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Summary

Salmonella enterica serotype 4,[5],12:i:- (STM) has become an increasing problem for food safety and has been often detected in pork products. For this study, weanling pigs were exposed to STM-contaminated feed, water, or air to determine possible STM transmission routes. An uninoculated control group of pigs was included. The STM was monitored daily in feces and rectal and nasal swabs. The STM colonization was most prevalent in tissues from tonsil, lower intestine, and mesenteric lymph nodes. No differences in lesion severity were observed between inoculated and control pigs. Contaminated feed, water, and aerosolized particles caused infection in weaned pigs; however, no STM colonization was observed in skeletal muscle destined for human consumption. Based on the results from this study, STM contamination in pork products most likely results from cross-contamination of meat by digesta or lymph node tissue during processing.

Introduction

From 2006 to 2015 there were a growing number of salmonellosis cases linked to pork products.⁵ 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, is among the most frequently reported. In 2013, STM was the third most common serotype linked to pigs, pork products, and clinical salmonellosis cases in Europe⁶ and one of the six most common

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⁵ Centers for Disease Control and Prevention. 2014. National Enteric Disease Surveillance: Salmonella Surveillance Overview. Available at: https://www.cdc.gov/nationalsurveillance/pdfs/2014_Salmonella-REPORT-508.pdf.

⁶ European Food Safety Authority. 2010. Scientific Opinion on monitoring and assessment of the public health risk of “*Salmonella* Typhimurium-like” strains. Available at: <https://efsa.onlinelibrary.wiley.com/doi/pdf/10.2903/j.efsa.2010.1826>.

serotypes isolated from foodborne illnesses in the United States.⁷ This pathogen has recently caused several outbreaks, some of which were traced back to contaminated pork or pork products. Because the pathogenicity gene repertoire of STM is highly similar to that of *Salmonella* Typhimurium but with greater antimicrobial resistance, researchers are working to understand the STM transmission route into the pork supply chain. The objective of this study was to assess and evaluate the transmission of STM when contaminated feed, water, and aerosolized particles were administered to weaned pigs.

Procedures

All protocols were reviewed and approved by the Kansas State University (K-State) Institutional Animal Care and Use Committee.

Cultures

Four strains of STM were selected for this study: RM 17306 (U.S. Department of Agriculture [USDA], California) isolated from a water source; FSLs 5-580 (Cornell University, Ithaca, NY) from an animal source; H20-01924 from a clinical source (Minnesota Department of Health); and KSU1966 from a feed mill facility.⁸ Strains were kept in cryovials (CryoCare Bacteria Preservers, Key Scientific Products, Stamford, TX) at -176°F until 48 h before the experiment then were individually plated on tryptic soy agar (Difco, BD, Sparks, MD) and incubated at 98.6°F for 24 h. A single colony was selected from each plate and transferred into 50 mL of Luria-Bertani Miller broth (Difco, BD) and incubated at 98.6°F for 12 to 18 h with shaking.⁹ On the day of the experiment, each culture was centrifuged at 4,000 rpm for 15 min at 41°F, and the pellet was resuspended in 5 mL of phosphate-buffered saline (VWR, Radnor, PA).⁶ The four STM strains were combined in equal amounts to obtain an inoculum of 6.7×10^9 CFU/mL.

Inoculum preparation

Because the intent of this study was to determine whether feed, water, or air could be a transmission route for STM into the pork supply chain, feed and water solutions experimentally contaminated with STM were prepared for subsequent animal inoculation. For the feed mixture, 10 mL of the freshly prepared 6.7×10^9 CFU/mL STM cocktail was added to 30 g of finely ground feed (K-State Phase 3 common diet with no special protein sources). The solution was allowed to absorb and then was hand homogenized for 2.5 min, and 60 mL of buffered peptone water (BPW; Difco, BD) was added to the feed mixture and gently mixed to create a slurry with an STM level of approximately 1.3×10^9 CFU/g.¹⁰ The contaminated water solution was created by adding 10 mL of the STM cocktail to 50 mL of BPW with gentle mixing to create an inoculum solution

⁷ Switt, A. I. M., Y. Soyer, L. D. Warnick, M. Wiedmann. 2009. Emergence, Distribution, and Molecular and Phenotypic Characteristics of *Salmonella enterica* Serotype 4,5,12:i:i. *Foodborne Pathog. Dis.* 6:407-15.

⁸ Trinetta, V., G. Magossi, M. W. Allard, S. M. Tallent, E. W. Brown, S. Lomonaco. 2020. Characterization of *Salmonella enterica* Isolates from Selected U.S. Swine Feed Mills by Whole-Genome Sequencing. *Foodborne Pathog. Dis.* 17:126-36.

⁹ Loynachan, A. T., J. M. Nugent, M. M. Erdman, D. L. Harris. 2004. Acute infection of swine by various *Salmonella* serovars. *J. Food. Prot.* 67:1484-8148.

¹⁰ Niederwerder, M. C., A. M. M. Stoian, R. R. Rowland, S. S. Dritz, V. Petrovan, L. A. Constance, J. T. Gebhardt, M. Olcha, C. K. Jones, J. C. Woodworth, Y. Fang, J. Liang, T. J. Hefley. 2019. Infectious dose of African swine fever virus when consumed naturally in liquid or feed. *Emerg. Infect. Dis.* 25:891-97.

of approximately 1.0×10^9 CFU/mL.⁷ No additional media were 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 K-State Large Animal Research Center (Manhattan, KS). Upon arrival, pigs were weighed (mean $\pm$ standard deviation = 13.8 ± 0.22 lb) and randomly allotted to one of four treatments, with three pigs per treatment: control (no STM), STM inoculation via feed slurry, STM inoculation via water source, and STM inoculation via aerosolization. Before inoculation, all pigs were confirmed negative for *Salmonella* shedding via rectal and nasal swabs.¹¹

Animal inoculation

After a 6-day acclimation period, animals were inoculated in separate treatment rooms. 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 g 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.¹² Before inoculation, the aerosol-inoculated pigs were sedated with tiletamine and zolazepam (2.2 to 4.4 mg/kg of body weight administered intramuscularly; Telazol, Zoetis, Kalamazoo, MI) 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 each pig's snout for approximately 4 min until no liquid remained in the holding chamber.¹³ Because of the speed of the nebulizer, 10 mL of solution would take too long for aerosol inoculation; therefore, only 1 mL of the cocktail was given to the aerosol-inoculated pigs. The feed- and water-inoculated pigs received an overall higher 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 days.

Data collection

Pigs were weighed daily, and health was evaluated for clinical signs of salmonellosis, specifically vomiting, diarrhea, or lethargy. Fecal samples, nasal and rectal swab samples, and environmental swab samples from the floor, feeder, and waterer were collected daily to monitor the presence of STM and quantify its excretion. A sterile cotton swab was used to stimulate the rectum, and approximately 10 g of fecal matter was collected directly into a stomacher bag.¹⁴ Rectal and nasal swabs were also taken with a sterile cotton swab and placed into 5 mL of phosphate buffered tryptic soy broth (TSB-PO₄;

¹¹ Smith, A. B., D. G. Renter, N. Cernicchiaro, X. Shi, T. G. Nagaraja. 2016. Prevalence and Quinolone Susceptibilities of *Salmonella* Isolated from the Feces of Preharvest Cattle Within Feedlots that Used a Fluoroquinolone to Treat Bovine Respiratory Disease. *Foodborne Pathog. Dis.* 13:303-08.

¹² Schumacher, L.L., J. C. Woodworth, C. K. Jones, Q. Chen, J. Zhang, P. C. Gauger, C. R. Stark, R. G. Main, R. A. Hesse, M. D. Tokach, S. S. Dritz. 2016. Evaluation of the minimum infectious dose of porcine epidemic diarrhea virus in virus-inoculated feed. *Am. J. Vet. Res.* 77:1108-13.

¹³ Dee, S. A., J. Deen, J. P. Cano, L. Batista, C. Pijoan. 2006. Further evaluation of alternative air-filtration systems for reducing the transmission of Porcine reproductive and respiratory syndrome virus by aerosol. *Can. J. Vet. Res.* 70:168-175.

¹⁴ Agga, G.E., T. M. Arthur, J. W. Schmidt, R. Wang, D. M. Brichta-Harhay. 2016. Diagnostic Accuracy of Rectoanal Mucosal Swab of Feedlot Cattle for Detection and Enumeration of *Salmonella enterica*. *J. Food. Prot.* 79:531-537.

Difco, BD) containing 2.13 g/L KH_2PO_4 and 12.54 g/L K_2HPO_4 (VWR).¹⁵ Environmental swabs were collected with a premoistened sponge (3M, St. Paul, MN). Samples were collected from a floor area (10 × 10 cm), the feeder, and waterers in each pen.¹⁶ Floor samples were collected from the same area each day regardless of the presence of fecal contamination in the sample area. All samples were transported on ice and immediately analyzed. At the completion of the study (day 7), pigs were sedated with tiletamine and zolazepam (2.2 to 4.4 mg/kg of body weight administered intramuscularly) and given a sodium pentobarbital overdose (85 to 100 mg/kg of body weight administered intravenously), and the carcasses were transported to the K-State Veterinary Diagnostic Laboratory for necropsy. Tissues (lungs; ileocolic, jejunal, and inguinal lymph nodes; tonsil; cecum; colon; ileum; right gluteal muscle; and gracilis muscle) were collected in duplicate. One sample of each organ or tissue was fixed in 10% buffered neutral formalin for histologic analysis, and the other sample (~20 g) was placed on ice and transported to the Food Safety and Microbiology Laboratory at KSU for microbial enumeration.

Microbial enumeration

A modified procedure was used to process fecal, rectal, and nasal samples.¹² The TSB- PO_4 (90 mL) was added to the fecal samples (when > 10 g was available, 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 made for all samples, and 1 mL of each dilution was plated onto Enterobacteriaceae (EB) Petrifilm (3M) and incubated at 37°C for 22 to 26 h. Colonies were counted, and for any sample that produced gas, the EB film was removed and pressed onto premade xylose lysine deoxycholate (XLD; Criterion, Santa Maria, CA) agar plates. These sample plates were incubated at 37°C for an additional 22 to 26 h, and black presumptive *Salmonella* colonies were counted. Environmental samples were processed following the USDA protocol;¹³ 50 mL of BPW was added to the sample, which was then hand homogenized, serially diluted, plated onto EB Petrifilm, and incubated. Petrifilm with gas-producing colonies was pressed onto XLD agar, and presumptive *Salmonella* colonies were counted as described in a previous research study.¹²

Tissue samples were trimmed and fat, fascia, and excreta were removed. Lymph nodes were placed into boiling water for 3 to 5 s for surface sterilization, transferred into a Whirl-Pak bag (Nasco, Ft. Atkinson, WI), and pulverized with a rubber mallet, and 80 mL of TSB- PO_4 was added. Serial dilutions were plated on EB Petrifilm as described in a previous research study.¹² For all plates with typical *Salmonella* colonies, identity was confirmed via PCR assay following a modified method developed in our laboratory.¹⁷

¹⁵ Chaney, W.E., G. E. Agga, S. V. Nguyen, T. M. Arthur, J. M. Bosilevac, E. Dreyling, A. Rishi, D. Brichra-Harhay. 2017. Rapid Detection and Classification of *Salmonella enterica* Shedding in Feedlot Cattle Utilizing the Roka Bioscience Atlas *Salmonella* Detection Assay for the Analysis of Rectoanal Mucosal Swabs. *J. Food. Prot.* 80:1760-67.

¹⁶ United States Department of Agriculture Food Safety and Inspection Service. 2019. Isolation and Identification of *Salmonella* from Meat, Poultry, Pasteurized Egg, and Siluriformes (Fish) Products and Carcass and Environmental Sponges. Available at: <https://s27415.pcdn.co/wp-content/uploads/2020/01/64ER20-7/Microbial/5-USDA-FSIS-4.10-Isolation-and-Identification-of-Salmonella.pdf>.

¹⁷ Magossi, G., J. Bai, N. Cernicchiaro, C. K. Jones, E. Porter, V. Trinetta. 2019. Seasonal presence of *Salmonella* spp., *Salmonella* Typhimurium and its monophasic variant serotype I 4,[5],12:i:-, in selected United States swine feed mills. *Foodborne Pathog. Dis.* 16:276-281.

Histopathology

Histopathology samples were collected and analyzed by a board-certified veterinary anatomic pathologist at the K-State Veterinary Diagnostic Laboratory. Each fixed tissue sample collected at necropsy was subjected to qualitative histologic analysis, including lesion analysis and scoring and the presence or absence of bacteria. Lesion scores were assigned for each tissue on a scale of 0 to 5, where 0 = samples with no inflammation (primarily neutrophils) and 5 = severe inflammation, replacement of the normal parenchymal tissue by inflammatory cells, fibrin, and/or tissue necrosis. In the small and large intestine, the presence of lymphocytes and plasma cells were considered normal.

Statistical analysis

Data were fit to a linear mixed model using the GLIMMIX procedure of SAS v. 9.4 (SAS Institute, Cary, NC). All calculations were completed in Excel (Microsoft, Redmond, WA) and logarithmically transformed. The limits of detection were calculated for all sample types, and plates on which no growth was observed were assigned this limit value in the statistical analysis. Fixed effects in the statistical model were treatment, day, and the associated interactions. 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 were treatment, tissue sample, and the associated interactions. Data were summarized using the FREQ procedure and reported as the percentage of observations within each sample by their lesion score.

Results and Discussion

For the duration of the experiment, no STM was detected in fecal, rectal, or nasal samples of control pigs (Table 1). Only one STM-positive fecal sample was obtained from aerosol-inoculated pigs, and this sample was collected on the last day of the experiment (day 7). At least one fecal sample was STM-positive from feed-inoculated pigs on 5 of the 7 days and from water-inoculated pigs on 6 of the 7 days. For the environmental samples, the highest prevalence of STM-positive samples was in the room housing feed-inoculated pigs followed by the room housing water-inoculated pigs. No positive environmental samples were identified from the rooms housing control or aerosol-inoculated pigs.

Overall, feed- and water-inoculated pigs excreted significantly more STM ($P = 0.0765$) (2.8 and 2.9 log CFU/g, respectively) than did aerosol-inoculated pigs (1.1 log CFU/g). However, the STM level in fecal samples was not impacted by sampling day or its interaction with treatment ($P > 0.05$). The STM level in rectal swabs also was not impacted by treatment, day, or their interaction ($P > 0.05$). The STM level in nasal swabs was not impacted by treatment or its interaction with day ($P > 0.05$) but was impacted by sampling day ($P = 0.0488$). The highest levels of STM were detected in nasal swab samples on day 3, and the levels gradually decreased through day 7 ($P < 0.05$). The STM

level in environmental samples was not impacted by treatment, day, or their interaction ($P > 0.05$).

Necropsy samples

At the end of the experiment, necropsies were conducted and the number of pigs with STM-positive tissue and organ samples was determined per treatment (Table 2). No necropsy samples from control pigs were positive for STM by bacterial culture. All treatment groups had similar numbers of STM-positive samples. Aerosol-inoculated pigs had the highest prevalence of STM-positive lung samples (all three pigs versus one and zero of three pigs in the feed and water inoculation treatment groups, 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 group had STM-positive tonsil, cecum or colon, or ileum samples. No control or inoculated pigs had STM-positive gluteal or gracilis muscles.

A significant treatment $\times$ sample interaction ($P < 0.05$) was found for STM in tissue samples collected at necropsy. The mesenteric lymph nodes (ileocolic and jejunal) and the lower digestive tract (cecum and colon) of the aerosol-inoculated pigs had higher levels of STM (4.0 and 4.0 CFU/g, respectively) ($P < 0.05$) than did those tissues of control pigs (no detectable STM). Tonsils from feed-inoculated pigs had a higher level of STM (4.7 CFU/g) ($P < 0.05$) than did control pigs (no detectable STM). Mesenteric lymph nodes (ileocolic and jejunal) and lower digestive tract tissues (cecum and colon) had higher levels of STM ($P < 0.05$) than did lungs, inguinal lymph nodes, and gluteal and gracilis muscles. All inoculated pigs, regardless of treatment group, had higher levels of STM than did control pigs ($P < 0.05$).

The cecum and colon had significantly higher numbers of lesions with higher scores (2.5 to 3) than did all other tissues (Figure 1). Gluteal and gracilis muscles lacked lesions (Figure 1) and were histologically normal, with no evidence of inflammation or degeneration. The cecum had significantly increased inflammatory cells (neutrophils) within the lamina propria, and cecal glands were often and variably filled with neutrophils and necrotic cellular debris ($P < 0.05$).

In conclusion, we demonstrated that feed, water, and aerosol STM-inoculation routes were associated not only with evidence for colonization of various tissues in weanling pigs, but also STM excretion in feces, rectal, and nasal samples during the initial 7 days following inoculation. Therefore, 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 1. Detection of STM positive samples per treatment each day¹

Day:	Control							Aerosol						
	1	2	3	4	5	6	7	1	2	3	4	5	6	7
Fecal	---	---	---	---	---	---	---	---	---	---	---	---	---	+-
Rectal	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Nasal	---	---	---	---	---	---	---	++	+++	-+	+-	---	-+	+-
Environmental ²	---	---	---	---	---	---	---	---	---	---	---	---	---	---

Day:	Feed							Water						
	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). The +/- denotes the STM status of each sample on a given day. Pig IDs are in the same order each day (i.e., pig 1 is in the same position every day for each sample).

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

Table 2. Detection of STM by bacterial culture in tissue samples collected during necropsy from each treatment¹

	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 d 7 and tissue and organ samples were collected at necropsy. Each treatment had 3 pigs. The +/- denotes the STM status of each pig for each tissue type. Pig IDs are in the same order each sample (i.e., pig 1 is in the same position each sample).

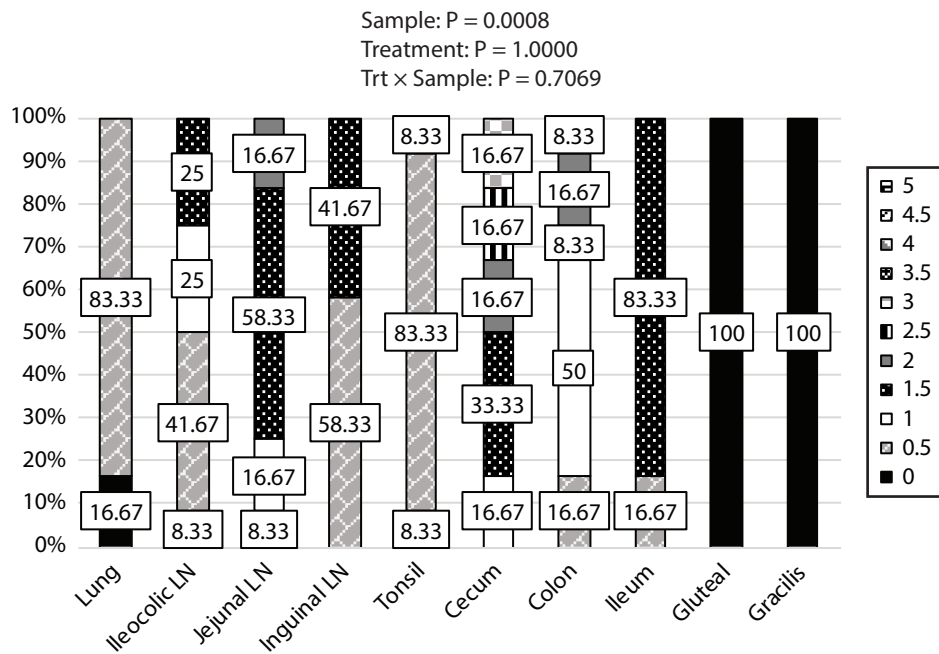


Figure 1. Frequency of histologic lesion scores for each tissue¹

¹ Lesions were 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 glands, replacement of glands by exudate, inflammatory cells, or surface epithelial damage.