

Assessing *Salmonella* prevalence and complexity through processing using different culture methods

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ABSTRACT Conventional *Salmonella* surveillance requires a week for isolation, confirmation, and subsequent serotyping. We previously showed that this could be reduced by 24 h by combining the pre-enrichment and enrichment steps into a single selective pre-enrichment step and was tested on directly after picking. The goal of this study was 2-fold: 1) to evaluate the use of selective pre-enrichment through each step of processing, including postintervention when the *Salmonella* load is reduced, and 2) to assess any changes in serovar populations in *Salmonella* positive samples. Duplicate carcass drip samples, each representative of 500 broiler carcasses, were collected by catching processing water drip under moving carcass shackle lines in each of three commercial broiler slaughter plants. Samples were collected post-pick, post-inside-outside bird wash (IOBW), and post-chill; duplicate wing rinses were performed pre- and post-antimicrobial parts dip. Each processing plant was sampled 6 times for a total of 180 samples collected. The number of *Salmonella* positives

identified with selective pre-enrichment conditions (48/180) was similar to traditional selective enrichment culture conditions (52/180), showed good concordance in recovery rate between the 2 culture methods (Fisher's exact test, $P = 0.72$). We also found that the incidence of *Salmonella* reduced dramatically after antimicrobial intervention (post-pick 66.7% vs. post chill 8.3%). When serovar populations were evaluated in *Salmonella* positive samples using CRISPR-SeroSeq, we detected four different *Salmonella* serovars, Kentucky, Infantis, Schwarzengrund, and Typhimurium, and their incidence rose between post-pick and post-IOBW. The relative abundance of Infantis within individual samples increased between post-pick and post-IOBW while the relative abundance of the other 3 serovars decreased. These results suggest that a selective pre-enrichment step reduces the time required for *Salmonella* isolation without negatively affecting detection and serovar profiles in culture positive samples were not altered between culture conditions used.

Key words: *Salmonella*, pre-enrichment, CRISPR-SeroSeq, broiler

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INTRODUCTION

Salmonella is a leading foodborne bacterial pathogen in the United States and is responsible for over 1 million illnesses each year (Scallan et al., 2011; Tack et al., 2019). A recent report noted that 17% of salmonellosis cases are attributed to chicken (Batz et al., 2021; IFSAC, 2021). *Salmonella* is often a commensal in the gastrointestinal tract (GIT) of chickens and mechanical interventions during processing such as scalding, plucking, evisceration, and parts cut-up might contribute to the cross-

contamination of *Salmonella* (Rouger and Zagorec, 2017; Ramirez-Hernandez et al., 2019; Vaddu et al., 2021b; Zeng et al., 2021), thus processing facilities could be a potential source of contamination for poultry meat (Rasschaert et al., 2007; Bourassa et al., 2015). Poultry processors usually employ multifaceted approaches to effectively reduce *Salmonella* during processing. These include several antimicrobial interventions used at different locations in the first processing area (New York rinse, inside-outside bird wash [IOBW], and immersion tanks pre- and post-chilling), while in the second processing areas parts (wings, drumsticks, and thighs) are treated with antimicrobial solutions (Kataria et al., 2020; Vaddu et al., 2021a). The major antimicrobial intervention during processing is immersion chilling of carcasses and the most commonly used antimicrobial solution in the poultry industry is peroxyacetic acid (PAA; Kumar et al., 2020).

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Salmonella isolation from carcass or parts rinses is a multiday process that includes a 24-h nonselective pre-enrichment step to resuscitate injured bacteria, followed by a 24-h selective enrichment step to inhibit the growth of non-*Salmonella* cells. The pre-enrichment is often performed in neutralized buffered peptone water (nBPW, also known as modified BPW), which contains sodium thiosulfate to neutralize the antimicrobial effect of residual PAA on the carcass (Gamble et al., 2017). Selective enrichment is performed in parallel in 2 *Salmonella* selective enrichment broths, Rappaport–Vassiliadis (RV) and tetrathionate (TT), to be able to capture *Salmonella* from high- and low-contaminated foods (Hammack et al., 1999; USDA-FSIS, 2021). Broth incubations are followed by plating onto selective indicator agar, such as Xylose lysine tergitol-4 agar (XLT4) and biochemical confirmation of presumptive *Salmonella* colonies. Given the time burden, there is significant interest in being able to reduce the time it takes to isolate *Salmonella*.

In our previous study, we showed that adding a selective component of RV broth (malachite green) and TT broth (bile salts), along with the antibiotic novobiocin to nonselective nBPW during the pre-enrichment step shortened the time required to isolate *Salmonella* by direct plating (Rasamsetti et al., 2021). The selective pre-enrichment conditions were able to identify all the positive samples (18/18) and the serovar profile analyzed was comparable to traditional culture conditions. Because this earlier work was a proof of principal, it was performed in broiler carcasses collected at post-pick, before any significant *Salmonella* interventions were used, so the *Salmonella* numbers were high. Also as part of that previous study, we used carcass drip sampling (Line et al., 2013) as a method of surveying hundreds of carcasses, rather than a small number (Rasamsetti et al., 2021).

Salmonella genomes contain 2 Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) loci, CRISPR 1, and CRISPR 2, which are located <10 kb apart (Touchon and Rocha, 2010). CRISPR spacer content is highly conserved within a serovar, and this property can be used to effectively discern *Salmonella* serovars (Fabre et al., 2012; Shariat et al., 2015; Bugarel et al., 2018) and map their relative serovar frequency within a single sample (Thompson et al., 2018; Cox et al., 2019; Rasamsetti et al., 2021; Siceloff et al., 2021). CRISPR-SeroSeq exploits this serotyping capability of *Salmonella* CRISPRs by amplifying the CRISPR spacers present in an individual sample and analyzing them by next-generation sequencing. By being able to identify all serovars in a particular sample, this technology overcomes a significant limitation of culturing, which typically only identifies the most abundant serovar(s) in a sample (Thompson et al., 2018). A previous study of ours identified an average of 5 serovars per carcass collected pre-chill using this methodology (Cox et al., 2019) and other studies also shown that carcass samples contaminated with *Salmonella* can consist of more than one serovar (Bourassa et al., 2015; Ramirez-Hernandez et al., 2019).

In the present work, we sought to investigate *Salmonella* prevalence by comparing the efficacy of selective pre-enrichment and selective enrichment to isolate *Salmonella* at 5 different points during processing from high to low *Salmonella* incidence from 3 different processing facilities. We cultured the samples using 3 types of selective pre-enrichment broths and compared the outcomes to conventional culture methods to determine their efficiency in detecting *Salmonella* at different stages of processing. To assess any changes in relative frequency of different serovars, we also used CRISPR-SeroSeq (Thompson et al., 2018; Cox et al., 2019) to characterize the serovar profiles of the positive samples, before and after antimicrobial interventions.

MATERIALS AND METHODS

Sampling

Samples were collected from 3 commercial broiler processing establishments, which each ran dual processing lines (Figure 1). One set of samples was collected from each line, and duplicate parts samples were collected before and after post-cut-up dip treatment. Carcass drip samples were collected as previously described (Line et al., 2013; Rasamsetti et al., 2021). Briefly, sanitized plastic tubs were placed beneath the moving shackle line following feather removal (post-pick), after inside-outside bird wash (post-IOBW), and after chilling (post-chill). The plastic tubs remained in place collecting all line drips for the time needed to allow approximately 500 carcasses to pass. The drip collection time ranged from 2 min and 50 s to 4 min and 4 s, depending on the line speed, and was independently calculated at each location within each plant on each visit with a timer. Carcass drip samples were transferred to sterile Nalgene bottles and immediately diluted 2-fold in 2x nBPW (Difco, Sparks, MD) containing 0.1% sodium thiosulfate (Acros Organics, NJ). Wing rinses were collected pre- and post-antimicrobial dip by placing 4 lbs of wings into a sterile bag and rinsing with 400 mL nBPW for 1 min. Wing rinse samples were placed in sterile Nalgene bottles. All samples were placed on ice and taken to the laboratory for bacterial culture on the same day.

Salmonella Isolation and Confirmation

In the lab, samples were incubated for 4 h at 37°C. Subsequently, 3 aliquots of 40 mL were placed into separate sterile plastic tubes and selective ingredients were added in different combinations to the following final concentrations: novobiocin 0.015 g/L; bile Salts 1 g/L; malachite green 0.1 g/L (Hardy Diagnostics, Santa Maria, CA). The following three pre-enrichment conditions were used: 1) nonselective pre-enrichment with nBPW as a control, 2) selective pre-enrichment with bile salts and novobiocin, and 3) selective pre-enrichment with malachite green and novobiocin. Samples were returned to the incubator for a further 20 h. After

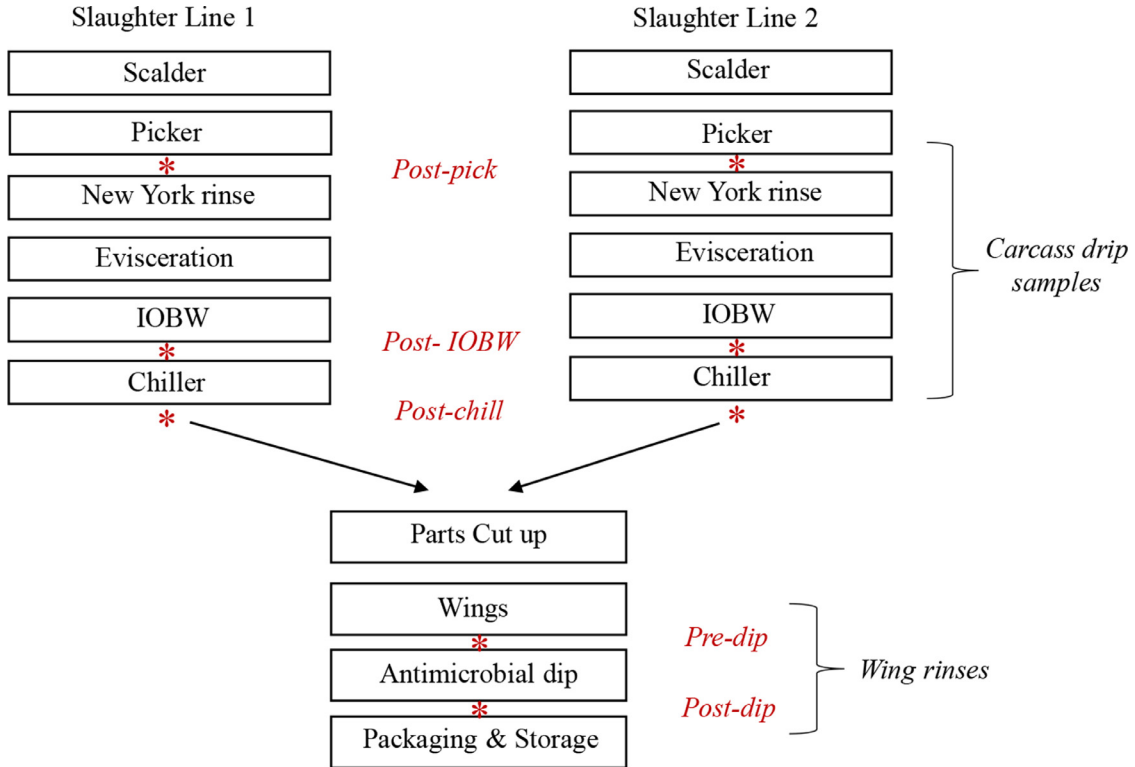


Figure 1. Diagram of a broiler processing establishment with relevant processing and intervention steps. Sample locations are indicated with an asterisk. Abbreviation: IOBW, inside-outside bird washer.

pre-enrichment, 10 μ L of all pre-enrichment broths were streaked for isolation onto XLT-4 agar (Hardy Diagnostics) and plates were incubated for 24 h at 37°C. To compare the selective pre-enrichment protocol with conventional *Salmonella* isolation, 0.1 mL and 1 mL of nBPW-only non-selective pre-enrichment cultures were subinoculated into RV and TT selective enrichment broths (Hardy Diagnostics), respectively. These were incubated for 24 h at 37°C before plating onto XLT-4 agar. XLT-4 plates were examined after 24 h for development of typical black H₂S colonies and up to 5 characteristic *Salmonella* colonies were picked and streaked for isolation onto new XLT-4 plates, which were incubated as described above. Presumptive *Salmonella* colonies were confirmed by serum agglutination with poly O serum (BD Biosciences, San Jose, CA).

DNA Isolation and CRISPR-SeroSeq

A total of 1 mL from each pre-enriched (selective and nonselective) and enriched broth after incubation was transferred into a sterile microcentrifuge tube and centrifuged at 21,000 $\times g$ for 2 min to pellet the bacteria. The supernatant was removed, and pellets were stored at -20°C. Genomic DNA was isolated from samples confirmed to be *Salmonella* positive using the Genome Wizard Kit (Promega, Madison, WI) according to the manufacturer's instructions. DNA pellets were resuspended in 200 μ L of molecular grade water and stored at -20°C until further use. A 2- μ L aliquot of DNA was used as a template in the first step CRISPR-SeroSeq PCR to amplify *Salmonella* CRISPR spacer sequences

with the following primers: forward primer, 5'-tcgtcgccagcgtcagatgtgtataagagacagcgcgccagcgggataaacc-3' and reverse primer 5'-gtctcgtgggctcggagatgtgtataagagacaggctggcgccgggaacac-3' (Thompson et al., 2018). A 5 μ L aliquot of each PCR product was analyzed by gel electrophoresis to confirm CRISPR amplification. The remaining ~28 μ L of PCR product was purified using the Ampure Bead system (Beckman Coulter, Indianapolis, IN) according to manufacturer's protocol and resuspended in 40 μ L of molecular grade water. From this, 2 μ L was used as a template in the second PCR with primers containing sequences for the addition of Illumina adaptors and dual index sequences as per the Illumina Nextera protocol (Illumina, San Diego, CA). The PCR products were analyzed by gel electrophoresis before purification as above and then individual samples were pooled in approximate equimolar proportions. The pooled sample was sequenced using an Illumina NextSeq with 150 cycles, single end read. The pooled sequencing sample had 2 negative controls (water used as template in PCR1 and then PCR2, and water used as a template for PCR2). *Salmonella* serovar Enteritidis genomic DNA with known CRISPR content was used as positive control. The sequencing reads without a 100% dual-indexed barcode sequence match were removed. The analysis of CRISPR-SeroSeq were performed using a R script that scans sequence reads and uses BLAST to match sequence reads to a database containing 135 serovars and wrote the output directly to Microsoft Excel (Thompson et al., 2018; Deaven et al., 2021; Siceloff et al., 2021). Serovars were confirmed only if they contained multiple CRISPR spacers unique to a

Table 1. *Salmonella* incidence at different locations in processing Plant 1, following pre-enrichment, selective pre-enrichment, and selective enrichment.

Sample location	Pre-enrichment	Selective pre-enrichment		Selective enrichment ^a		Cumulative		Total
		Novobiocin, bile salts	Novobiocin, malachite green	RV	TT	Selective pre-enrichment	Selective enrichment	
Post-pick	4/12	3/12	7/12	7/12	10/12	9/12	10/12	10/12
Post-IOBW	3/12	3/12	4/12	8/12	5/12	5/12	8/12	8/12
Post-chill	0/12	0/12	1/12	0/12	0/12	1/12	0/12	1/12
Pre-dip	0/12	0/12	0/12	0/12	1/12	0/12	1/12	1/12
Post-dip	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12
Total	7/60	6/60	12/60	15/60	16/60	15/60	19/60	20/60 ^b

Abbreviations: IOBW, inside/outside bird washer; RV, Rappaport-Vassiliadis broth; TT, tetrathionate broth.

^aSelective enrichment in RV and TT broth followed 24-h incubation in nonselective neutralized buffered peptone water.

^bMcNemar's test between selective pre-enrichment and selective enrichment for all samples, $P > 0.05$.

specific serovar. Where we had sequence files for RV and TT enrichments from the same sample, this data was combined and presented as one selective enrichment population. Similarly, where we had sequence files following malachite green/novobiocin and bile salts/novobiocin culture, we combined the data into one selective pre-enrichment population. The relative frequency of each specific serovar was calculated based on the amount of spacer reads present in an individual sample and presented as a heatmap, which was made in Microsoft Excel.

Statistical Analysis

To compare the *Salmonella* incidence between the 3 types of culture conditions (nonselective BPW, selective pre-enrichment, and selective enrichment) McNemar's test was used. Fisher's exact test was used to compare the *Salmonella* incidence between the sample locations within each processing facility. Both of these analyses were performed using Stata (version 17.0; StataCorp LP, College Station, TX). Bray-Curtis dissimilarity

metric (Bray and Curtis, 1957) was used to compare serovar populations and considered both serovar identities and the relative abundance of each serovar within a population. The Bray-Curtis metric was calculated using Qiime2 (2020.11) (Bolyen et al., 2019) and ranged between 0 (identical populations) and 1 (no serovars in common).

RESULTS

Salmonella Recovery in BPW With Selective Ingredients

The overall *Salmonella* incidence at Plant 1 was 33% (20/60) (Table 1). The highest *Salmonella* incidence in Plant 1 was seen at post-pick 83% (10/12), followed by post-IOBW 67% (8/12), and there was not a statistical difference in the incidence between the 2 locations (Fisher's exact test, $P = 0.64$) (Figure 2A). A single post-chill (1/12) sample was positive, and this was only detected using selective pre-enrichment with novobiocin and malachite green (Table 1 and Figure 2B). A single pre-dip

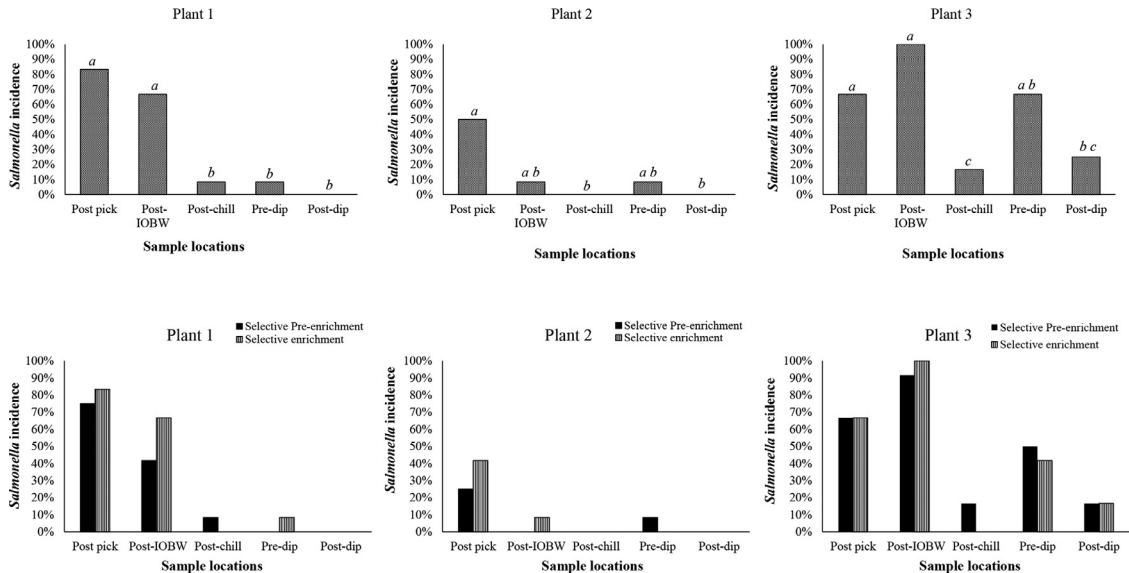


Figure 2. *Salmonella* incidence at different processing stages from Plants 1-3 and comparison of different enrichment cultures. A total of 12 samples were collected at each location in each plant. The top panel indicates overall *Salmonella* incidence for all the plants. The bottom panel indicates *Salmonella* incidence following selective pre-enrichment (black bars) and selective enrichment individually (black line bars). Italicized alphabets are used to demonstrate significantly different groups according to a Fisher's exact test. Abbreviation: IOBW, inside-outside bird washer.

Table 2. *Salmonella* incidence at different locations in processing Plant 2, following pre-enrichment, selective pre-enrichment, and selective enrichment.

Sample location	Pre-enrichment	Selective pre-enrichment		Selective enrichment ^a		Cumulative		Total
		Novobiocin, bile salts	Novobiocin, malachite green	RV	TT	Selective pre-enrichment	Selective enrichment	
Post-pick	1/12	0/12	3/12	3/12	4/12	3/12	5/12	6/12
Post-IOBW	0/12	0/12	0/12	0/12	1/12	0/12	1/12	1/12
Post-chill	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12
Pre-dip	1/12	1/12	1/12	0/12	0/12	1/12	0/12	1/12
Post-dip	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12
Total	2/60	1/60	4/60	3/60	5/60	4/60	6/60	8/60 ^b

Abbreviations: IOBW, inside/outside bird washer; RV, Rappaport-Vassiliadis broth; TT, tetrathionate broth.

^aSelective enrichment in RV and TT broth followed 24-h incubation in nonselective neutralized buffered peptone water.

^bMcNemar's test between selective pre-enrichment and selective enrichment for all samples, $P > 0.05$.

wing rinse sample was *Salmonella* positive, and no *Salmonella* was detected after the dip. In total, *Salmonella* incidence at Plant 2 was lower than Plant 1, 13% (8/60) (Tables 1 and 2), and no *Salmonella* was detected in post-chill samples at Plant 2 (Table 2). *Salmonella* incidence in Plant 2 was highest at post-pick 50% (6/12) and was slightly lower at post-IOBW 8.3% (1/12) but there was not a significant difference (Fisher's exact test, $P = 0.06$; Figure 2A). As observed in Plant 1, one pre-wing dip rinse sample was *Salmonella* positive, and no post-dip wing rinses were positive (Table 2). At Plant 3, the overall *Salmonella* incidence was higher than Plants 1 and 2, 55% (33/60) (Tables 1–3), with 67% (8/12) incidence at post-pick and 100% (12/12) incidence at post-IOBW (Figure 2A). This was not a significant increase in incidence (Fisher's Exact Test, $P = 0.09$). Two post-chill (2/12) samples were positive and were only detected using selective pre-enrichment conditions (Table 3 and Figure 2B). Eight pre-dip wing rinses 67% (8/12) and 3 post-dip wing rinses 25% (3/12) were *Salmonella* positive (Table 3). There was a significant increase in incidence from post-chill 17% (2/12) to pre-dip 67% (8/12) (Fisher's Exact Test, $P = 0.036$), followed by significant reduction in post-dip samples (25%) (Fisher's Exact Test, $P = 0.045$; Figure 2A).

We next sought to compare the *Salmonella* incidence between selective pre-enrichment (addition of selective ingredients to the nBPW pre-enrichment step) and selective enrichment in RV and TT broths. At Plant 1, the incidence between selectively pre-enriched and

selectively enriched samples was similar at 25% (15/60) and 32% (19/60) and there was no significant difference between the 2 culture methods used (McNemar's test, $P = 0.37$; Table 1). Similar observations were made for Plant 2 ($P = 0.68$) and Plant 3 ($P = 0.75$; Tables 2 and 3). Therefore, across the whole study, the isolation of *Salmonella* by selective pre-enrichment and selective enrichment was comparable. The *Salmonella* incidence between nonselective pre-enrichment (nBPW alone) and selective enrichment was significant at Plants 1 and 3 (McNemar's test, $P = 0.003$ and $P = 0.004$, respectively), demonstrating that some enrichment was required for *Salmonella* detection and that the congruence between selective pre-enrichment and selective enrichment was not simply because of a higher initial *Salmonella* load in the samples. At Plant 2, the overall low incidence of *Salmonella* limited appropriate statistical analysis to compare nonselective pre-enrichment with selective enrichment.

Overall, the *Salmonella* incidence in all the samples from the processing plants was 34% (61/180) (Tables 1–3). Direct plating from the selective pre-enrichments identified 27% (48/180) while plating from the selective enrichments identified 29% (52/180), and these 2 approaches were not statistically different (Fisher's exact test, P -value = 0.72). When the culture conditions were considered individually, enrichment in TT broth yielded 26% (47/180) positive samples compared to enrichment in RV broth (23%; 41/180). No selection was provided in samples plated directly from the 24-h

Table 3. *Salmonella* incidence at different locations in processing Plant 3, following pre-enrichment, selective pre-enrichment, and selective enrichment.

Sample location	Pre-enrichment	Selective pre-enrichment		Selective enrichment ^a		Cumulative		Total
		Novobiocin, bile salts	Novobiocin, malachite green	RV	TT	Selective pre-enrichment	Selective enrichment	
Post-pick	2/12	3/12	7/12	6/12	7/12	8/12	8/12	8/12
Post-IOBW	10/12	11/12	10/12	10/12	12/12	11/12	12/12	12/12
Post-chill	0/12	1/12	1/12	0/12	0/12	2/12	0/12	2/12
Pre-dip	3/12	4/12	5/12	5/12	5/12	6/12	5/12	8/12
Post-dip	1/12	2/12	1/12	2/12	2/12	2/12	2/12	3/12
Total	16/60	21/60	24/60	23/60	26/60	29/60	27/60	33/60 ^b

Abbreviations: IOBW, inside/outside bird washer; RV, Rappaport-Vassiliadis broth; TT, tetrathionate broth.

^aSelective enrichment in RV and TT broth followed 24-h incubation in nonselective neutralized buffered peptone water.

^bMcNemar's test between selective pre-enrichment and selective enrichment for all samples, $P > 0.05$.

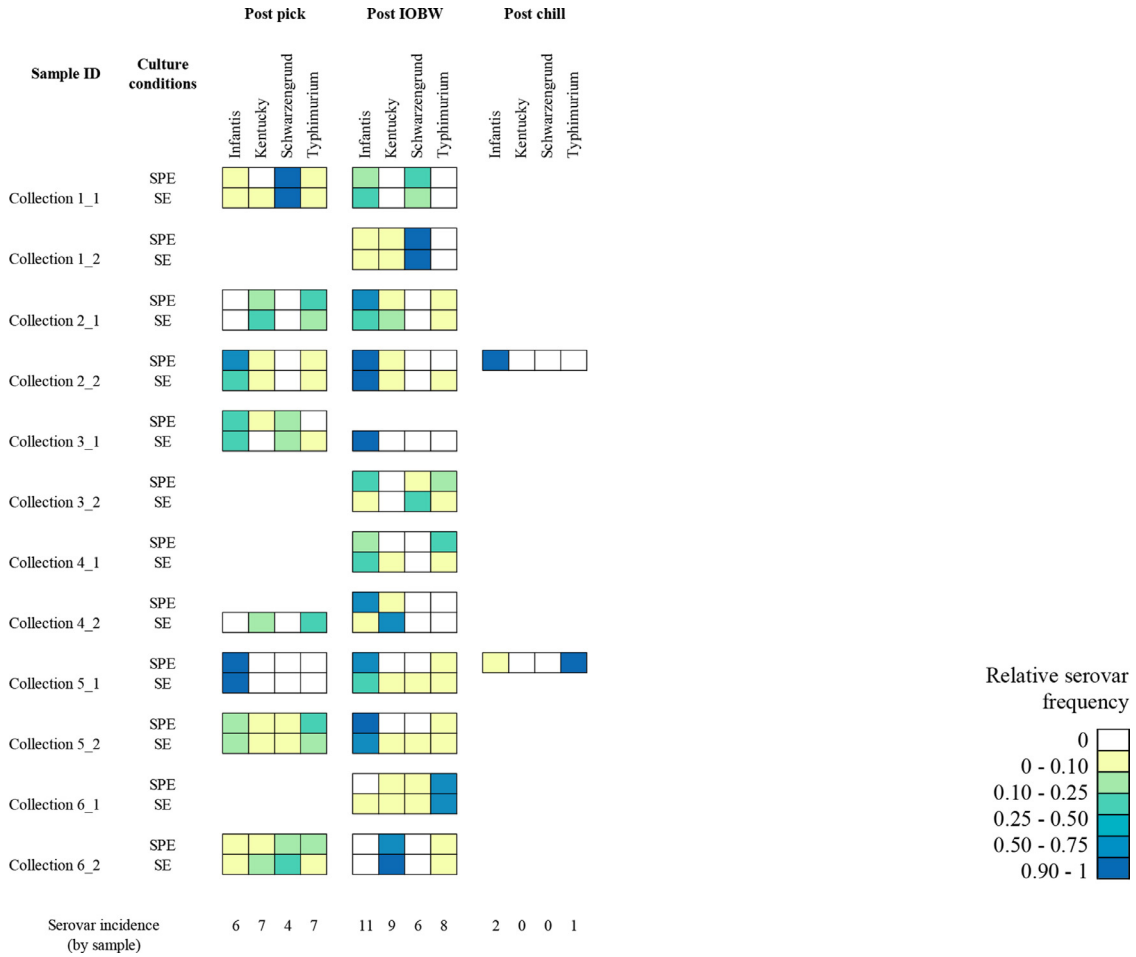


Figure 3. *Salmonella* serovar population profiles in carcass drip samples collected at post-pick, post-IOBW and post-chill locations in Plant 3 and cultured under selective pre-enrichment (SPE) and selective enrichment (SE) conditions. The sample ID field indicates when and where the sample was collected, for example, Collection 1_1 refers to the first of six sample collections and was performed on the first of two processing lines. The relative abundance of each serovar is indicated as a heatmap according to the key shown on the bottom right, such that the darker the color, the greater the proportion of that serovar in a sample. Abbreviation: IOBW, inside-outside bird washer.

nBPW culture and this resulted in 14% (25/180) *Salmonella* positive samples. For the selective pre-enrichments, addition of malachite green and novobiocin yielded 22% (40/180) positive samples and addition of bile salts and novobiocin in 15% (28/180) positives.

We performed CRISPR SeroSeq analysis on the *Salmonella* positive samples obtained from Plant 3 because for this plant we were able to isolate *Salmonella* at each processing step, albeit over multiple sampling visits. Overall, we identified 4 different serovars: Infantis, Kentucky, Schwarzengrund, and Typhimurium (Figures 3 and 4). The most common serovar was Infantis, which was found in 23/33 *Salmonella* positive samples. This analysis was only performed on the selectively pre-enriched and selectively enriched samples, and not the nonselectively enriched samples (nBPW alone) as the latter often have a high background due to contaminating DNA of non-*Salmonella* that precludes a full population analyses (Cox et al., 2019). In total, we analyzed all 33 *Salmonella* positive samples, which included 22 samples where we could pair *Salmonella* profiles from selective pre-enrichment and selective enrichment, and 11 samples where we had singles of either selective pre-enrichment or selective enrichment conditions. There

was one culture-positive sample [Collection 4_2 (fourth collection drip, second processing line), selective pre-enrichment condition] that we were unable to analyze by CRISPR-SeroSeq due to low read depth. To determine whether the media had any influence on the *Salmonella* serovar profiles we detected, we calculated the Bray-Curtis metric for the 22 paired samples. This provides a measure of population similarity by taking into account the presence of community members and their relative frequency. In total 68% (15/22) paired samples showed a good match (Bray Curtis <0.3), and 27% (6/22) a moderate match (Bray Curtis >0.3, <0.7) and a single paired sample had no match (Collection 2_1 pre-dip wing rinsate; Bray-Curtis = 0.79) (Supplemental Table 1). We next compared the serovars found at post-pick with those found at post-IOBW. All 4 serovars were detected at both post-pick and post-IOBW. The incidence of each serovar also increased between these 2 stages. For example, serovar Infantis was found in 6 samples at post-pick and in 11 samples at post-IOBW. Similarly, serovar Schwarzengrund was detected 4 times at post-pick and 6 times at post-IOBW. In considering the relative abundance of each serovar, serovar Infantis is particularly interesting as it was found as the

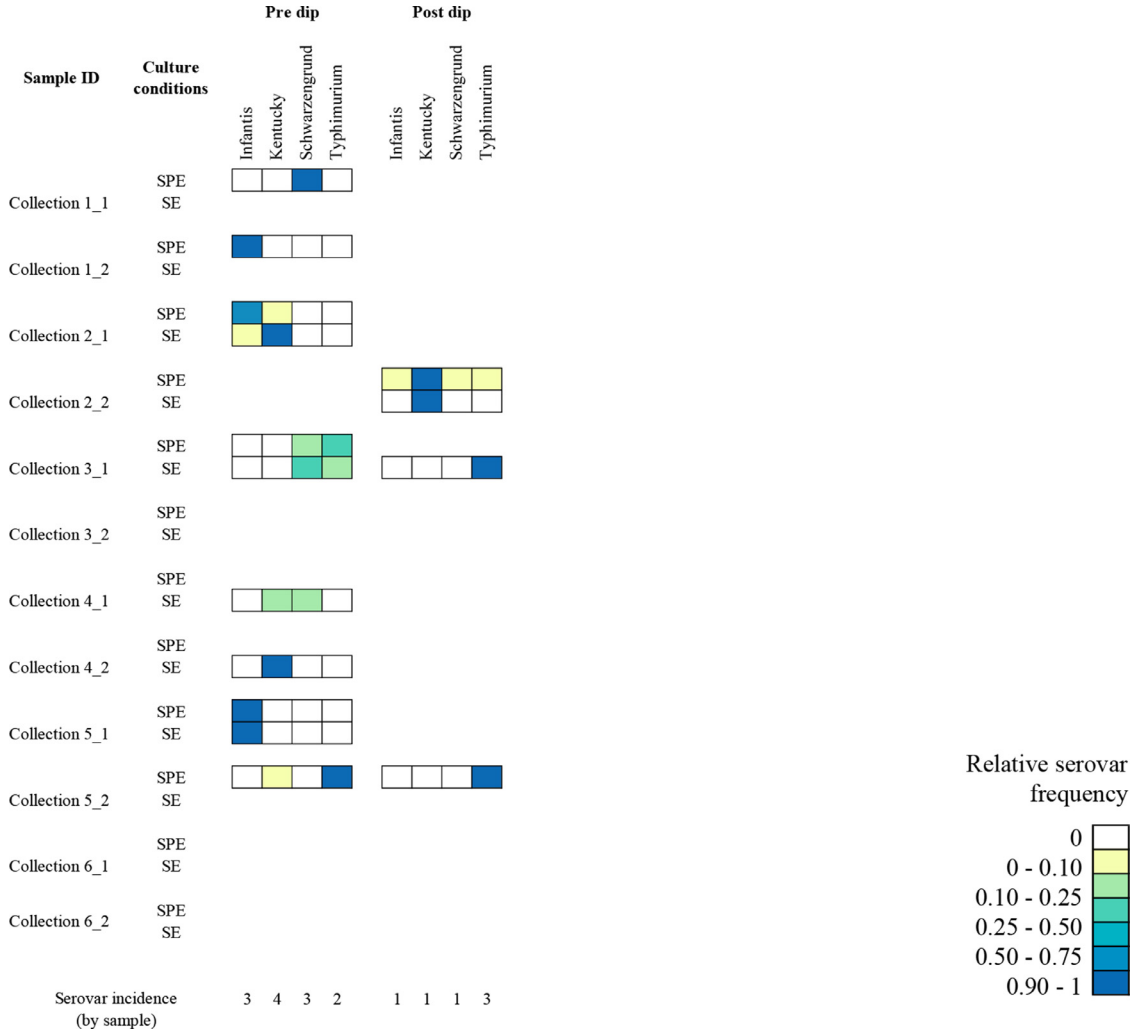


Figure 4. *Salmonella* serovar profiles in wing rinses collected at pre- and post-dip in Plant 3 and cultured under selective pre-enrichment (SPE) and selective enrichment (SE) conditions. The sample IDs are as explained in Figure 3. The relative abundance of each serovar is indicated as a heatmap according to the key shown on the bottom right.

dominant serovar (dark green to blue shading) in three post-pick samples (Collections 2_2, 3_1, and 5_1) but was found to be the dominant serovar in 4 post-IOBW samples following both selective pre-enrichment and selective enrichment (Collections 2_1, 2_2, 5_1, and 5_2) plus 2 of the selective pre-enrichment samples (Collection 3_2, and 4_2) and 3 of the selectively enriched samples (Collections 1_1, 3_1, and 4_1). For serovar Schwarzengrund, its relative abundance in each sample decreased between post-pick and post-IOBW. Both serovars Kentucky and Typhimurium also decreased in relative abundance between post-pick and post-IOBW except for in 2 samples (Collections 4_2 and 6_2 and Collections 4_2 and 5_1, respectively). At post-chill, *Salmonella* positive samples were identified, and these contained serovars Infantis and Typhimurium as major serovar. Both serovars were identified in the respective post-IOBW samples from the corresponding collection.

In the pre- and post-dip wing rinses, the same 4 serovars were also detected. Since we only had 2 paired pre- and post-dip samples (Collections 3_1, selective enrichment and 5_2, selective pre-enrichment), it was not

possible to draw a meaningful conclusion across the dataset with respect to serovar dynamics before and after intervention. The incidence of serovars Infantis, Kentucky, and Schwarzengrund decreased between pre-dip and post-dip wing rinses. For example, serovar Schwarzengrund was found in four samples at pre-dip and in a single sample at post-dip. In contrast, serovar Typhimurium was detected twice in pre-dip rinses and three times in post-dip rinses. Considering the relative abundance of each serovar, three serovars such as Infantis Kentucky, Schwarzengrund were found as the dominant serovar (dark green to blue shading) in 6 (6/8) pre-dip samples but were lost in all the relative post-dip samples. Serovar Typhimurium remained the dominant serovar in the paired pre- and post-dip samples. A single post-dip sample (Collection 2_2) had serovar Kentucky as dominant one with relative pre-dip sample as negative.

DISCUSSION

Nearly a fifth of all salmonellosis cases are linked to chicken (IFSAC, 2021) and there is a need to mitigate

this pathogen during broiler processing. In this study, we sought to address this need in 2 different ways. First, *Salmonella* isolation is the cornerstone of all *Salmonella* surveillance and monitoring programs but since it takes several days to complete, we sought to determine if the culture protocol could be shortened by 24 h. Second, it is likely that future *Salmonella* monitoring programs will be focused on certain serovars, such as those most frequently linked with human salmonellosis. It is becoming clear that *Salmonella* in poultry often exists as mixed serovar populations (Thompson et al., 2018; Cox et al., 2019; Rasamsetti et al., 2021). Culture-based *Salmonella* isolation and subsequent characterization is limited to analyzing the most abundant serovars in such mixed populations, which is a challenge in terms of understanding how different serovars can respond to different antimicrobial controls used during processing. Thus, we used population analyses by CRISPR-SeroSeq to investigate differences among serovars at different stages of processing. We also used this approach as an additional comparison of the 2 culture methods used in this study.

In this study, we used carcass drip sampling, as previously described (Line et al., 2013) to be able to capture *Salmonella* incidence in a larger number of carcasses than is logistically possible with carcass rinses. We intended to collect drip samples for the pre- and post-dip parts samples, however, this wasn't possible at the 3 plants that we visited. Therefore, for the parts analysis, we used wing rinses as pre-dip and post-dip samples. Previous work has identified *Salmonella* at each critical step in processing, with significantly lower incidence post interventions (i.e., after immersion chilling and after parts were submersed into an antimicrobial dip) (Boubendir et al., 2021). For example, Boubendir and colleagues observed that the *Salmonella* incidence dropped from 43.6% pre-evisceration to 5.1% post-immersion chilling containing PAA identified (Boubendir et al., 2021). This and other studies show that the chiller treatments with chemical solutions such as PAA effectively reduce the *Salmonella* incidence (Nagel et al., 2013; Ramirez-Hernandez et al., 2019). At Plant 3, *Salmonella* incidence increased between post-pick and post-IOBW, which suggests cross-contamination had occurred during that stage of processing, most likely during evisceration (Rivera-Pérez et al., 2014; Gu et al., 2020). This is supported by our serovar data, as the incidence of each serovar increased from post-pick to post-IOBW. The higher incidence following evisceration is consistent with the other studies, although our sampling pattern is different (Rivera-Pérez et al., 2014; Park et al., 2015; Gu et al., 2020) and but in contrast, another study observed reduction in *Salmonella* incidence after evisceration (Ramirez-Hernandez et al., 2019). These discrepancies are likely driven by the use of different antimicrobials at different processing steps. A recent study suggested that the use of chlorine at defeathering and PAA at multiple intervention steps could be most effective in reducing *Salmonella* incidence (González et al., 2019).

Overall, the *Salmonella* incidence between selective pre-enrichment and selective enrichment was similar across all 3 plants in our study, encompassing 18 visits and 180 total samples. CRISPR-SeroSeq analysis of samples collected from Plant 3 also demonstrated that 95% of paired selective pre-enrichment and selective enrichment had good or moderate concordance at the population level. Despite this overall congruence, there were a few individual samples where we were able to isolate *Salmonella* from the selective pre-enrichment but not the selective enrichment, and this only occurred in post chill samples (one sample from Plant 2, two samples from Plant 3) and one pre-dip sample (Plant 3). This is somewhat perplexing; a potential explanation is that the cells were damaged during antimicrobial treatment and despite the 24-h recovery in nonselective nBPW, they were unable to withstand the stronger selective pressures provided by RV and TT broths but were able to survive the reduced selective pressure in the selective pre-enrichment broths. Cumulatively, we only had four *Salmonella* positive samples collected at post-chill, so a larger study is required to determine whether this relationship is true, and these would be important studies to perform as they would have implications on current culture methods used to monitor for *Salmonella*. Further, *Salmonella* PCRs can be inhibited by RV and TT broths but not BPW or other media (Stone et al., 1994; Hyeon et al., 2010). Therefore, a potential benefit of our approach could be that the selective pre-enrichment avoids this inhibition and this would have additional utility to be used directly as a qPCR template. Similarly, we found some reciprocal instances with isolation from selective enrichment but not selective pre-enrichment, which most notably occurred during early processing stages (post-pick at Plants 1 and 2 and post-IOBW at all 3 plants). It is possible that the high overall microbial load present on carcasses pre-chill required a full enrichment in RV or TT broths to limit background flora and allow *Salmonella* to be isolated, and therefore, the lower selective pressure provided by the selective pre-enrichment was not sufficient. In some cases, we were able to isolate *Salmonella* following selective pre-enrichment in novobiocin and bile salts but not with novobiocin and malachite green, and vice versa. This is not unexpected given the differences sometimes seen in isolation between TT and RV broths (Singer et al., 2009; Gorski, 2012; Cox et al., 2019; Larsen et al., 2021), therefore, and similarly to current culture protocols, we suggest performing these selective pre-enrichment steps in parallel to be able to best capture *Salmonella*.

At the *Salmonella* population level, preintervention serovar diversity decreased from 3.00 to 2.83 serovars per sample at post-pick and post-IOBW, respectively, to 1.50 serovars per sample at post-chill, demonstrating that the interventions used effectively reduced *Salmonella* incidence and that this was accompanied by a reduction in the number of serovars found in each sample postintervention. While this trend is interesting and expected, a larger sample number is needed to confirm this relationship. A recent study, showed a similar

trend by identifying 2 different serovars on 4 pre-chill carcasses compared to zero carcasses post chill that contained more than one serovar (Boubendir et al., 2021).

Across our data, serovars Infantis and Kentucky were the most prevalent and were found in 69.7 and 63.6% of *Salmonella* positive samples, respectively. This is not unexpected as both these serovars are commonly found in poultry (Antunes et al., 2016; Shah et al., 2017; Ramirez-Hernandez et al., 2019; Richards et al., 2020). These serovars differ in their association with human illness: serovar Infantis is the sixth most common serovar isolated by the CDC (Tack et al., 2019), and ser. Kentucky is less clinically significant (Shah et al., 2017). Studies have demonstrated that serovar Infantis can effectively colonize chickens, has increased tolerance to antimicrobials, and that some strains are multi drug resistant (Asai et al., 2006; Shah et al., 2017; Pate et al., 2019; Drauch et al., 2020; Drauch et al., 2021; Zeng et al., 2021). Using CRISPR-SeroSeq, we determined that serovar Infantis was present at all sampling locations. Recent studies (Park et al., 2015; Zeng et al., 2021) have also reported the persistence of serovar Infantis in processing facilities. Importantly, between post-pick and post-IOBW, serovar Infantis increased in relative abundance in most of the samples. This suggests that it might have tolerated the antimicrobials used in the New York rinse than the other serovars that were present. Studies have shown that cleaning and disinfection may not eliminate serovar Infantis (Zeng et al., 2021) and FSIS *Salmonella* monitoring shows that this serovar is increasingly found in plants across the United States, which suggests that it is more tolerant to antimicrobials such as peroxyacetic acid (McMillan et al., 2020). While this is may be occurring here, we evaluate our data with caution because 1) serotyping was only performed at one plant, 2) only a few of our postintervention samples from all 3 plants were *Salmonella* positive, 3) the cross contamination between post-pick and post-IOBW confounds our understanding of serovar Infantis persistence.

Collectively, the data presented herein demonstrate that selective pre-enrichment is comparable to selective enrichment in effectively isolating *Salmonella* and does not impact the serovars that are detected. Selective pre-enrichment shortens *Salmonella* culture protocol by 24 h, which is beneficial for any *Salmonella* monitoring system.

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DISCLOSURES

The authors declare that they have no competing financial interests.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.101949.

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