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Evaluation of two commercially-available *Salmonella* vaccines on *Salmonella* in the peripheral lymph nodes of experimentally-infected cattle

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Abstract

Background: *Salmonella* is a common inhabitant of the ruminant gastrointestinal tract, where it often resides asymptomatically and may be shed into the feces. More recently it was discovered that *Salmonella* may be contained within the peripheral, non-mesenteric lymph nodes, where it is impervious to in-plant pathogen control interventions and may serve as a source of *Salmonella*-contamination of ground beef. Over the past 10 years considerable research effort has been expended at understanding how this pathogen gets to these lymph nodes, the duration of infection, and, most importantly, screening and developing potential intervention strategies that may be employed on farm prior to the animal being presented for slaughter.

Methods: Utilizing an experimental model of *Salmonella* inoculation of bovine peripheral lymph nodes (PLNs), two pilot vaccine experiments were conducted to evaluate two *Salmonella* vaccines: *Salmonella* Newport Bacterial Extract (Experiment I) and Endovac-Bovi® (Experiment II) on preventing *Salmonella* acquisition by these nodes. In Experiment I, 4 months following the booster vaccination, 30 steers were inoculated with three *Salmonella* serotypes intradermally: Newport, Montevideo, and Anatum administered to the right legs, left legs, and to the caudal thorax and abdomen, respectively. Cattle were inoculated every other day over the course of five days (three total inoculation events) and 6 and 12 days following the final *Salmonella* inoculation, 16 and 14 head in each treatment were euthanized, respectively. In Experiment II, 12 head of Holstein steers were utilized. Seven days following the booster and weekly thereafter for 3 weeks (four total inoculation events), cattle were inoculated as above and euthanized 7 days following final inoculation. Right and left sub-iliac, popliteal and pre-scapular lymph nodes were collected in each experiment, weighed and cultured for *Salmonella*.

Results: In Experiment I, no treatment differences were observed in *Salmonella* prevalence 6 days post-inoculation (necropsy 1). However, in vaccinated cattle at the second necropsy, a reduction ($p = 0.05$) in *Salmonella* prevalence was observed in the sub-iliac and pre-scapular lymph nodes as well as when all nodes were evaluated collectively ($p = 0.04$). In Experiment II, the vaccine reduced ($p = 0.03$) *Salmonella* prevalence in the right popliteal and tended ($p = 0.09$) to decrease prevalence in both popliteal lymph nodes.

Conclusion: Under these experimental conditions, the data generated provide evidence of a partial vaccine effect on *Salmonella* within PLNs and indicate that further research may be warranted.

Keywords: cattle, lymph node, *Salmonella*, vaccine

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Introduction

Recent research indicates that the lymphatic system, and peripheral lymph nodes (PLNs) in particular, may be a significant contributor to the contamination of ground beef with *Salmonella*.[1] *Salmonella* prevalence in these nodes varies significantly and is influenced by cattle type [feedlot fattened *versus* those removed (or culled) from herds for productivity reasons], region, and season.[2,3] Others[4] reported that lymph nodes collected from cattle slaughter plants had an overall *Salmonella* prevalence of 1.6% while research examining *Salmonella* prevalence in lymph nodes of cattle originating from different feedlots reported a wide range in prevalence (0–88%) among the different operations.[5] In a study in which 3300 sub-iliac lymph nodes were collected across the United States from feedlot-fattened and culled cattle, the authors reported a median *Salmonella* prevalence of 11.8% and 0.65%, respectively.[2] Further, for *Salmonella*-positive lymph nodes, concentration of *Salmonella* varied from 0.1 to greater than 3.8 CFU ($\log_{10}$) g^{-1} of lymph node.[2] Conventional wisdom suggests that *Salmonella* within the PLN originates in the gastrointestinal tract, likely escaping into systemic circulation, where it is captured in the lymph and transported to the regional lymph node. Researchers, utilizing wild type isogenic tag-strains of *Salmonella*, noted that transmission of *Salmonella* from the gastrointestinal system to the lymphatic system was frequently observed.[6] However, in other experimental-challenge studies, researchers had very little success in producing *Salmonella*-positive PLNs following oral dosing of *Salmonella*.[7]

As PLNs are below the surface of the carcass, and frequently encased in adipose tissue, they are protected from currently used in-plant pathogen interventions that focus on preventing or removing surface contamination. Based on a preliminary risk assessment, *Salmonella*-harboring PLNs are likely the primary contributor to *Salmonella* in ground beef,[1] and in lieu of removal during slaughter, solutions will need to be implemented on the pre-slaughter side of production. Currently, few pre-harvest interventions are available for controlling *Salmonella* in cattle and are limited to vaccines and the feeding of direct fed microbials. In previous research, whole-herd vaccination with a vaccine containing siderophore receptors and porin proteins (SRPs) from *Salmonella* Newport was associated with a reduced prevalence of fecal *Salmonella* (8.0% *versus* 36.8%) when compared with herds that did not vaccinate.[8] Others reported there was no evidence of a reduction in the fecal shedding of *Salmonella* in sub-clinically infected dairy cows[9,10] although an improvement in milk production was observed in one study.[10] Similarly, no vaccine effect was observed on fecal prevalence of *Salmonella* in feedlot cattle.[11] In prior work, we reported modest efficacy of a vaccine administered to control *Salmonella* in the PLN.[12] Therefore the objective of the current research was to use an experimental model of *Salmonella* infection of the PLN[12,13] to examine the efficacy of two commercially-available *Salmonella* vaccines, *Salmonella* Newport SRP vaccine and a *Salmonella* Typhimurium bacterin-toxoid vaccine, in pilot studies to reduce *Salmonella* in the PLN.

Methods

All animal care and experimental procedures were reviewed and approved by the Animal Care and Use Committee of the Food and Feed Safety Research Laboratory, USDA (ACUC No. 2013001). All research below was conducted at this same laboratory. Thirty and 12 Holstein and Holstein-cross steers (average body weight = 137 kg and 103 kg ± approximately 15 kg) for Experiments I and II, respectively) were purchased from a single supplier on two occasions and transported to our laboratory in College Station, TX, where they were maintained on pasture and supplemented with a commercial non-medicated calf starter. Upon arrival, steers were weighed, identified with an ear tag, and metaphylactically administered tulathromycin (Draxxin®, Zoetis Inc., Kalamazoo, MI, USA), and an anthelmentic (Cydectin, Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO, USA) per label directions.

Experiment I

Cattle were brought to the laboratory in the summer of 2012 and maintained on native pasture prior to acclimation to pen and diet. Animals were randomly assigned to treatment (control or vaccine; 15 head per treatment). For randomization to treatment, steers were assigned a random number generated from a random number table, those numbers ranked, and the first 15

assigned to the control treatment. Vaccinated steers ($n = 15$) were administered a commercially-available *Salmonella* vaccine (2 ml subcutaneously (s.c.); *Salmonella* Newport Bacterial Extract vaccine with SRP® Technology, Zoetis Inc. Kalamazoo, MI, USA) on days 0 and 21 per label directions while control animals ($n = 15$) received a sham-injection of sterile saline (2 ml s.c.). In between the initial (July 2012) and booster vaccination (August 2012), animals were group housed on approximately 5-acre grass paddocks and supplemented with a commercial beef feed that in combination with the pasture, met or exceeded their recommended nutrient requirements. Approximately 4 months following the booster vaccination (January 2013), steers were moved to a single outdoor pen where they were group housed for the remainder of the study and fed the commercial beef feed and Bermuda grass hay through to completion of experiment. All steers were inoculated with *Salmonella* intradermally as described previously[10] using the ComforTen® Multiple Skin Test Device (Hollister-Steir Allergy, Spokane, WA, USA). Briefly, the administration device was dipped into tryptic soy broth (TSB) containing the overnight culture of *Salmonella* and then applied medially and laterally above the metacarpus and metatarsus of the steer, providing 20 applications of *Salmonella*/leg. Applications were likewise made on each side of the back, just below the shoulders, and on each side of the abdomen. The device was dipped into the *Salmonella* broth prior to each administration and a new device used for each animal and each serotype. *Salmonella* Newport (4.6×10^6 CFU ml^{-1}) was administered to the right legs, *S.* Montevideo (6.5×10^6 CFU ml^{-1}) to the left legs, and *S.* Anatum (4.7×10^6 CFU ml^{-1}) to the abdomen and back. Cattle were inoculated every other day over the course of five days (three total inoculation events). Six and 12 days following the final *Salmonella* inoculation, one half of the cattle in each treatment were euthanized (Euthasol® euthanasia solution; Delmarva Laboratories, Inc., Midlothian, VA, USA) and the right and left sub-iliac, popliteal, and pre-scapular lymph nodes collected, weighed and cultured for *Salmonella* as described previously.[14] Briefly, immediately following collection, lymph nodes were trimmed of excess fat and surface sterilized by immersion in boiling water for 3 s prior to placement in a sterile whirl pak bag and the node pulverized using a rubber mallet. Eighty milliliters of tryptic soy broth (TSB) were added to the pulverized lymph node followed by thorough mixing using a laboratory blender. For the quantitative culture, 1 ml of the lymph node–TSB homogenate was plated in duplicate onto EB Petrifilm (3M, St. Paul, MN, USA) and incubated at 37°C for 18–24 h. Gas forming colonies were counted prior to transfer of the colonies from the film to XLD plates and incubated (37°C, overnight). For prevalence determination, 1 ml from each enrichment was subjected to anti-*Salmonella* immunomagnetic separation. Each 1 ml aliquot was mixed with 20 µl of anti-*Salmonella* beads (Invitrogen, Carlsbad, CA, USA) and incubated with shaking at room temperature for 15 min. The beads were then extracted and washed twice prior to plating onto BGA agar with sulfadiazine (80 mg/l). Plates were incubated at 37°C for 18–24 h prior to visual confirmation and serogrouping of *Salmonella*-positive samples (three colonies per sample), using slide agglutination with *Salmonella* antiserum (Difco Laboratories, Detroit, MI, USA) to determine whether the recovered isolates were of the same serogroup as the challenge strain (Montevideo – C_1; Newport – C_2; Anatum – E_1).

Experiment II

Twelve head of Holstein steers were utilized and group housed as above in a large outdoor pen and fed a commercial beef feed and Bermuda grass hay (50:50). One-half of the steers were administered Endovac-Bovi® with Immune Plus® [commercially available *Salmonella* Typhimurium bacterin-toxoid vaccine (IMMVAC Inc., Columbia, MO, USA)] on day 0 (October 2012) followed by a booster vaccination 14 days later. Control steers received a sham injection of sterile saline (equal volume). Seven days following the booster and weekly thereafter for 3 weeks (four total inoculation events), all cattle were inoculated intradermally with the three strains of *Salmonella* [Newport (3.3×10^6 CFU ml^{-1}); Montevideo (8.5×10^6 CFU ml^{-1}); and Anatum (3.3×10^6 CFU ml^{-1})], euthanized and necropsied (7 days following final *Salmonella* inoculation; November 2012), as described above. One control steer died unexpectedly during the experimental period of an unknown cause. Six isolates from each *Salmonella*-positive samples were serogrouped as above.

Table 1. The percentage of *Salmonella* positive lymph nodes (popliteal, pre-scapular, and sub-iliac; by node and combined) in cattle vaccinated with *Salmonella* Newport Bacterial Extract vaccine (SRP) prior to experimental inoculation with *Salmonella.* Cattle necropsied 6 or 12 days post-inoculation (Experiment I).

Node	Necropsy 1 (day 6)			Necropsy 2 (day 12)		
	Control	Vaccine	*p*-value	Control	Vaccine	*p*-value
Popliteal						
Right	62.5	37.5	0.32	57.1	14.3	0.09
Left	50	50	1	71.4	42.9	0.28
Both	56.3	43.8	0.48	64.3	28.6	0.06
Pre-scapular						
Right	62.5	87.5	0.25	57.1	14.3	0.09
Left	12.5	50	0.11	57.1	28.6	0.28
Both	37.5	68.8	0.08	57.1	21.4	0.05
Sub-iliac						
Right	75	50	0.3	57.1	14.3	0.09
Left	50	50	1	57.1	28.6	0.28
Both	62.5	50	0.48	57.1	21.4	0.05
All nodes	52.2	55	0.89	62.3	19.9	0.04

SRP, siderophore receptor and porin protein

Statistical analysis

Data were analyzed using the commercially available software (SAS version 9.4 software, SAS Institute Inc., Cary, NC, USA). Qualitative data (proportion of positives) by node type (popliteal, pre-scapular and sub-iliac) were subjected to chi-square analysis using the PROC Frequency procedure. The effect of vaccine on *Salmonella* prevalence in all the PLNs combined was examined using logistic regression techniques and the model adjusted with animal as the co-variate to account for potential clustering of the outcome within animal. Non-zeros, that is, those instances where *Salmonella* was recovered from the PLN, yet was below the limit of quantification, were assigned a concentration that varied from 0.1 to 0.5 log CFU g^{-1} depending on size of the lymph node. Quantitative data (log transformed, base-10) were analyzed using analysis of variance techniques. Individual animal served as the experimental unit and differences were considered significant at a 5% level of significance.

Results and discussion

In the first experiment utilizing the SRP vaccine, no treatment differences were observed in *Salmonella* prevalence of the PLNs on the first necropsy, 6 days post-inoculation (Table 1). There was a trend ($p = 0.08$) for more *Salmonella*-positive pre-scapular nodes in the vaccinated steers compared with control animals. Overall, 52.2% and 55% of the PLNs were *Salmonella*-positive in control and vaccinated steers, respectively (Table 1). Four steers were culture negative for *Salmonella* in all of the lymph nodes examined. Increasing the time from inoculation to necropsy was associated with a detectable effect of the vaccine. A reduction ($p = 0.05$) in *Salmonella* prevalence was observed in the vaccinated cattle in the sub-iliac and pre-scapular lymph nodes as well as when all nodes were evaluated collectively ($p = 0.04$). Similar associations were observed for the popliteal lymph nodes ($p = 0.06$) and for the lymph nodes (sub-iliac, pre-scapular, and popliteal) from the right side of the body ($p = 0.09$; Table 1). *Salmonella* concentrations in the PLN were insufficient [0.1–1.5 CFU ($\log_{10}$) g^{-1} lymph node], meaning very few lymph nodes across both necropsies contained quantifiable concentrations of the challenge strains of *Salmonella*, for statistical analysis. This has been encountered previously by the authors even with multiple applications of *Salmonella* and

suggests differences in experimental *Salmonella* strains *versus* those acquired naturally and/or a different response by the host animal upon exposure.

Multiple *Salmonella* strains were inoculated into these experimental animals for two reasons. First, as cattle frequently harbor multiple serogroups within their gastrointestinal tract, it was deemed important to evaluate the vaccines against multiple *Salmonella strains*. Second, as this research was some of the early research following the discovery of *Salmonella* in the PLNs, it was unknown whether different serotypes varied in their likelihood of finding their way into a PLN. Serogroup distribution (data not shown) among those isolates examined from Experiment I was similar among treatments. All isolates matched one of the three challenge strain serogroups. The majority of recovered isolates were identified as serogroup E_1 (76% and 60% in control and vaccinated cattle, respectively). Serogroup C_1 accounted for 18% and 30%, and C_2 for 6% and 11% of the recovered isolates in control and vaccinated cattle, respectively. As the *Salmonella* strains were applied using similar concentrations and an equal number of applications, the over-representation of the serogroup E_1 (Anatum) compared with C_1 (Montevideo) and C_2 (Newport) is something of a surprise. Further, E_1 isolates were recovered from all lymph nodes, not just the sub-iliac lymph nodes as might be anticipated given the site of Anatum administration (abdomen and caudal thorax). That said, four applications of the device were made to both the right and left sides of the abdomen and caudal thorax, both locations that are served by the sub-iliac lymph nodes, which may explain in part the abundance of recovered E_1 isolates observed in this study.

According to the manufacturer, the SRP vaccine induces an antibody response against SRPs produced by *Salmonella* to acquire iron and thereby limiting iron acquisition. The vaccine was developed using SRPs specifically from *Salmonella* Newport, and while SRPs are utilized by the vast majority of *Salmonella* serotypes, to date there is only anecdotal evidence that the vaccine is effective against other serotypes. This, however, does not explain the relative low frequency of recovery of C_2 isolates compared with C_1 and E_1 in the current study, as there were few isolates (6% and 11%) recovered from cattle in both treatments. A more likely explanation of these differences likely lies in serotype differences and/or the location of administration. These data are somewhat consistent with field data in that the SRP vaccine has been associated with mixed results when fecal shedding of *Salmonella* was evaluated. To date, however, it is unknown to what extent gastrointestinal populations of *Salmonella* are associated with the prevalence of *Salmonella* in the PLNs. If they are associated, these field data may lend support of serotype-specific efficacy. For example, an apparent lack of effect of the SRP vaccine on serogroup E_1 (Anatum) was reported[11] when fecal shedding of *Salmonella* in feedlot cattle was examined. Previously, we reported a modest effect of this same vaccine when Newport and Montevideo were used to challenge cattle[12] and therefore were optimistic that a similar response would be seen in this study with Montevideo and Anatum. Subsequent research, conducted in part as a result of the results herein, evaluated the same SRP vaccine on *Salmonella* in the PLNs of fed cattle in a commercial production setting.[15] Those researchers reported no difference between vaccinated and control cattle and noted that the high *Salmonella* prevalence in this particular feedlot may have overwhelmed any beneficial effect exerted by the vaccine.

In the second experiment, the Endovac-Bovi® vaccine was associated with reduced ($p = 0.03$) *Salmonella* prevalence in the right popliteal lymph nodes and tended ($p = 0.09$) to decrease prevalence in both popliteal lymph nodes when evaluated together. While not statistically significant, some evidence for a lowered ($p = 0.14$) *Salmonella* prevalence in the right pre-scapular and left sub-iliac nodes was observed (60% *versus* 16.7% for control and vaccinates, respectively; Table 2). One animal was culture negative for *Salmonella* in all of the nodes examined. It is important to note that fewer animals were included in Experiment II than in Experiment I; therefore, we lacked statistical power to detect what appeared to be substantial differences between vaccinated and control animals. *Salmonella* concentrations above the limit of detection were observed in only four steers and eight total nodes, ranging from 1.9 to 2.4 CFU ($\log_{10}$) g^{-1} of lymph node, and did not appear to be associated with treatment status (data not shown). Forty-five percent of the examined isolates belonged to serogroup C_1

Table 2. The percentage of *Salmonella* positive lymph nodes (popliteal, pre-scapular, and sub-iliac; by node and combined) in cattle vaccinated with Endovac-Bovi® vaccine prior to experimental inoculation with *Salmonella*. Cattle necropsied 7 days post-inoculation (Experiment II).

Node	Control	Vaccine	*p*-value
Popliteal			
Right	60	0	0.03
Left	60	50	0.74
Both	60	25	0.10
Pre-scapular			
Right	60	16.7	0.14
Left	80	83.3	0.89
Both	70	50	0.34
Sub-iliac			
Right	40	50	0.74
Left	60	16.7	0.14
Both	50	33.3	0.43
All nodes	60	36.1	0.30

(Montevideo), while 35% and 19% belonged to groups E_1 (Anatum) and C_2 (Newport), respectively (data not shown).

The Endovac-Bovi® vaccine used in this experiment is made utilizing a genetically engineered *Salmonella* Typhimurium that has temporarily or permanently lost its ability to produce part, or all, of the capsular O side-chain carbohydrate. This impairment reportedly exposes the inner aspects of the bacterial cell wall that may serve as antigens to the host immune system. If this core is indeed common to all *Salmonella*, cross-protective immunity might be possible across most serotypes. If so, this may explain why the distribution of recovered serotypes was more similar in Experiment II when compared with Experiment I, in which E_1 was the predominant serogroup recovered. As in Experiment I, it is possible that increasing the time from inoculation to necropsy, and thus allowing more time for the vaccine to work, may have produced more significant results.

While these pilot vaccine studies observed significant vaccine effects, the failure of the SRP vaccine to work under commercial conditions in subsequent research[15] and the challenges of incorporating a vaccine program into the cattle feeding industry such that it does not require additional processing of the cattle to administer a booster vaccine suggest that significant improvements need to be made in this technology prior to further evaluation and ultimate adoption by the cattle industry. That said, vaccines are viewed by many as viable pre-harvest intervention strategies and research is on-going to develop effective vaccines for *Salmonella* within the PLNs of cattle.

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Conflict of interest statement

The authors declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: GHL has provided scientific advice to Zoetis (formerly Pfizer Animal Health, the manufacture of certain products used in this study) and has on occasion billed for this service. GHL has also received honoraria for service on advisory boards and presentations. No other financial conflicts of interest are reported.

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