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Salmonella prevalence of lymph nodes and synovial fluid of orally inoculated swine

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ABSTRACT

Salmonella is a foodborne pathogen that may be associated with the consumption of meat products. Failure of current interventions to control *Salmonella* in the food supply of the U.S. has led researchers to believe that atypical carcass reservoirs may be partially responsible for harboring this pathogen. In this two phase study, pigs (n = 36/Phase 1; n = 38/Phase 2) were experimentally infected orally with *Salmonella* Typhimurium to monitor the spread of the organism within the animal body. Fecal samples were collected 24, 48, and 72 h post-infection and tested for the presence of the *Salmonella*. After the pigs were euthanized, Ileocecal, subiliac, popliteal, and mandibular lymph nodes were collected, and synovial fluid was collected from the coxofemoral, shoulder, and stifle joints at the same post-infection timepoints to test for the experimentally inoculated bacteria. Fecal prevalence tended to be greater in Phase 1 (P = 0.06; 52.8 versus 31.6%). Ileocecal lymph node prevalence was 41.67% for Phase 1 and 37.00% for Phase 2. Both mandibular and subiliac lymph node prevalence was determined to be 2.78% in Phase 1; however, no *Salmonella* were detected in Phase 2. Examination of synovial fluid yielded a prevalence of 2.63% in all locations (from a single pig) in Phase 2 but was not different from Phase 1 (P = 0.30) in which no samples were positive for *Salmonella*. These results suggest that it is possible for orally contracted *Salmonella* to migrate to musculoskeletal lymph nodes. Contamination in these areas may lead to cross-contamination of meat products. Further research is needed to determine routes and migration patterns of *Salmonella* from the gastrointestinal tract to peripheral tissues to further elucidate how these infections impact food safety.

Keywords: swine, *Salmonella*, lymph node, synovial

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INTRODUCTION

Salmonella was the most commonly reported foodborne bacterial infection in 2011 (16.42 cases per 100,000 people), thus failing to meet the objectives to reduce the incidence of foodborne *Salmonella* illness set forward by the 2010 national health objective (6.8 cases per 100,000 people; Centers for Disease Control and Prevention; CDC, 2012). The "Healthy People 2010" report, a publication of the United States Department of Health and Human Services, indicated a failure to mitigate *Salmonella* illness (Johnston, 2012). A broader understanding of etiological and ecological characteristics and strategies to reduce and control the prevalence of this pathogen remains a priority in all meat producing species.

Recent estimates suggest that among foodborne illness in the U.S., 9 to 15% of all *Salmonella* infections, and 7.5% of *Salmonella enterica* serotypes Enteritis and Typhimurium infections, are associated with the consumption of pork or pork products (Hald *et al*., 2004; Pires *et al*., 2010). The risk of *Salmonella* infection from pork consumption is often considered minimal when compared to *Salmonella* infections stemming from the consumption of other food products (especially poultry). However, the commonality in serotypes isolated from pigs and human infection (USDA, 2013) combined with *Salmonella*'s ubiquitous nature in swine production settings makes the pathogen an area of focus for the pork industry.

With regard to *Salmonella* in the food supply, lymph nodes have recently become a harbor of interest. Lymph nodes in musculoskeletal tissues of beef carcasses are often included in retail cuts and/or ground beef, and have been targeted as an atypical reservoir of *Salmonella* (Arthur *et al*., 2008; Gragg *et al*., 2013). Lymph nodes collected from the gastrointestinal tract and head of pigs at harvest have also been reported to harbor *Salmonella* (Vieira-Pinto *et al*., 2005); however, there has been minimal research conducted to evaluate the occurrence of *Salmonella* in peripheral lymph nodes of seemingly healthy swine that are more likely to be introduced into the food supply.

In addition to lymph nodes, synovial fluid may also harbor *Salmonella*, thus serving as another potential source of contamination within food products. While investigations into *Salmonella* and other foodborne pathogens in pork synovial fluid are limited, an increased prevalence of *Salmonella* in human joints after trauma or invasive procedures has been reported, and supports the hypothesis that joint synovial fluids can harbor *Salmonella* (Fihman *et al*., 2007). Nairn (1973) associated *Salmonella* and other pathogenic bacteria with osteomyelitis and synovitis in commercial turkeys. Additionally, 55 to 93% of commercial swine suffer from hind foot lesions, abrasions, and/or infections (Gentry *et al*., 2002; Mouttotou *et al*., 1999). Thus, the prevalence of hind foot lesions/infections combined with the ubiquitous nature of *Salmonella* supports the theory of *Salmonella* manifestation in bone joints of infected and/or sick animals, making synovial fluid a possible contamination vector.

There is limited information within the literature to elucidate the mechanisms of translocation of *Salmonella* from the gastrointestinal tract into the circulating lymph system and ultimate migration to musculoskeletal lymph nodes in apparently healthy animals. Thus, the overall objective of this study was to determine if pigs that were orally inoculated with *Salmonella* would harbor the pathogen in mesenteric and musculoskeletal lymph nodes, as well as synovial fluid.

MATERIALS AND METHODS

All procedures in this study were reviewed and approved by the USDA-ARS, Livestock Issues Research Unit's Institutional Animal Care and Use Committee (IACUC protocol 2010-10-JAC8).

Animals

Pigs, diet, and experimental design

This experiment was conducted in two phases in which Yorkshire/Duroc crossbred pigs (n = 36 for Phase 1; n = 38 for Phase 2; average 10 ± 1.4 kg BW) were purchased from a commercial swine producer

and transported to the USDA Livestock Issues Research Unit's Swine Facility in Lubbock, TX. Pigs were fed a non-medicated commercial diet composed of (dry matter basis): ground corn 56.2%, soybean meal 23.25%, rice bran 9%, fish meal 4%, soyhulls 3.3%, Pork Flex 110 2.75%, tallow 1%, L-threonine 0.2%, L-lysine 0.15%, and methionine 0.15%. The diet was formulated according to Nutrient Requirements of Swine recommendations and pigs were allowed *ad libitum* access to water. Pigs were individually penned and housed in an environmentally controlled facility with an average air temperature of 28.2 ± 0.4°C. Fecal samples were collected from each pig upon arrival and each subsequent day (for 5 d) during the dietary/facility adaptation period to verify that no growth occurred on novobiocin (20 µg/ml) and nalidixic acid (25 µg/ml) supplemented Brilliant Green agar (BGA_{NN}). Prior to inoculation, no colonies grew on any of the BGA_{NN} plates. Fecal samples were also analyzed during the adaptation period by enrichment for the presence of bacteriophages that could lyse the *Salmonella* Typhimurium (Callaway *et al*., 2010), strain used in the present inoculation study.

In phase one, pigs were supplemented via the diet with phosphate buffered saline (PBS) or PBS with *Enterobacter cloacae*. In phase 2, pigs were fed with and without the inclusion of yeast cell wall products. For both phases, at the end of the 5 d adaptation period, each pig was inoculated with *Salmonella* Typhimurium (2×10^{10} CFU/pig) via oral gavage (10 mL total volume per pig) at 0 h. The concentration of *Salmonella* Typhimurium was utilized to ensure relatively elevated concentrations of *Salmonella* in the gastrointestinal tract to further enhance the possible transfer of the pathogen into systemic lymph.

Bacterial cultures

Salmonella enterica serotype Typhimurium (ATCC BAA-186) from the USDA Food and Feed Safety Research Unit culture collection was repeatedly grown (4 passages) by 10% (vol/vol) transfer in anoxic (85% N_2, 10% CO_2, 5% H_2 atmosphere) Tryptic soy broth (TSB) medium at 37°C to adapt the culture for growth in the anaerobic intestinal tract. This strain was made resistant to novobiocin and nalidixic acid (20 and 25 µg/mL, respectively) by repeated transfer and selection in the presence of sub-lethal concentrations of each antibiotic. This resistant phenotype was stable through multiple unselected transfers in batch culture and through repeated culture vessel turnovers in continuous culture (data not shown). Overnight cultures (1 L) contained populations of *Salmonella* Typhimurium that were determined to be 4×10^9 CFU/ml by serial dilution and plating.

Gastrointestinal sample collection

Pigs (n = 12/d) in Phase 1 of the study were humanely euthanized at 24, 48, and 72 h after inoculation with *Salmonella*. For Phase 2, all pigs were euthanized 72 h post-inoculation in an effort to increase the possibility of finding *Salmonella* in peripheral lymph nodes and joints based on results from Phase 1. Ileocecal lymph nodes were aseptically collected and enriched following maceration (Figure 1). Digesta and epithelial tissues from the terminal rectum were also aseptically collected upon necropsy.

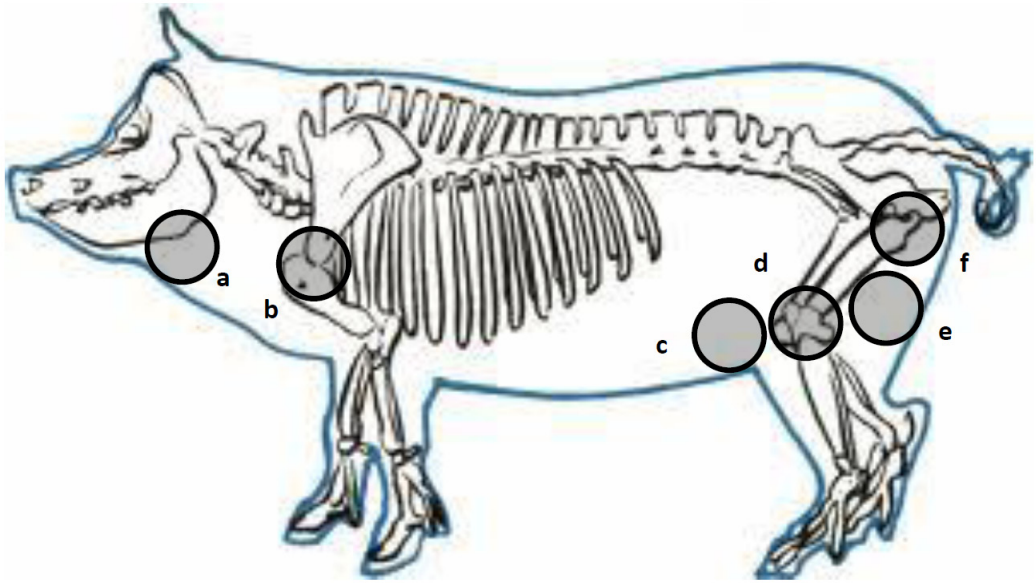
Lymph node collection

Mandibular, subiliac, and popliteal lymph nodes were collected from each animal following collection of gastrointestinal contents. Each lymph node was collected aseptically from the right side of the carcass. Samples were subjected to a surface disinfectant dip with 70% ethanol and were subsequently macerated and placed in tetrathionate broth for enrichment. Isolation of the inoculated *Salmonella* strain was conducted as described below.

Synovial Fluid Collection

Synovial fluid was collected from each pig (n = 74) at three anatomical locations (i.e., shoulder, coxofemoral, and stifle) on the right side of the carcass. These joints were selected because each of these anatomical locations represent an area of the carcass that may be included in a retail cut or a joint that may

Figure 1. Graphical representation of lymph node and synovial sampling locations.
a. Mandibular lymph node, b. Shoulder joint, c. Subiliac lymph node, d. Stifle Joint, e. Popliteal lymph node, f. Hip joint



be exposed during fabrication that could lead to possible cross contamination. Joints were exposed using leverage on one side of the joint along with a sterile scalpel cutting the skin on the opposite side of the joint. Both the skin of the animal and the instruments used were disinfected with 70% ethanol prior to incision. Expressed synovial fluid was collected with environmental sponges (EZ 10BPW; World Bioproducts, Woodinville, WA) that were placed in 10 mL buffered peptone water (BPW). Sponges were stomached at 230 RPM for 2 min. (Stomacher 400 Circulator, Seward, Davie, FL), and the enrichment was incubated at 37°C prior to detection.

Salmonella Detection

To qualitatively confirm the presence of inoculated *Salmonella* Typhimurium in lymph nodes, synovial fluid, rectal contents, and epithelial samples, macerated samples and sponges were incubated overnight in tetrathionate broth at 39°C and a 200 µL aliquot was transferred to Rappaport-Vassiliadis R10 Broth which was subsequently incubated at 42°C for 24 h. Following this secondary enrichment, samples were streaked on BGA_{NN} plates. Plates that exhibited colonies after 24 h incubation were classified as positive for experimentally innoculated *Salmonella* Typhimurium. Unless otherwise noted, all media and agar were from Difco Laboratories (Sparks, MD). Reagents and antibiotics were obtained from Sigma Chemical Co., St. Louis, MO. Post-enrichment synovial samples were subjected to real-time PCR analysis (BAX ®; Dupont, Wilimington, DE; AOAC 100201) to confirm the presence of *Salmonella*.

Statistical Analysis

The experimental unit in both phases was the individual pig. Pigs were randomly assigned to a harvest day for Phase 1. Data from pigs positive for *Salmonella* were analyzed using Pearson Exact Chi Square analysis of SAS (v. 9.3 SAS Inst. Inc., Cary, NC). Interactions between fecal and illeocecal lymph node prevalence were analyzed using binomial logistic regression in PROC GLIMMIX of SAS. For Phase 1, day was considered a random variable. Significance was determined at $P < 0.05$ for all data.

Table 1. The prevalence of *Salmonella* detected in four different lymph nodes from pigs experimentally inoculated with *Salmonella*.

	% Positive for *Salmonella*				
	Feces	Illeocecal	Popliteal	Mandibular	Subiliac
Phase 1[1]	52.7	41.6	0.0	2.7	2.7
Phase 2[2]	31.5	37.0	0.0	0.0	0.0
P-value[3]	0.0	0.6	1.0	0.3	0.3

[1] n = 36 pigs
[2] n = 38 pigs
[3] SEM = 2.34

RESULTS AND DISCUSSION

Animals

Pigs did not demonstrate any visual signs or symptoms of disease during this short term infection study. However, quantified immunological markers were consistent with an infection. Additionally, a small, yet significant, febrile response was observed after the *Salmonella* challenge (data not shown).

Fecal

Fecal prevalence of the experimentally inoculated *Salmonella* in Phase 1 was 52.8% (85%, 46%, and 30% for 24, 48, and 72 h post-inoculation, respectively) and 31.6% in Phase 2 of the study (Table 1). There was a tendency (P = 0.06) for Phase 1 fecal prevalence to be greater than Phase 2 across all collection timepoints. This tendency is understandable due to Phase 2 sample collection only occurring at 72 h post-infection. Also, there was no fecal x day interaction (P = 0.18). The presence of *Salmonella* in the feces of pigs plays a major role in the cross-contamination of pork carcasses and ultimately the food supply. While the pigs in this study were in a controlled environment, many factors can influence infection and fecal shedding of *Salmonella* such as transportation (Hurd *et al*., 2002), lairage (Hurd *et al*., 2001), and co-mingling with other animals and new environments (Hurd *et al*., 2001). Fecal prevalence in pigs raised in commercial swine production operations has been reported to be between 1 and 33% (Davies *et al*., 1998; Rodriguez *et al*., 2006; Barber, 2002; Foley *et al*., 2008). Gebreyes *et al*., (2004) reported that swine herds with a greater prevalence of fecal *Salmonella* had the greatest incidence of carcass contamination at harvest, further solidifying the correlation between fecal and carcass contamination. Furthermore, Ojha and Kostrzynska (2007) stated that pigs may shed 10 million cells/g in feces during a *Salmonella* infection. *Salmonella* shedding in feces may transfer to other pigs or lead to cross-contamination via lairage, feed, or water.

Salmonella in Lymph Nodes

In Phase 1 of the study, a total of 41.8% of pigs (n = 15) experimentally inoculated were positive for *Salmonella* in ileocecal lymph nodes at necropsy. The pigs were euthanized at three timepoints (24, 48, and 72 h post-inoculation; n = 13, 13, 10, respectively) in Phase 1, and ileocecal lymph node prevalence of *Salmonella* at these time points was 46.15%, 46.15%, and 30.00%, respectively (Table 1). *Salmonella* prevalence in ileocecal lymph nodes was less than expected considering the concentration and dosage of innocula introduced into the gastrointestinal tract of the animal. Callaway and colleagues

(2011) reported that in a trial in which pigs were inoculated with a similar dose of *Salmonella*, 77 to 83% of ileocecal lymph nodes were positive for *Salmonella*. In Phase 1 of the present study, all popliteal lymph nodes collected tested negative for *Salmonella*, but subiliac and mandibular lymph nodes were positive for the experimentally infected *Salmonella* strain at a prevalence of 2.78% (Table 1). Interestingly, all of these positive peripheral lymph nodes were isolated from pigs necropsied only at 48 h after inoculation.

A total of 36.80% of ileocecal lymph nodes collected from pigs in Phase 2 (at 72 h post-inoculation) of the study were positive for the inoculated *Salmonella* strain; however, no peripheral lymph nodes collected from Phase 2 pigs harbored *Salmonella* (Table 1). These results are consistent with data reported by Gray *et al.*, (1996) which indicated that lymph nodes near the ileocolic junction were the lymph nodes with the greatest *Salmonella* prevalence at harvest. While there were numerical differences, there were no statistical differences in lymph node prevalence of *Salmonella* between the two phases of the present study for ileocecal (P = 0.67), popliteal (P = 1.00), mandibular (P = 0.30), and subiliac (P = 0.30) lymph nodes.

Our results associated with the presence of *Salmonella* in subiliac and mandibular lymph nodes are of great importance to food safety as these lymph nodes are anatomically located in areas of the pork carcass that are commonly included in trim and retail cuts. These lymph nodes may also be exposed and evaluated during post-mortem USDA inspection and ultimate fabrication of the carcass. Exposure of infected lymph tissues during fabrication could potentially lead to cross contamination of equipment and/or other carcasses. The presence of *Salmonella* in the feces of live pigs has been previously examined in conjunction with subiliac lymph nodes; however, little association was present to suggest feces as a predictor of lymph node contamination (Wang *et al.*, 2010). Studies conducted by Hurd *et al*. (2001, 2002) reported less incidence of *Salmonella* in feces from swine at the farm when compared to ileocecal lymph nodes positive for *Salmonella* at harvest. Wood *et al*. (1989) reported the prevalence of *Salmonella* infected ileocecal lymph nodes to be between 30 and 50% less than the prevalence of *Salmonella* in feces from the same animals. In the present study, there was no interaction between fecal and ileocecal lymph node prevalence (P = 0.15). These previously reported data as well as data from the current study help elucidate a possible disconnect between fecal shedding of *Salmonella* from pigs and the incidence of *Salmonella* harbored in the lymphatic system (Foley *et al.*, 2008).

Berends *et al*. (1996) stated that the gastrointestinal tract and lymph nodes may be major sources of *Salmonella* carcass contamination and subsequent transfer to human consumers. Positive correlations have been reported between *Salmonella* in feces and on carcasses (Berends *et al.*, 1997) and between *Salmonella* in the intestinal tract and carcasses (Swanburg *et al.*, 1999). A study conducted by Vieira-Pinto *et al*, (2005) sampled carcasses that swabbed positive for *Salmonella*, and the researchers reported that 18.8% of ileocecal lymph nodes from those carcasses contained *Salmonella*. Additionally, pig mandibular lymph nodes and tonsils have also been reported to harbor *Salmonella* (12.9 and 9.9%, respectively; Vieira-Pinto *et al*. 2005). Based on data from the current study, we can infer that there is a relatively miniscule possibility that *Salmonella* (contracted orally) will be harbored in musculoskeletal lymph nodes at 72 h post-inoculation. While the *Salmonella* prevalence of these lymph nodes was very limited at 72 h post-infection, further investigation should be conducted to elucidate the timeframe of migration of oral *Salmonella* infections as well as other factors that may impact pathogen migration in the lymphatic system. While musculoskeletal lymph node prevalence was not great, a single contaminated lymph node could potentially contaminate thousands of pounds of product when comminuted together with other trim. Given that *Salmonella* can be internalized within the lymph nodes of pigs, the bacterial cells are not as susceptible to topical in-plant pathogen reduction systems implemented by most packers and processors. For these reasons, some packers excise lymph nodes as a routine part of the fabrication process in an effort to reduce potential contamination of pork trim.

Table 2. The prevalence of *Salmonella* detected in synovial fluid from three different joints from pigs experimentally inoculated with Salmonella.

	% Positive for *Salmonella*		
	Shoulder	Hip	Stifle
Phase 1[1]	0.0	0.0	0.0
Phase 2[2]	2.6	2.6	2.6
P-value[3]	0.3	0.3	0.3

[1] n = 36 pigs
[2] n = 38 pigs
[3] SEM = 1.32

Synovial

Synovial fluid prevalence was 0 and 2.63% for Phase 1 and 2 respectively. There was no statistical difference (P = 0.30; Table 2) between *Salmonella* positive synovial samples in Phase 1 and Phase 2 of the study. Of the synovial swabs collected (n = 222; 3/pig), only three swabs were positive for *Salmonella*, all of which were from the same pig. This particular pig was noted to have a large abscess on the abdomen at the time of harvest. While this phenomenon was only observed in one animal, we hypothesize that the immunocompromised state of the pig during the experimental infection may have played a role in the transmission of *Salmonella* into the synovia. This hypothesis is supported by the fact that no visible lesions were noted on this particular animal at any of the joint sampling locations. While little is known about *Salmonella* in the joints of swine, Varley and Wiseman (2001) suggest that immunosuppression due to porcine reproductive and respiratory syndrome (PRRS) may predispose the swine to synovial infections by *Haemophilus parasuis*. More research is needed to determine if immunocompromised pigs translocate infections from the gastrointestinal tract to other peripheral tissues. Similar to lymph nodes, *Salmonella* in synovial fluid is not susceptible to post-harvest topical pathogen reduction interventions applied to the carcass. The original hypothesis of this study stated that synovial fluid may harbor *Salmonella* and be a possible vector for potential cross-contamination when exposed during fabrication. However, based on the data from the current study, we can infer that the possibility of *Salmonella* cross-contamination via synovial fluid from pigs that orally acquire this pathogen is relatively low.

CONCLUSIONS

Overall, this study determined that experimental oral inoculation of pigs with *Salmonella* may result in *Salmonella* penetrating the lymphatic system and reaching peripheral lymph nodes. While most of the infection was localized to the illeocecal lymph nodes and feces, the infection was also shown to reach peripheral lymph in some animals. While only being observed in one animal, we hypothesize that oral infection with *Salmonella* may be able to influence *Salmonella* prevalence in the joints of immunocompromised animals. Elucidating the transmission routes of *Salmonella* to peripheral tissues in the carcasses of pigs that enter the food chain is vital to the establishment of interventions and control points to prevent foodborne illness and cross contamination in the pork production process. More research needs to be conducted to determine how *Salmonella* infections with different routes of entry migrate through the lymphatic system and how these infections impact animal health and food safety.

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