples were collected. Furthermore, a metadata analysis

study evaluated *Salmonella* prevalence between 1999-2005 which estimated 59% of farms to be *Salmonella* positive at a given time (Sanchez et al., 2007).

The reduced prevalence of *Salmonella* in our study is likely due to the sampling sites selected. Most of the previous research has focused on shedding of *Salmonella* in the feces with little emphasis on its prevalence in the environment. The few studies investigating farm environment focused on soil, lagoons, boots, and dust as sites of sample collection (Rajic et al., 2005; Rodriquez et al., 2006; Keelara et al., 2013). These samples seem to have a lower *Salmonella* prevalence (5-20%) than fecal samples (30-67%; Rajic et al., 2005; Rodriquez et al., 2006; Sanchez et al., 2007; Keelara et al., 2013). Furthermore, when feed or water samples were investigated for the presence of *Salmonella,* again a low percentage was reported: 0-15% and 3%, respectively (Barber et al., 2002; Funk et al., 2004).

It is important to note the large difference between the culture positive samples and the *Salmonella* positive samples confirmed by PCR as analytical technique could be another reason for the difference in prevalence in our study compared to others in the literature. Magossi et al. (2019b) observed similar discrepancies where 62.2% of samples were culture positive while only 19.8% were *Salmonella* positive. The false positives were confirmed to be other *Enterobacteriaceae*, such as *Citrobacter*, by biochemical tests (Magossi et al., 2019b). *Citrobacter,* as well as other *Enterobacteriaceae,* can commonly be found within swine environment and could be falsely identified as *Salmonella* when only culture plates are analyzed (Liu et al., 2015; Mollenkopf et al., 2017). Pławińska-Czarnak et al. (2021) identified *Citrobacter braakii* and *Proteus mirabilis* from isolates initially thought to be *Salmonella enterica* based off XLD and BGS culture isolation. Similarly, *Citrobacter freundii* and *Proteus*

mirabilis produced colonies similar to *Salmonella enterica* when plated on XLD (Park et al., 2012).

Conclusions

The main objective was to evaluate *Salmonella* presence in the environment. To our knowledge, no other studies have evaluated environmental areas not commonly prone to fecal contact. The current research sampled not only fecal samples and locations prone to fecal contact such as the floor, gate, and walls, but also feeders and waterers. Culture samples identified suspect *Salmonella* positive locations; however, without PCR confirmation, *Salmonella* prevalence would have been overestimated. Overall, *Salmonella* was ubiquitous throughout one farm, regardless of sample location. Feeders and waterers were not often *Salmonella* positive as fecal contact is considered low on these surfaces.

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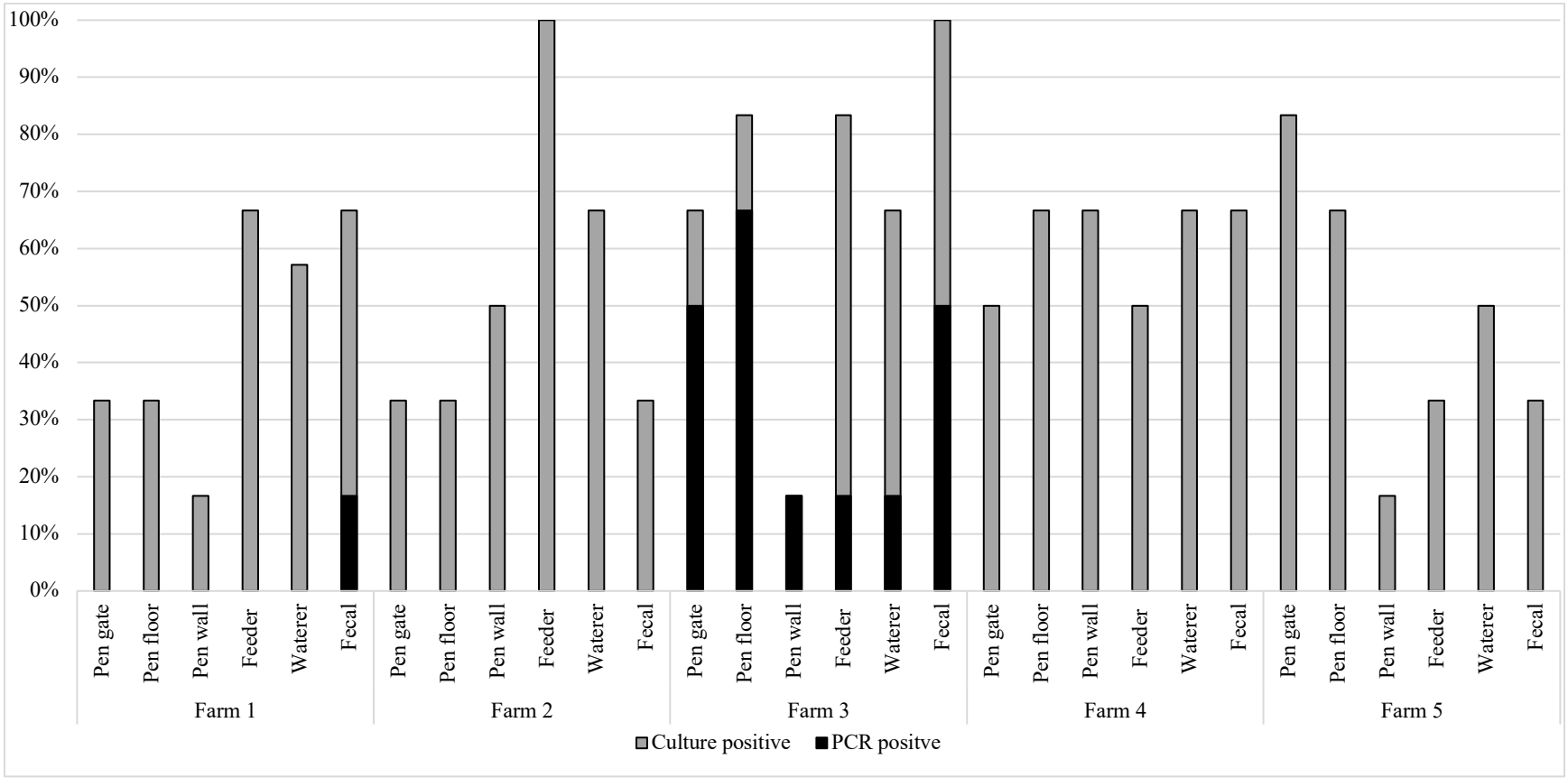
Table 2-1 Presence of culture positive and *Salmonella* positive (PCR confirmed) samples by farm and sampling location.[1]

	Culture positive	***Salmonella* positive**
Farm		
P-value	0.2482	0.1023
Farm 1	45% (17/38)	6% (1/17)
Farm 2	51% (19/37)	0% (0/19)
Farm 3	68% (25/37)	52% (13/25)
Farm 4	59% (22/37)	0% (0/22)
Farm 5	46% (17/37)	0% (0/17)
Sampling location		
P-value	0.2307	0.5733
Pen gate	53.3% (16/30)	18.8% (3/16)
Pen floor	56.7% (17/30)	23.5% (4/17)
Pen fence	33.3% (10/30)	10.0% (1/10)
Feeder	66.7% (20/30)	5.0% (1/30)
Waterer	61.3% (19/31)	5.3% (1/19)
Fecal	60.0% (18/30)	22.2% (4/18)
Fan	0% (0/5)	0% (0/0)

[1] Farm × sampling location was not significant ($P > 0.05$) for either culture *or Salmonella* positive samples.

Figure 2-1 Percentage of culture positive and PCR[1] confirmed positive sampling sites by farm.



[1] PCR percentage = number of PCR positive sampling sites/total number of samples (n=6)

Chapter 3 - Inoculation of weaned pigs by feed, water, and airborne transmission of *Salmonella enterica* Serotype 4,[5],12:i:-

Abstract

Salmonella enterica Serotype 4,[5],12:i:- (*STM*) has become an increasing burden for food safety. This strain has been often associated with swine products. Inoculated feed, water and air were given to weanling pigs in order to determine possible *STM* transmission routes. A control group of pigs was also sampled. *STM* was monitored daily through feces, rectal and nasal swabs. *STM* colonization was observed in the tissues with the greatest amount in the tonsils and lower in the digestive tract, and mesenteric lymph nodes. No differences in abnormal lesions were observed between the inoculated and the control pigs. Contaminated feed, water, and aerosolized particles are able to cause infection in weaned pigs; however, no *STM* colonization was observed in meat destined for human consumption. Contaminated pork products most likely stem from cross-contamination from digesta or lymph nodes during pork processing.

Introduction

Pork is the most consumed meat in the world, and even so has lower association with human foodborne illness compared to beef or chicken (Delgado et al., 2001). From 2006 to 2015, the number of clinical cases of *Salmonella* linked to pork products has grown (CDC, 2014). Among the clinically and economically relevant *Salmonella* serotypes linked to contaminated pork products, *Salmonella enterica* Serotype 4,[5],12:i:- (*STM*), a monophasic variant of *S. enterica* Typhimurium, has been among the most frequently reported. One of the first isolates of *STM* was obtained from chicken carcasses in Portugal around 1986/87 (Machado and Bernardo,

1990), but recently *STM* has been progressively implicated in human diseases worldwide. Since 1995, the reported cases of *STM* in the US has increased (Moreno Switt et al., 2009). In 2013, *STM* was the third most common serotype linked to pig, pork products and clinical cases in Europe (EFSA, 2010), and one of the six most common serotypes isolated from foodborne illnesses in the US (Moreno Switt et al., 2009). This pathogen has recently caused several outbreaks of which a number were traced back to contaminated pork or pork products. In 2006, *STM* was responsible for two cases likely associated to pork products in Luxemburg, causing 24 hospitalizations and one death (Mossong et al., 2007). In 2015, a large recall from whole roaster hogs contaminated with *STM* in Graham, WA was followed by investigations that traced the source of the outbreak to a pork slaughter establishment where inadequate employee handwashing practices and poor cleaning conditions of utensils and surfaces could have caused contamination in the pork products (Kawakami et al., 2016).

Researchers have demonstrated the role of pigs as a *Salmonella* reservoir (De Knegt et al., 2015). Animals can become infected with pathogens through contaminated feed, water, aerosol and contact with other animals (Proux et al., 2001; Crump et al., 2002; Davies et al., 2004; de Oliveira et al., 2005; Maciorowski et al., 2006). When animals consume contaminated feed or water or have contact with the pathogen indirectly, they can harbor bacteria without manifesting clinical signs. As asymptomatic carriers, the animals will still shed the organisms in their feces, promoting a cycle of pathogen spread within the farms and herds (Rostagno and Callaway, 2012). When animals are harvested, the fabrication into pork cuts can result in the contact of contaminated gastrointestinal contents from infected pigs to its carcass and others around, via fomites such as knives, processing tables and workers (Olsen et al., 2001; Vieira-Pinto et al., 2005). The resulting contaminated pork products can then be sold to the final

consumer and cause human illness by improper cooking or by cross contamination between the raw pork and other food surfaces (Carrasco et al., 2012). Since the pathogenicity gene repertoire of *STM* is highly similar to that of *S.* Typhimurium, but with even greater antimicrobial resistance, it is essential to understand *STM* transmission route into the pork supply chain. Therefore, the objective of this study was to assess and evaluate *STM* contamination of feed, water and aerosol in weaned pigs.

Materials and Methods

All protocols were reviewed and approved by Kansas State University Institutional Animal Care and Use Committee.

Cultures

Four strains of *Salmonella* 4,[5],12:i:- (*STM*) were selected for this study: RM 17306 (USDA-California), isolated from a water source, FSLS 5-580 (Cornell University) from an animal source, H20-01924 from a clinical source (Minnesota Department of Health), and KSU1966 from a feed mill facility (Trinetta et al., 2020). Strains were kept in cryovials (CryoCare Bacteria Preservers, Key Scientific Products, Stamford, Texas) at -80ºC until 48 hours prior to the experiment, then individually plated on tryptic soy agar (TSA; BD Difco, Sparks, MD) and incubated at 37°C for 24 hours. A single colony was selected from each plate and transferred into 50 mL Luria-Bertani Miller (LB) broth (BD Difco, Sparks, MD) at 37° for 12-18 hours while shaking (Loynachan et al., 2004). On the day of the experiment, each culture was centrifuged at 4,000 rpm for 15 min at 5°C and the pellet was resuspended in 5 mL phosphate-buffered saline (PBS; VWR, Radnor, PA). The four different strains were equally combined to reach a starting inoculum of 6.7×10^9 CFU/mL (Loynachan et al., 2004).

Inoculum Preparation

Because the intent of this study was to understand if feed, water or air could potentially be a transmission route for *STM* into the pork supply chain, an experimentally contaminated feed mixture and water solution were prepared for the subsequent animal inoculation. For the feed mixture, 10 mL of the 6.7×10^9 CFU/mL freshly prepared cocktail (equal combination of 4 *STM* strains) was added to 30 g of finely ground feed (KSU Phase 3 common diet with no special protein sources). The solution was allowed to absorb and then hand homogenate for 2.5 min. Sixty mL of buffered peptone water (BPW; BD Difco, Sparks, MD) was added to the feed mixture and gently mixed to create a slurry with a *STM* concentration of approximately 1.3×10^9 CFU/g (Niederwerder et al., 2019). The contaminated water solution was created similarly by adding 10 mL of the cocktail to 50 mL of BPW and gently mixing to create a solution of approximately 1.0×10^9 CFU/mL (Niederwerder et al., 2019). No additional media was added to the cocktail to contaminate the air by aerosolization (6.7×10^9 CFU/mL).

Animals

Twelve pigs (24 days of age) were transported to the Large Animal Research Center (LARC) at Kansas State University, Manhattan, Kansas. Upon arrival, pigs were weighed and randomly allotted to one of four treatments (avg weight 6.24 ± 0.01 kg) with 3 pigs per treatment. Treatments were: 1 – control (no *STM*), 2 – *STM* inoculation via feed slurry, 3 – *STM* inoculation via water source, and 4 – *STM* inoculation via aerosolization. All pigs were confirmed negative for *Salmonella* via rectal and nasal swabs prior to inoculation (Smit et al., 2016).

Animal Inoculation

After a 6-d acclimation period, animals were inoculated based on the treatment room where they were previously allocated. No *STM*-inoculated feed, water or air was offered to pigs in the control room. For the feed inoculation, a freshly-prepared *STM* feed slurry (10 mL at 1.3×10^9 CFU/g) was administered to each individual pig while holding the animal to allow feed intake and swallowing reflex to be monitored. The contaminated water solution (10 mL at 1.0×10^9 CFU/mL) was administered by orogastric gavage with an 8F catheter and a 60 mL catheter syringe (Schumacher et al., 2016). Prior to inoculation, the aerosol-inoculated pigs were sedated with tiletamine and zolazepam (Telazol, Zoetis, Kalamazoo, MI; 2.2-4.4 mg/kg BW administered intramuscularly) to reduce stress and allow easier handling. Next, 1 mL of cocktail (6.7×10^9 CFU/mL) was added to a portable nebulizer (Mayluck, Amazon, Seattle, WA) and held to the pig's snout until no liquid remained in the holding chamber, approximately 4 minutes (Dee et al., 2006). Due to the speed of the nebulizer, a 10 mL solution would be too time consuming for aerosol inoculation. Therefore, only 1 mL of solution (6.7×10^9 CFU/mL) was given to the aerosol inoculated pigs. The feed and water inoculated pigs received an overall greater dose of *STM* due to the nature of the inoculation method. After the one-time inoculation, pigs were housed in their allotted rooms and provided *ad libitum* access to *STM*-free feed and water for 7 d.

Data collection

Pigs were weighed daily and health was evaluated to monitor for clinical signs of Salmonellosis. Specifically, presence of vomiting, diarrhea, or lethargy were recorded. Fecal, nasal, rectal, and environmental swabs were collected daily from the floor, feeder, and waterer to monitor and quantify the excretion of *STM*. A sterile cotton swab was used to stimulate the rectum, and approximately 10 g of fecal matter was collected directly into a stomacher bag

(Agga et al., 2016). Rectal and nasal swabs were also taken with a sterile cotton swab and placed into 5 mL of phosphate buffered tryptic soy broth (TSB-PO_4; BD Difco, Sparks, MD) containing 2.13 g/L KH_2PO_4 (VWR, Radnor, PA) and 12.54 g/L K_2HPO_4 (VWR, Radnor, PA) (Chaney et al., 2017). Environmental swabs were collected with a pre-moistened sponge (3M, St. Paul, MN). A floor area (10 cm × 10 cm), the feeder, and waterers in each pen were sampled (USDA-FSIS, 2019). All samples were transported on ice and immediately analyzed. At the completion of the study (d 7), pigs were sedated with tiletamine and zolazepam (2.2-4.4 mg/kg BW administered intramuscularly) and given a sodium pentobarbital overdose (85-100 mg/kg BW administered intravenously) prior to transportation to the Kansas State University Veterinary Diagnostic Laboratory for necropsy. Tissue samples from the lungs, ileocolic, jejunal, and inguinal lymph nodes, the tonsils, the cecum, colon, and ileum, and the right gluteal and gracilis muscle were collected in duplicate. One sample was fixed in 10% buffered neutral formalin for histological analysis while the other sample (approximately 20 g) was placed on ice and transported to the Kansas State Food Safety and Microbiology Laboratory for microbial enumeration.

Microbial Enumeration

A modified procedure was used to process fecal, rectal, and nasal samples (Brichta-Harhay et al., 2012; Chaney et al., 2017). Briefly, 90 mL of TSB-PO_4 was added to the fecal samples (if less than 10 g were collected a 1:9 ratio of fecal matter: TSB-PO_4 was used) and homogenized by hand. No additional TSB-PO_4 was added to the nasal or rectal samples. Serial dilutions were performed for all samples, and a 1 mL aliquot plated onto Enterobacteriaceae (EB) petrifilm (3M, St. Paul, MN). Petrifilm were incubated at 37°C for 22-26 hours. Colonies were counted, and if any were gas producing, the EB film was removed and pressed onto pre-

made Xylose Lysine Desoxycholate (XLD; Criterion, Santa Maria, CA) agar plates. These samples were incubated at 37°C for an additional 22-26 hours and black *Salmonella* presumptive colonies were counted. Environmental samples were processed following the USDA-FSIS protocol (USDA-FSIS, 2019), where 50 mL of BPW were added to the sample and hand homogenized prior to serial dilution on EB petrifilm and incubated. Petrifilm with gas producing colonies were pressed onto XLD and presumptive colonies counted, as previously described (Brichta-Harhay et al., 2012; Chaney et al., 2017).

Tissue samples were trimmed or cleaned from any fat, fascia or excreta. Lymph nodes were placed into boiling water for 3-5 s for surface sterilization, transferred into a whirl-pak bag and pulverized using a rubber mallet before adding 80 mL of TSB-PO_4. Serial dilutions were plated on EB petrifilm as described above (Brichta-Harhay et al., 2012; Chaney et al., 2017; Webb et al., 2017). All plates with typical *Salmonella* colonies were confirmed via PCR, following a modified method developed in our laboratory (Pendergast et al., 2013; Magossi et al., 2019).

Histopathology

Histopathology samples were collected and analyzed by a veterinary anatomic pathologist at the Kansas State University Veterinary Diagnostic Laboratory. A qualitative analysis of presence and absence of bacteria in the samples collected at necropsy was conducted. Lesion scores were rated on a scale from 0 to 5, with a score of 0 representing samples with no observed neutrophils and a score of 5 representing moderate-to-severe neutrophilic inflammation, replacement of the crypts/glands, presence of inflammatory cells, or severe surface damage. Lymphocytes and plasma cells were considered normal inhabitants of the GI mucosa.

Statistical analysis

Data were fit using a linear mixed model using the GLIMMIX procedure of SAS, v 9.4 (SAS Institute Inc., Cary, NC). All calculations regarding CFU per mL or g were completed in Excel and logarithmically transformed. Non-detectable limit values were calculated for all sample types, any plates where no growth was observed were given this value in the statistical analysis. Fixed effects in the statistical model included treatment, day, and the associated interaction. A Kenward-Roger denominator degrees of freedom adjustment was used, and a Tukey-Kramer multiple comparison adjustment was used. Data were modeled using repeated measures and the variance-covariance structure was taken as either heterogeneous first-order autoregressive or first-order ante-dependence according to the model fitting criteria.

Histopathology data were also fit using the GLIMMIX procedures of SAS, v 9.4 and were analyzed as ordinal outcomes using a generalized linear model with a multinomial distribution and a cumulative logit link function. Fixed effects included treatment, tissue sample, and the associated interaction. Data was summarized using the FREQ procedure and reported as percentage of observations within each sample by their lesion score.

Results

Fecal, rectal, nasal, and environmental samples

For the duration of the experiment, control pigs had no *STM*-positive fecal, rectal, or nasal samples (Table 1). Aerosol-inoculated pigs had only one positive fecal sample, which occurred late in the experiment (d 7). Conversely, at least one fecal sample was *STM*-positive among the feed-inoculated pigs on 5 of the 7 days, and on 6 or the 7 days among the water-inoculated pigs. Among the environmental samples, the greatest prevalence of *STM*-positive samples were from the room housing feed-inoculated pigs, followed by the room housing water-

inoculated pigs. No positive environmental samples resulted from the rooms housing the control or aerosol-inoculated pigs.

Overall, feed- and water-inoculated pigs excreted a greater quantity of *STM* ($P = 0.0765$), compared to aerosol-inoculated pigs (2.8 and 2.9 log CFU/g vs 1.1 log CFU/g, respectively). However, there was no evidence that day or its interaction with treatment impacted the quantification of *STM* in fecal samples ($P > 0.05$). There was no evidence that treatment, day, or its interaction impacted the quantification of *STM* in rectal swabs ($P > 0.05$). Neither treatment nor its interaction with day impacted ($P > 0.05$) the quantification of *STM* in nasal swabs, but there was evidence that it was impacted by sampling day ($P = 0.0488$). The greatest levels of *STM* were detected from nasal swabs on day 3, and the quantity gradually decreased through d 7 ($P < 0.05$). There was no evidence that treatment, day, or its interaction impacted the quantification of *STM* in rectal swabs or environmental samples ($P > 0.05$).

Necropsy samples

At the end of the experiment, necropsy was conducted and the number of pigs with *STM* positive tissue and organ samples per treatment determined (Table 2). No control pigs had necropsy samples positive for *STM*. All treatments had similar number of positive samples. As expected, aerosol-inoculated pigs had the greatest prevalence of *STM*-positive lung samples (3 of 3 vs. 1 of 3 or 0 of 3 for feed or water-inoculated pigs, respectively). All inoculated pigs, regardless of method, had *STM*-positive ileocolic and jejunal lymph nodes, but only one feed-inoculated pig had an *STM*-positive inguinal lymph node. At least one pig per inoculation treatment had *STM*-positive tonsil, cecum/colon, or ileum samples. No pigs, even those inoculated with *Salmonella*, had *STM*-positive gluteal or gracilis muscles.

There was a significant treatment × sample interaction ($P < 0.05$) for the necropsy samples. The mesenteric lymph nodes (ileocolic and jejunal) and the lower digestive tract (cecum and colon) of the aerosol inoculated pigs had greater quantities of *STM* ($P < 0.05$) than the control pigs (4.0 and 4.0 CFU/g, respectively vs. no detectable *STM* in the control). The tonsils from the feed inoculated pigs had a greater presence of *STM* ($P < 0.05$) than the control pigs (4.7 CFU/g vs no detectable *STM* the control). The mesenteric lymph nodes (ileocolic and jejunal) and the lower digestive tract (cecum and colon) had a greater colonization of *STM* ($P < 0.05$) than the lungs, inguinal lymph node, the gluteal and gracilis muscles in the hind limb. Overall, all inoculated pigs, regardless of treatment, had a greater presence of *STM* than the control pigs ($P < 0.05$).

Histopathology

The cecum and colon had a greater frequency of lesion scores between 2.5 and 3 than the other tissue samples (Figure 1). Gluteal and gracilis muscles did not display any signs of lesions compared to the other sample types (Figure 1). In fact, the gluteal muscle was histologically-normal, with no inflammation or degeneration (Figure 2a). The cecum, however, had increased inflammatory cells and glands were observed to be filled with neutrophils and debris ($P < 0.05$; Figure 2b).

Discussion

This research demonstrates for the first time that *STM* consumed via contaminated feed or water or inhaled via aerosolized droplets may lead to intermittent excretion of *STM* in their feces and nasal excretions. In this experiment, pigs intermittently shed *STM* throughout the seven days of the trial. Other research with *STM* has also found intermittent shedding in the feces at 21

days post inoculation (dpi); however, Cevallos-Almeida et al. (2018) saw continuous fecal shedding at three different sampling points up to 84 dpi (Cevallos-Almeida et al., 2018; Naberhaus et al., 2020). Greater *Salmonella* excretion and continuous fecal shedding was observed when pigs were inoculated with *STM* compared to pigs in the same study inoculated with either *S.* Typhimurium or *S.* Derby (Cevallos-Almeida et al., 2019). Continuous fecal shedding and increased quantities of *STM* could be indicative of better attachment and replication in the intestines causing increased spread and contamination of the monophasic serotype.

Intermittent shedding of *S.* Typhimurium has been observed in rectal swabs from pigs intranasally inoculated (Oliveira et al., 2006). This is consistent with the intermittent shedding observed in the feed and water inoculated pigs of our study. Rectal swabs were not as sensitive to *STM* detection as fecal samples. Fecal samples less than 5 grams have been found to reduce the sensitivity of *Salmonella* detection in other work; therefore, the quantity of fecal matter collected from a rectal swab may not have been enough for accurate *Salmonella* detection (Bonardi, 2017). Aerosol inoculated pigs had fewer positive fecal and rectal samples than the other inoculated pigs. The lack of fecal shedding may point to different modes of infection and circulation when pigs inhale high levels of *Salmonella* than those that ingest it via their feed or water. However, trial duration may also explain the different shedding patterns between the inoculation methods. Oliveira et al. (2005) observed more *S.* Typhimurium positive samples from 8-19 dpi than 1-7 dpi when pigs when introduced to contaminated air. It is likely that airborne *STM* colonized and was excreted at a slower rate than *STM* introduced directly to the digestive system.

Nose-to-nose transmission of *Salmonella* has been observed in experimental conditions with *S.* Typhimurium (Oliveira et al., 2007). It has been difficult to prove nose-to-nose

transmission in commercial settings as pigs have constant contact with other animals, feces, and equipment. Due to the natural rooting behavior of pigs, nasal samples can entail a combination of nasal secretions and feed or feces from the environment. While this may lower the accuracy of detecting *Salmonella* in the nasal samples themselves, it can provide evidence to an alternate transmission route other than a fecal-oral one. In our study, nasal samples were able to detect *STM* throughout the trial in all inoculated treatments. This was also the only sample type where aerosol inoculated pigs consistently tested positive for *STM.* The continuous shedding observed in these samples alludes to *STM* secretion from the nose and does point towards nose-to-nose transmission as another *Salmonella* infection route.

Positive environmental samples were found consistently in the feed and water inoculated pens throughout the seven days of our trial. Most of the positive samples came from the plastic flooring of the pens, probably due to traces of fecal matter. Other surveillance and controlled studies have also found *Salmonella* traces on the pen floors (Proux et al., 2001; Dorr et al., 2009). As pen floors are areas constantly in contact with feces, the presence of *Salmonella* from environmental samples is not surprising. Other surfaces within the pen are not as consistently contaminated with *Salmonella* as they are less likely to be in contact with fecal matter (Gosling et al., 2017). Feeders and waterers had fewer positive *STM* samples than the pen floor in our study and positive samples were inconsistently observed. Few studies focus on feeders and waters as possible sites for contamination. Those studies which include environmental samples from these locations use them as surveillance measures during disinfection and do not report the presence or absence of *Salmonella* prior to disinfection (Martelli et al., 2017).

Our study observed a greater *STM* colonization in the tonsils of the feed inoculated pigs. Increased quantities of *STM* in the tonsils was also observed in previous research where the

inoculum was given similarly to the water inoculated pigs in this study (Cevallos-Almeida et al., 2018; Cevallos-Almeida et al., 2019). Contact between the tonsils and the inoculum may factor into the increased quantities of *STM* found within the tonsils. However, in pigs intranasally infected with *STM,* where the inoculum would once again come into contact with the tonsils, we observed decreased quantities of *STM* than the other tissues (Shippy et al., 2018). Shippy et al. (2018) observed increased quantities of *STM* present in the ileocecal lymph nodes and regions in the lymph nodes. Our study also found increased quantities of *STM* in the ileocolic and jejunal lymph nodes, especially for the aerosol inoculated pigs. While the tonsils were the most highly colonized samples, the mesenteric lymph nodes (ileocolic and jejunal) and the lower digestive tract (cecum and colon) were the most consistently positive samples. *STM* colonization is commonly found in the mesenteric lymph nodes and is a good indicator of infection (Cevallos-Almeida et al., 2018; Shippy et al., 2018; Cevallos-Almeida et al., 2019; Naberhaus et al., 2020). Contamination in the cecum was also observed in two studies by Cevallos-Almeida et al. and can indicate presence of *Salmonella* in the feces (Cevallos-Almeida et al., 2018; Cevallos-Almeida et al., 2019). Interestingly, the aerosol inoculated pigs were the only treatment to have *STM* positive lungs from all pigs. The presence of *STM* in the lungs suggests successful colonization and infection from the nebulized inoculum and not fecal-oral transmission from one carrier pig. Directional airflow has been used to infect pigs with airborne *Salmonella* in previous trials; however, to the author's knowledge this was the first study to find *STM* within the lungs (Proux et al., 2001; Martelli et al., 2017).

Our study observed successful *STM* colonization in a number of lymph nodes and tissues; however, the likelihood of those contaminated tissues being included in pork products must also be considered. Lymph nodes such as the ileocolic and jejunal are often associated with

Salmonella contamination in swine (Vieira-Pinto et al., 2005; Bessire et al., 2018). These lymph nodes, however, are easily removed from the carcass and do not pose equal amounts of risk to product contamination as other lymph nodes. Deep tissue lymph nodes, such as the subiliac or peripheral, are more difficult to isolate and remove during processing and could remain in the finished product. Specifically, these products pose a risk in ground products as they are derived from multiple carcasses and have greater risk of *Salmonella* contamination than whole products (Zhang et al., 2019).

Studies that compared lesions from *STM* or *S.* Typhimurium inoculated pigs found more severe lesions in the *S.* Typhimurium tissue samples than *STM* originating from *STM* inoculated pigs (Arruda et al., 2019). In our study, lesion scores did not differ between the control pigs and the inoculated pigs. Lesion scores differed between tissues as increased epithelial damage and neutrophil presence were observed in the cecum, jejunal lymph node, colon, and ileum compared to the gluteal and gracilis muscles. More severe lesions and epithelial damage have been observed early on after inoculation of *S.* Typhimurium; however, slow recovery was observed to begin around 6 dpi (Bellido-Carreras et al., 2019). As our pigs were not necropsied until 7 dpi, any lesions or damage may have already been repaired to an extent similar to the control pigs.

Conversely to our study, pigs intranasally infected with *S.* Choleraesius had more severe lesions and shed a greater amount of *Salmonella* than the gastric-inoculated pigs (Gray et al., 1995). This difference was probably due to the use of a nebulizer for aerosol inoculation in our study. Our goal was to simulate a more natural inhalation of the cocktail compared to the intranasal inoculation used in other studies.

In conclusion our study demonstrates that feed, water, and aerosol inoculation routes were successfully able to cause *STM* excretion in feces, rectal and nasal samples, and

colonization in tissues of weanling pigs. *STM* could be introduced into pork products if cross-contamination and improper handling of lymph nodes and digestive tissue were to occur.

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Table 3-1 Detection of *STM* positive samples per treatment each day[1]

	Control							Aerosol							Feed							Water						
Day	1	2	3	4	5	6	7	1	2	3	4	5	6	7	1	2	3	4	5	6	7	1	2	3	4	5	6	7
Fecal	---	---	---	---	---	---	---	---	---	---	---	---	---	+--	++-	---	+--	++-	---	++-	++-	++-	+--	++-	+--	+--	+--	---
Rectal	---	---	---	---	---	---	---	---	---	---	---	---	---	---	+--	++-	+--	+--	+--	---	+--	++-	++-	+--	+--	+--	+--	---
Nasal	---	---	---	---	---	---	---	++-	+++	+--	+--	---	+--	+--	+--	+++	+--	++-	+--	++-	+--	++-	+--	+++	+--	---	---	---
Environmental[2]	---	---	---	---	---	---	---	---	---	---	---	---	---	---	++-	++-	++-	+--	+--	---	--+	+--	---	+-+	+--	---	---	---

[1] Fecal, rectal, and nasal samples were taken from each pig daily (n=3). Environmental samples were taken daily from the floor, feeder, and waterer (n=3). +/- denotes the *STM* status of each sample on a given day.

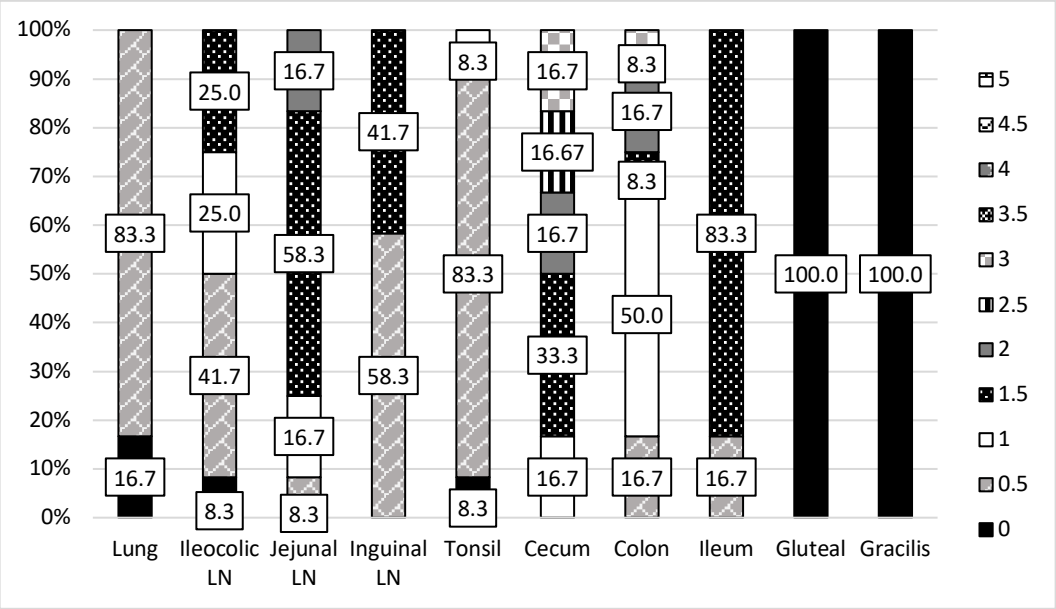
[2] Arranged in order 1) plastic flooring, 2) feeder, 3) waterer.

Table 3-2 Detection of *STM* positive necropsy samples from each treatment[1]

	Control	Aerosol	Feed	Water
Lung	---	+++	+--	---
Ileocolic and Jejunal lymph node	---	+++	+++	+++
Inguinal lymph node	---	---	+--	---
Tonsil	---	+--	+++	++-
Cecum and Colon	---	+++	++-	++-
Ileum	---	++-	++-	++-
Gluteal muscle	---	---	---	---
Gracilis muscle	---	---	---	---

[1] Pigs were euthanized on d7 and tissue and organ samples were collected at necropsy. Each treatment had 3 pigs. +/- denotes the *STM* status of each pig for each tissue type.

Figure 3-1 Frequency of the histopathology scores for each sample[1]



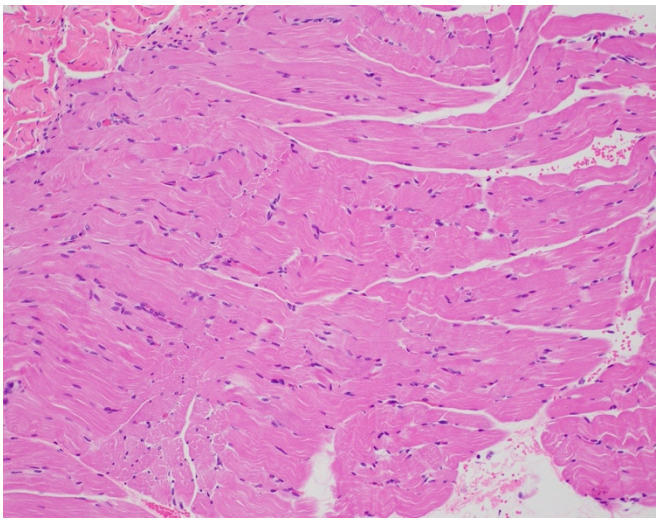
Sample: $P = 0.0008$

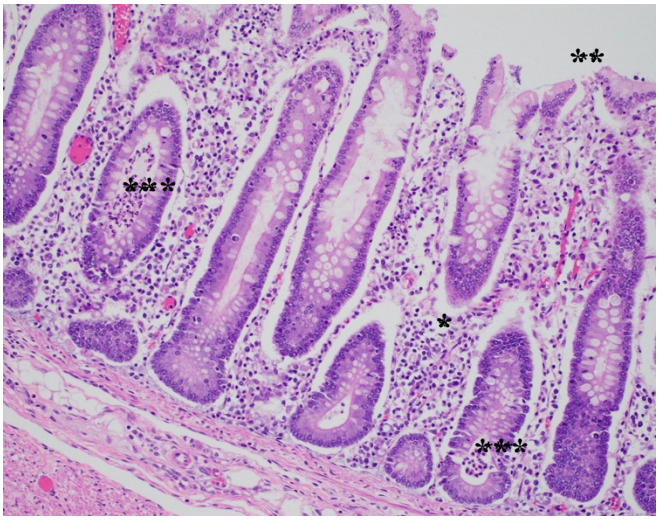
Treatment: $P = 1.0000$

[1] Scored between 0-5 where 0 = no neutrophils were observed; lymphocytes and plasma cells were considered normal inhabitants of the GI mucosa and 5= moderate to severe neutrophilic inflammation/clusters in propria and in glands; there was replacement of crypts/glands by exudate, inflammatory cells; there was more severe surface damage with exudate (erosive or ulcerative colitis).

Figure 3-2 Example of tissue collected at necropsy and assigned lesion scores:(A) Gluteal sample taken from a water inoculated pig. No inflammation or degeneration was observed; (B) Cecal tissue sample taken from a feed inoculated pig.

*Increased inflammatory cells in the lamina propia
**Artifactually damaged epithelium cells
***Glands filled with neutrophils and debris





Chapter 4 - Evaluation of biosecurity measures on a swine operation using Glo Germ powder as a visible learning aid[2]

Abstract

Glo Germ powder was used to determine the efficacy of common biosecurity practices to prevent the powder from spreading to other areas within a farm. Pictures from four locations were taken before and after personnel movement to observe any differences in Glo Germ coverage. The percentage of Glo Germ coverage in the pictures was evaluated by 47 panelists and averaged. The area without biosecurity measures had more Glo Germ coverage than the three areas with biosecurity measures ($P < 0.0001$). The use of Glo Germ can be used as a learning aid to demonstrate the efficacy of common biosecurity practices.

Introduction

Farm biosecurity is an integral aspect in maintaining the health of the herd. Movement and isolation of animals, human traffic, and pests can all pose a risk of a biosecurity infraction. Viruses such as porcine reproductive and respiratory syndrome virus (PRRSV) can be transferred from boots into vehicles and into other farms (Dee et al., 2002; Dee et al., 2003). Under simulated conditions, low biosecurity measures have been shown to increase the spread of

[2] This work has been submitted for publication to *Journal of Swine Health & Production*. Harrison OH, PL Dahmer, JT Gebhardt, CB Paulk, JC Woodworth, CK Jones. Evaluation of Biosecurity Measures on a Swine Operation Using Glo Germ Powder as a Visible Learning Aid. Submitted 10/21/21.

porcine epidemic diarrhea viruses (PEDV) compared to higher biosecurity measures such as showers and changing clothes (Kim et al., 2017).

Upholding the health of the farm is reliant on the ability of workers to continuously implement existing biosecurity protocols. Lapses in biosecurity compliance, especially in times of perceived low infection risk or during worker shortages, can cause biosecurity breaches. During simulated games, players were more likely to break biosecurity in order to earn a higher payout when they were more certain animals would not become infected (Merrill et al., 2019; Trinity et al., 2020). Frequent biosecurity breaches have also been found when eight poultry farms were surveyed using hidden cameras. During the surveillance time, 44 types of biosecurity errors were made, with 2 to 7 events occurring per day per farm (Racicot et al., 2011). Biosecurity breaches tend to happen due to rushing through work and are often done unintentionally. It is difficult for employers to visually demonstrate a biosecurity breach without endangering the farm when teaching new employees or visitors.

Glo Germ Company manufactures fluorescent gels or powders which can simulate germs or other contaminants under UV light. Glo Germ has been used in research settings to compare handwashing techniques and as a demonstration for aseptic technique in hospitals (Turner et al., 1994; Mittal et al., 2011). Spreading Glo Germ throughout a deli revealed areas of cross-contamination between the original equipment and the doors, meat products, and prep equipment (Maitland et al., 2013). Similarly, Glo Germ has been used to evaluate biosecurity exit protocols when applied to lab coats and gowns (Guo et al., 2014). The different applications have all demonstrated Glo Germ's ability to be used as a teaching aid to improve biosecurity aptitude of individuals; however, it is not routinely used in swine facilities to teach biosecurity principles. Therefore, the objective of this study was to use Glo Germ within a swine operation to

demonstrate the efficacy of common biosecurity protocols which can be used as a visible teaching aid for future students and farm personnel.

Materials and Methods

The Kansas State University Institutional Review Board approved the protocol used in this experiment. The study was conducted concurrently with the spring 2021 Swine Undergraduate Research class (UGR). Prior to the start of their trial, all undergraduate students were taught the biosecurity protocols of the farm. Students were not made aware how the biosecurity protocols were being evaluated or why there was powder in key areas throughout the farm.

Glo Germ Coverage

Four different locations at the KSTRC were photographed weekly for 7 weeks to provide an assessment of the efficacy of the biosecurity measures to prevent movement of the Glo Germ powder (Glo Germ Company, Moab, Utah). All pictures were taken on a standard iPhone mounted onto a PVC frame with attached blacklights (Figure 4.1) which could be transported to each location. The PVC frame measured 2 × 2 × 2 ft and was wrapped in a large black trash bag to block light from the surroundings. Two LED flashlights (Rayovac, Energizer Brands, LLC, St. Louis, MO) were mounted equal distance apart on the center beam of the frame.

The locations were 1) the clean side of the entry bench into the farm, 2) the flooring within the shower, 3) the clean side of the locker room after completing the required shower, and 4) within the barn (control – no biosecurity measure). Glo Germ was spread in areas preceding the clean areas such as outside the entry door, the dirty side of the locker room, and the feed room used in the barn. The clean areas were cleared of any remaining Glo Germ from the prior

week in the evening before the UGR's heaviest traffic day and pictures were taken of these areas to serve as "before" pictures. Following student movement, "after" pictures were taken of the same areas. These before and after pictures were blindly evaluated by 47 untrained panelists to determine the quantity of Glo Germ coverage visible within each picture on a scale from 0 to 100% coverage; each picture was assessed once per panelist (n=47). The assessed quantity of Glo Germ visible was than averaged across all panelists, so that each photograph was represented by a single value. Before and after Glo Germ averages were then aligned and the difference between those averages for each location within a given day was calculated. These average differences would represent the increased quantity of Glo Germ visible between the before and after pictures.

Statistical Analysis

Data were analyzed using a linear model fit using the GLIMMIX procedure of SAS, v 9.4 (SAS Institute Inc., Cary, NC). Location on a given day was the experimental unit, and data were analyzed as the average change in panelist-assigned Glo Germ coverage between the before and after traffic images at each location on each day of evaluation. Location was considered a fixed effect in the statistical model. Least squares means were reported using a Tukey multiple comparison adjustment.

Results

The control location had increased Glo Germ coverage compared to the three other locations ($P < 0.0001$) as would be expected considering no biosecurity measures were in place to prevent to movement of Glo Germ onto the surface evaluated. On average the three locations with biosecurity measures in place did not have increased Glo Germ coverage above 1% following movement of students through the three locations. The average difference in Glo Germ

coverage of the control, however, was 19.5% across the 7 weeks (Figure 4.2). There was no evidence of a difference in Glo Germ coverage between the entry bench, shower floor, or clean side of the shower ($P > 0.05$).

Visual evidence of a biosecurity breach was evident during week 2 of this experiment. Figure 4.3a is the floor of the clean side of the locker room prior to any student and personnel movement. Figure 4.3b is of the same area after a biosecurity breach with increased coverage of orange Glo Germ visible. In contrast, Figure 4.3c shows the same location from week 3 with little to no visible Glo Germ after all student and personnel successfully showered through and stopped the spread of Glo Germ.

Discussion

Fomites, such as boots and coveralls, have been identified as sources of viral transmission in previous studies (Otake et al., 2002; Pitkin et al., 2009; Kim et al., 2017). These studies have found that a lack of handwashing and not changing clothing and shoes between groups of animals will lead to infection and cross-contamination of pathogens. However, like most viral work, the research was conducted in a biosecure facility and is hard to replicate on a commercial farm or alongside farm personnel.

Implementing Glo Germ at the farm allowed students and personnel to see the difference biosecurity measures can make. In the control area, increased quantities of Glo Germ could be seen without UV light and could be tracked throughout the barn. However, areas where biosecurity measures were followed greatly reduced the quantity of Glo Germ visible and predominantly stopped the spread of Glo Germ altogether. Similarly, Anderson et al. (2018) included an entry bench prior to the showers at a commercial swine farm and saw reduced coverage of Glo Germ following the bench and no visible Glo Germ after the bench and shower.

Julien and Thomson (2011) also used Glo Germ as a teaching aid for poultry producers. Producers were impressed by the quick visual Glo Germ was able to provide and how efficiently Glo Germ was able to demonstrate the gaps in biosecurity.

One biosecurity breach was observed during our trial. Glo Germ was observed on the clean side of the locker room following the shower. It is most likely due to personnel undressing on the dirty side of the locker room, stepping through the Glo Germ powder, walking across the shower without washing off, and stepping onto the clean side. The reasoning behind this breach is largely unknown but could have been caused by someone rushing into the farm late or assuming they were not at a risk to bring pathogens onto the farm and decided to skip the shower. Time constraints have previously been cited as the reason for a lapse in biosecurity even if the worker was aware of the necessary protocols (Millman et al., 2017).

Biosecurity continues to be a difficult subject for employers to teach and for farm personnel and visitors to continuously uphold. Breaches of varying extremes are common in farms; however, the risk of pathogen introduction remains a constant threat. Demonstrating the potential spread and contamination of a pathogen will help reiterate the need for biosecurity protocols on farms. Visual aids, such as Glo Germ, are easy and effective ways to exhibit biosecurity compliance and highlight any breaches within a farm.

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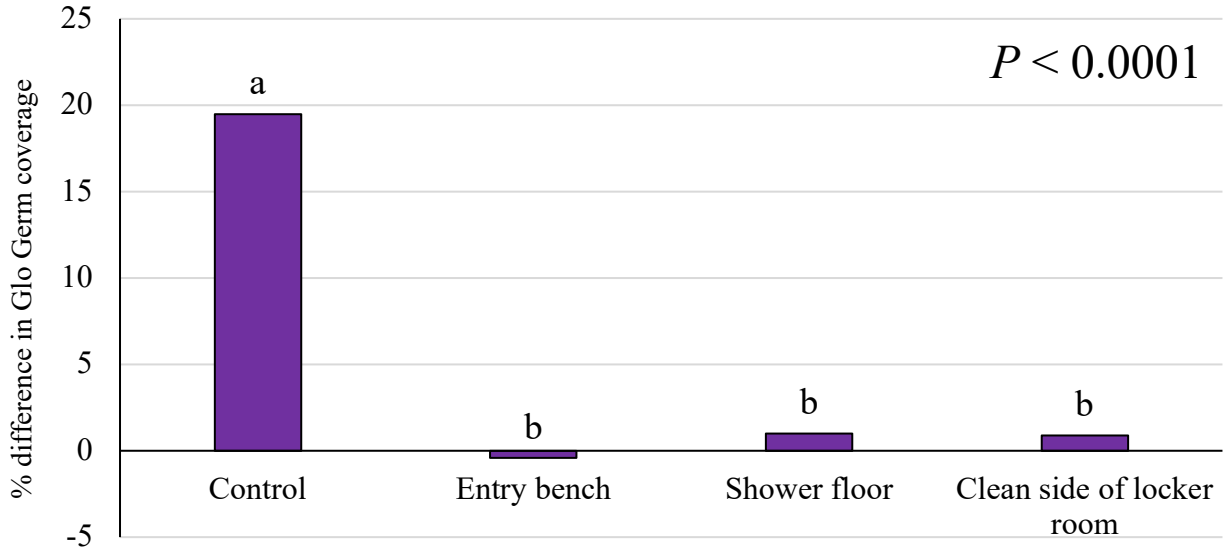
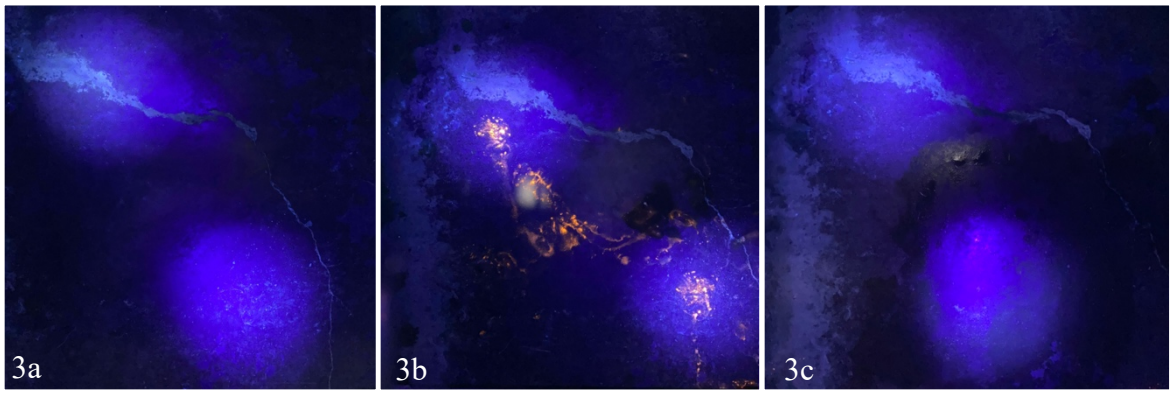
Figure 4-1 Image of PVC frame with attached blacklights used for all pictures taken

Figure 4-2 Utilizing Glo Germ to assess biosecurity principles on a swine farm[1]



[1] Measures the average increase in Glo Germ coverage between before people traffic and after people traffic; a higher percentage represents more Glo Germ visible after personnel movement. Control area = no biosecurity measures; entry bench, shower, and locker room = biosecurity measure. Means with differing superscripts differ significantly ($P < 0.05$).

Figure 4-3 Images detailing a biosecurity breach in a swine operation[1]



[1] Glo Germ powder was orange in this location

Figure 3a. Clean side of the locker room before student and personnel movement in week 2.
3b. Clean side of the locker room after student and personnel movement in week 2.
3c. Clean side of the locker room after student and personnel movement in week 3.