Samples collected from United States meat processing facilities contain complex multiserovar *Salmonella* populations

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Objectives

The goals of this project are to both analyze trends in *Salmonella* competition within multiserovar, post-processing meat and poultry samples as well as to determine the efficacy of CRISPR-SeroSeq as a molecular tool to identify these populations. The importance of this research lies in the shortcomings of current serotyping techniques, which pose the concern of overlooking outcompeted serovars and subsequently misidentifying associated public health risks.

Abstract

Despite efforts to reduce *Salmonella* during processing, meat and poultry products remain a considerable source of salmonellosis, constituting 42.4% of annual outbreaks. *Salmonella* can be characterized into different serovars, each with varied capacity to cause human illness. Conventional *Salmonella* monitoring often relies on picking a single colony for serotyping. However, where mixed serovar populations occur, minority serovars remain undetected. An important knowledge gap is the extent to which these minority populations contribute to human illness. CRISPR-SeroSeq is a PCR-based approach that detects and determines relative abundances of multiple serovars within a single sample by utilizing sequence reads for the serovar-specific CRISPR spacers. We applied CRISPR-SeroSeq to 256 post-antimicrobial intervention samples collected from chicken, pork, turkey, and beef slaughter plants across the United States. In total, 48 serotypes were detected, with eleven in the CDC top 10. Amongst samples, 35.7% contained multiserovar populations, averaging 1.55 serovars per sample with a maximum of seven detected within a single sample. Serovars of highest concern, as denoted by the CDC, were found in the background 33.7% of the time. Collectively, these data demonstrate that high-resolution analysis provided by CRISPR-SeroSeq improves *Salmonella* surveillance and the understanding of serotype dynamics in the meat and poultry processing.

Introduction

Salmonella is a genus of facultative, Gram-negative bacteria that can cause high morbidity as well as mortality within a range of hosts, including livestock and humans (Giannella 1996). Its ubiquity in nature and exceptional host adaptation makes them among the most persistent, zoonotic pathogens to date (Bäumler et al. 1998). Non-Typhoidal *Salmonella enterica* is one of the leading causes of foodborne illness in the United States, causing an estimated 1.35 million infections and 26,500 hospitalizations annually as of 2022 (CDC 2019). Human salmonellosis infections can be generally characterized by acute gastroenteritis, with an estimated 5% of the population experiencing more severe symptoms including bacteremia and meningitis (Acheson and Hohmann 2001). The economic effects imposed by *Salmonella* are wide ranging, with an estimated $4.1 billion USD lost annually in productivity, medical, and premature deaths (USDA 2021). As well, within the agricultural industry, contamination events can be detrimental, leading to possible trade declines and recalls (Dey et al. 2013).

Though the ubiquity of *Salmonella* spp. in nature allows it to proliferate in many environments (Giannella 1996), incidences of human salmonellosis are often associated with the ingestion of contaminated meat products. An estimated 42.4% of incidences of human salmonellosis in the United States are attributed to chicken, pork, turkey, and beef products, with chicken making up the majority of cases at 17.3% (IFSAC 2022). With global meat consumption rising, particularly in poultry, the need to greater refine surveillance and interventive strategies becomes more significant (Henchion et al. 2014).

Salmonella spp. are known to exist as normal flora within the intestinal tracts of a wide range of food animals and frequently act as asymptomatic infections (Wiedemann et al. 2015). Transmission and cross-contamination events can occur at multiple timepoints during the preslaughter process, including at the farm, during transportation, and during lairage (Zamora-Sanabria and Alvarado 2017). Contaminated feed, stress-induced shedding, fecal contact, and many other determinants can result in a relatively high level of *Salmonella* persisting on and within animals at slaughter (Zamora-Sanabria and Alvarado 2017). Contamination of *Salmonella* on the processing line can also be influenced by several means, including transmission from the environment, biofilm formation on equipment, and redistribution within a carcass during evisceration or cutting (Obe et al. 2021, Rouger et al. 2017). In the United States, the Food Safety and Inspection Service (FSIS), a branch of the United States Department of Agriculture (USDA), surveils all meat and poultry processing and formulates guidelines for microbial reduction interventions (USDA n.d.). However, despite extensive surveillance, sanitation, and preventive implementations, human salmonellosis cases remain high.

Salmonella spp. can be further defined into over 2500 serovars. Discrimination of serovars has often been attributed to variations in their H (flagellar) and O (lipopolysaccharide) antigens, yet these serovars also house considerable phenotypic variations including virulence, host preference, antimicrobial resistance, and capacity to infect humans (Grimont and Weill 2007). Most incidences of human salmonellosis are linked to a small subset of serovars within *Salmonella enterica* subsp. *enterica.*, including serovars Enteritidis, Newport, and Typhimurium, with ten serovars making up 58.5% of incidences (CDC 2016).

Since several clinically significant *Salmonella* serovars are known to asymptomatically inhabit livestock, and many display varying resistances to intervention, serotyping within processing is of incredible importance. Currently, the FSIS utilizes a colony picking approach to serotyping, where preenriched samples are enriched for *Salmonella* in Tetrathionate broth and subsequently streaked onto BGS (Brilliant Green Sulfur) and DMLIA (Double Modified Lysine Iron Agar). From the DMLIA plate, 1-3 colonies are picked for subsequent serotyping (USDA-FSIS 2021). This poses a major issue, as in samples with mixed-serovar populations, those in less abundance have a significant probability of remaining undetected. In situations where serovars of clinical significance are overlooked, food safety concerns may be artificially deflated. As well, interventions targeted towards the majority population (ie. antimicrobials or acids) may be less effective towards the minority serovar and could select for their survival within samples. These concerns can be addressed by utilizing a sequencing-based approach that surveils samples wholistically. *Salmonella* spp. house a clustered regularly interspaced short palindromic repeat (CRISPR)-Cas system with two distinct loci. While *Salmonella* no longer adapts new spacers, the sequences have remained, and it has been shown that CRISPR spacer content is specific to serovar (Fabre et al. 2012). CRISPR-SeroSeq is a sequencing-based technology that utilizes relative spacer content to determine relative frequencies of multiple serovars within a single sample, with abilities to detect serovars of 0.003% abundance (Thompson et al. 2018).

In this study, we applied CRISPR-SeroSeq technology to post-antimicrobial intervention samples collected by the FSIS from chicken, pork, turkey, and beef slaughter plants across the United States. We initiated this study to compare the serovars identified by CRISPR-

SeroSeq to the culture-based serotyping approach used by the FSIS, and to see how often multi-serovar populations arose and which serovars were being underrepresented by the current methodologies. We also sought to understand *Salmonella* population trends between food animal groups to greater identify and highlight further product-specific interventions. Amongst our samples, we identified a significant portion that housed multi-serovar populations, which many clinically important serovars being found as the minority serovar in samples. In addition, this study shows the diverse array of *Salmonella* serovars identifiable within these food products.

Materials and Methods

DNA Amplification and Sequencing

A subset of *Salmonella*-positive sample isolates collected by the FSIS-USDA from poultry, pork and beef slaughterhouses were selected for CRISPR-SeroSeq analysis. Samples were enriched after collection and stored by the FSIS. Enrichments were sent to Dr. Dayna Harhay, a research microbiologist at the USDA, for re-enrichment, isolation, and culture-based serotyping. Doubly enriched, Salmonella-positive samples were sent to our lab for CRISPR-SeroSeq analysis. Isolates were stored at -20 °C. A One-Step PCR was performed at 25 cycles with a total reaction volume of 35 µl (2 µl template DNA, 4 µl each forward and reverse primers, 0.25 µl *Taq* polymerase (New England Biolabs), 0.25 µl deoxyribonucleotide triphosphates, 3.5 µl 10x *Taq* buffer, 21 µl molecular-grade water) using primers localized to the direct repeat sequences within the *Salmonella* CRISPR arrays (Thompson et al.). Primers contained Illumina adapters and dual index barcodes to facilitate multiplexing and sample identification. Amplification products were visualized by gel electrophoresis, and samples

with minimal to no banding were reamplified with 4 µl template at 30 cycles. Products were purified using Ampure (Beckman-Coulter) according to the manufacturer's guidelines. Prior to sequencing, 90 samples were pooled together in approximate equimolar amounts, and each sequencing run contained a negative, non-template water control from the PCR and a positive control containing genomic DNA of *Salmonella enterica* subsp. *enterica* serovar Enteritidis, a serovar with a well characterized CRISPR profile. Pooled libraries were sequenced on an Illumina NextSeq by Wright Labs with 150 cycles.

Serovar Population Analysis by CRISPR SeroSeq

Sequencing results were run through the CRISPR-SeroSeq Pipeline, an R (v. 4.04) script which scans sequence reads and uses BLAST to search for sequence similarity to characterized serovar spacer genomes within a database. Possible serovars were called based on total corresponding spacer content and outputs exported through Excel. Files were further analyzed to eliminate improbable serovars, such as serovars with no or few unique spacers (ie. all shared with present serovars) or those in too low abundance to call. Samples with <1000 total reads and serovars with <0.5% abundance were not called for this study. Relative serovar abundances were determined for each sample and compiled into a heatmap for visualization.

Data Analysis

Serovar abundance data of samples were paired and assessed for Bray Curtis dissimilarity indexes. Indexes were compiled and visualized by heat map, with dissimilarity being determined as in Table 1. Analysis was performed using R (v. 4.04) software.

Results

A total of 256 *Salmonella*-positive samples originating from chicken, pork, turkey, and beef slaughterhouses were collected for serotyping. CRISPR-SeroSeq analysis of these samples revealed 35.2% (90/256 samples) contained multiserovar populations, with an average of 1.55 serovars detected per sample (Fig. 1). Within this study, 48 distinct serovars were identified, with one sample (873) containing seven unique serovars: Anatum, Infantis, Liverpool, Muenchen I, Ohio, Reading I, and Uganda, being the highest diversity recovered (Fig. 2). Three serovars, Infantis, Typhimurium[1], and Kentucky I, were most frequently detected, constituting 48.7% of all serovar incidences within the sample set (Fig. 3).

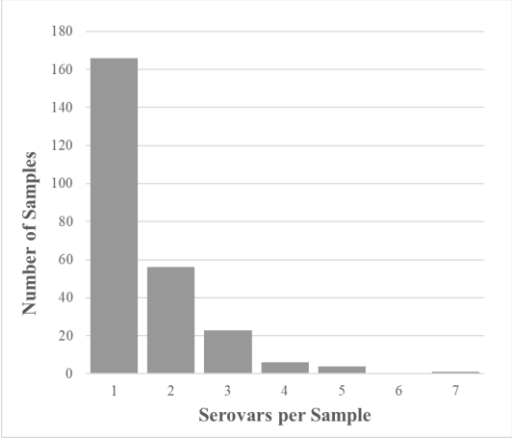
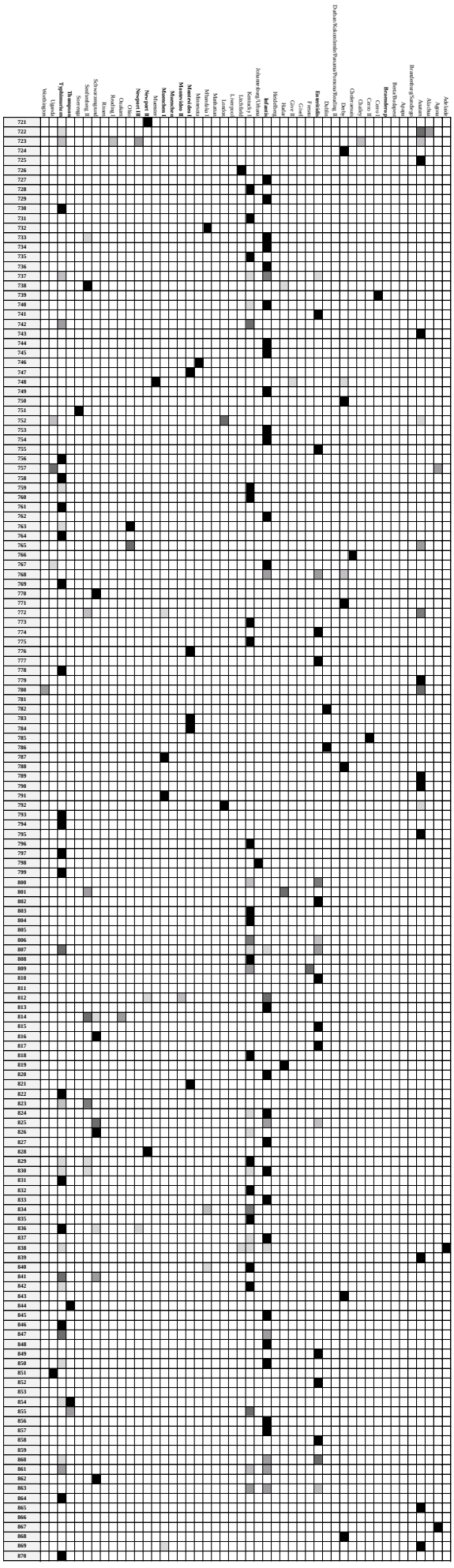


Figure 1. Multiserovar populations were recovered from 35.2% of samples. *Samples contained an average of 1.55 serovars with a maximum of seven serovars recovered from a single sample.*

Serovars of human clinical importance, as designated by the CDC in the *Salmonella* Annual Report (16.), were recovered from 64.8% (166/256) of samples (Fig. 2). A total of eleven clinically significant serovars were identified: Braenderup, Enteritidis, Infantis, Montevideo I, Montevideo II, Muenchen, Muenchen I, Newport II, Newport III, Thompson, and Typhimurium. Notably, these serovars were present as the minority group in 33.7% of total involved incidences (Fig. 4).

[1]*CRISPR-SeroSeq does not distinguish between serovar Typhimurium and its monophasic variant.*



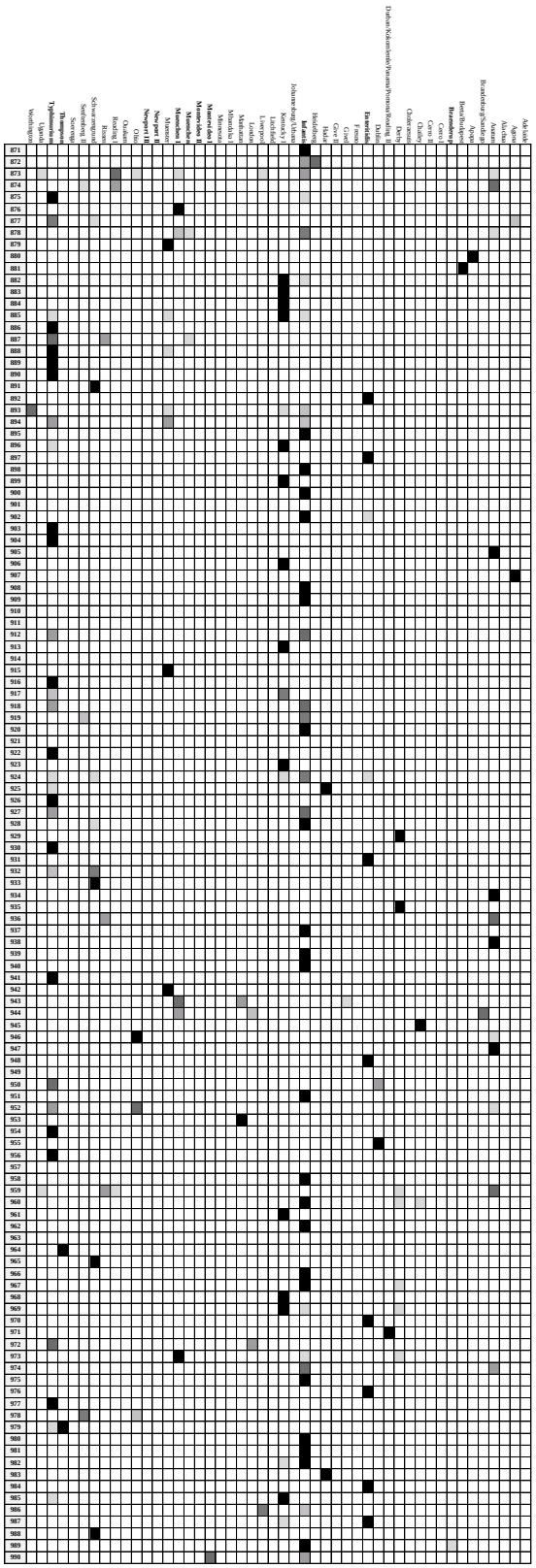


Figure 2. Detection of 48 unique serovars by CRISPR-SeroSeq deep serotyping. *Lefthand column of heatmap depicts sample ID while the top row represents serovars, with serovars of human clinical importance, as denoted by the CDC, are bolded. Serovar abundances are represented by darkened boxes following the legend below:*

0	
0 - 0.10	
0.10 - 0.25	
0.25 - 0.50	
0.50 - 0.75	
0.75 - 0.90	
0.90 - 1	

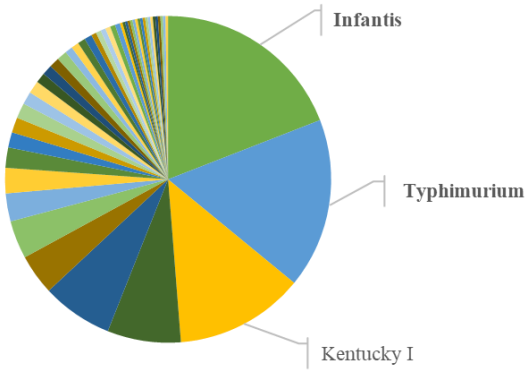


Figure 3. Three serovars encompass 48.7% of all detected incidences. *Serovars Infantis, Typhimurium, and Kentucky I comprised 19.1%, 16.8%, and 12.8% of incidences respectively. Serovars Infantis and Typhimurium are bolded, representing their importance to human health as denoted by the CDC.*

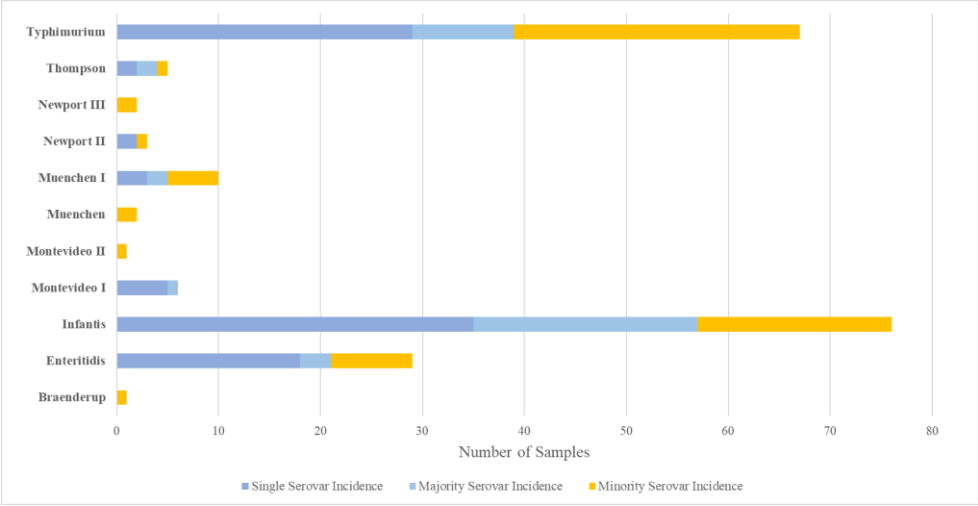


Figure 4. Serovars of human clinical importance were found in the background 33.7% of involved samples. *Serovars designated by the CDC as clinically important are depicted on the left*

Bray-Curtis indexes between sample pairings determined 4.6% of samples had identical serovar abundances, while 78.0% of samples were completely different, representing the high variance in recovered serovar abundances.

Table 1. Samples showed high relative dissimilarity in Bray-Curtis pairings. *Relative similarity of index values is represented as the top row, and frequency of index values returned amongst pairings is depicted in the bottom row.*

Bray-Curtis Index	**0 (Identical)**	**0-0.3 (Similar)**	**0.3-0.7 (Moderately Similar)**	**0.7-1 (Dissimilar)**	**1 (No Similarities)**
Frequency of pairings	0.046017	0.047089	0.038786765	0.08817402	0.7799326

Discussion

Processed poultry and meat products are often associated with cases of human Salmonellosis (IFSAC 2022). Given the inherent diversity of *Salmonella* serovars in their host preference, antimicrobial resistance, and capabilities to cause human illness, it becomes increasingly clear that current serotyping methodologies do not fully assess the risks posed by multiserovar populations (Grimont and Weill 2007). By picking only one or few colonies, as in current methodologies, there is a significant possibility of neglecting minority serovars where multiserovar populations exist and undershooting the public health risks of certain contaminated meat and poultry products.

In our study, the isolation of multiserovar *Salmonella* populations from post-processing poultry and meat samples yielded an overall prevalence of 35.2%, with 48 unique serovars being detected amongst 256 *Salmonella* positive samples (Fig. 2). Despite extensive antimicrobial interventions, it is apparent by these data that certain serovars are evading knockdown. A study surveying *Salmonella* positive, pre-harvest samples collected from chicken breeder flocks between July 2020 and June 2021 recovered multiserovar populations from 32.09% of samples (Siceloff et al. 2022). Though these results follow a similar trend to the observed data, another recent study surveying *Salmonella* serovar dynamics within cattle feed returned an impressive 56% multiserovar prevalence (Shariat et al. 2022), showing the possible discrepancies based on animal product type. These concerns will be addressed in future extensions of this study when animal source data is incorporated, and analysis of trends between different meat products will be incorporated.

Serovars of human clinical significance were found in the background in 33.7% of related incidences (Fig. 4). Notably, Typhimurium, a serovar of human concern which had a total incidence of 67, was found in the minority of 41.8% (28/67) of related incidences. This supports the concern that clinically significant serovars may remain in the background and be overlooked by current, colony-picking methodologies for serotyping. This concern is exemplified when considering the dynamic nature of *Salmonella* serovar prevalence, which is emphasized in a 2018 longitudinal study evaluating serovar dynamics in swine from 1997-2015, where significant changes in serovar frequency can be noted not only in the surveyed swine populations, but in proximal human and veterinary clinical cases (Yuan et al. 2018). The evident fluidity in serovar dynamics coupled with clinically significant serovars being underrepresented in current surveillance brings the concern of population shifts towards these concerning serovars due to ecological and antimicrobial interventions.

Despite the depth of study, limitations still exist. For one, all enrichments were performed using Tetrathionate (TT) broth, which has been known to produce false positives in secondary enrichment as well as having significant biases towards certain serovars (Gorsi et al. 2011, Cox et al. 2019). Without sample pairings, possible deviations from true serovar abundances and prevalence may occur. However, this does not discount the importance of serotyping these samples. An additional limitation lies in the current blindness of the study. The lack of animal source data, processing facility information (ie. location, antimicrobials used, time of year, etc.), as well as FSIS-USDA serotyping data limit the deeper analytical abilities of this study. Without an understanding of sample origins and relevant factors affecting serovar prevalence, it is difficult to fully represent the proficiency of CRISPR-

SeroSeq as a serotyping tool over the current methodology. However, the future implementation of these data will allow for these interpretations.

Salmonella contamination, despite interventions, remains a prolific threat in the food processing industry. With processing being the most direct link to the consumer in the meat production timeline, the implementation of thorough surveillance and serotyping measures holds great importance, as miss-assessing risks associated with these products poses deep concern to public health. With current serotyping measures unable to provide a wholistic analysis of *Salmonella* populations, we sought to show the proficiency of CRISPR-SeroSeq as a deep serotyping tool for identifying multiserovar populations within a single sample. The recovery of these populations within post-intervention processing samples and the effective detection of minority serovars of clinical significance highlights the importance of greater depth of surveillance in *Salmonella* serotyping within our food products. Future extensions of this research will include the implementation of animal source data as well as FSIS-USDA colony-based serotyping data, allowing for derivation of serovar trends within specific products as well as comparative analysis of the depth and accuracy between the current serotyping methodologies and CRISPR-SeroSeq.

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