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FOOD MICROBIOLOGY

Regional *Salmonella* Differences in United States Broiler Production from 2016 to 2020 and the Contribution of Multiserovar Populations to *Salmonella* Surveillance

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ABSTRACT Poultry remains a considerable source of foodborne salmonellosis despite significant reduction of *Salmonella* incidence during processing. There are multiple entry points for *Salmonella* during production that can lead to contamination during slaughter, and it is important to distinguish the serovars present between the different stages to enact appropriate controls. National *Salmonella* data from the U.S. Department of Agriculture–Food Safety Inspection Service (USDA-FSIS) monitoring of poultry processing was analyzed from 2016 to 2020. The overall *Salmonella* incidence at processing in broiler carcasses and intact parts (parts) decreased from 9.00 to 6.57% over this period. The incidence in parts was higher (11.15%) than in carcasses (4.78%). Regional differences include higher proportions of serovars Infantis and Typhimurium in the Atlantic and higher proportion of serovar Schwarzengrund in the Southeast. For Georgia, the largest broiler-producing state, USDA-FSIS data were compared to *Salmonella* monitoring data from breeder flocks over the same period, revealing serovar Kentucky as the major serovar in breeders (67.91%) during production but not at processing, suggesting that it is more effectively removed during antimicrobial interventions. CRISPR-SeroSeq was performed on breeder samples collected between 2020 and 2021 to explain the incongruence between pre- and postharvest and showed that 32% of samples contain multiple serovars, with up to 11 serovars found in a single flock. High-resolution sequencing identifies serovar patterns at the population level and can provide insight to develop targeted controls. The work presented may apply to other food production systems where *Salmonella* is a concern, since it overcomes limitations associated with conventional culture.

IMPORTANCE *Salmonella* is a leading cause of bacterial foodborne illness in the United States, with poultry as a significant *Salmonella* reservoir. We show the relative decrease in *Salmonella* over a 5-year period from 2016 to 2020 in processed chicken parts and highlight regional differences with respect to the prevalence of clinically important *Salmonella* serovars. Our results show that the discrepancy between *Salmonella* serovars found in pre- and postharvest poultry during surveillance are due in part by the limited detection depth offered by traditional culture techniques. Despite the reduction of *Salmonella* at processing, the number of human salmonellosis cases has remained stable, which may be attributed to differences in virulence among serovars and their associated risk. When monitoring for *Salmonella*, it is imperative to identify all serovars present to appropriately assess public health risk and to implement the most effective *Salmonella* controls.

KEYWORDS *Salmonella*, serovars, poultry, monitoring, CRISPR-SeroSeq, salmonellosis

Despite efforts to mitigate *Salmonella* during slaughter, poultry remains a significant cause of human salmonellosis and is responsible for approximately 23% of salmonellosis cases each year, 17% of which are directly linked to chickens (1). Isolates belonging to

Editor Christopher A. Elkins, Centers for Disease Control and Prevention

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The authors declare no conflict of interest.

Received 1 February 2022
Accepted 18 March 2022
Published 6 April 2022

Salmonella enterica subsp. *enterica* are most frequently associated with human illness, and this subspecies is represented by more than 1,500 distinct serovars that are characterized by their lipopolysaccharide (O) and flagellar (H) antigens (2). Serovars can inhabit different niches and show host tropism with altered capacities to cause illness in humans and animals (3–6). Of serovars frequently found in poultry in the United States, serovar Kentucky has a low association with human illness, while serovars Enteritidis, Typhimurium, and Infantis are often responsible for human salmonellosis (7, 8).

The United States produces over nine billion broilers each year (9), with the Southeast producing 5.01 billion, followed by the South Central region (~1.95 billion) and the Atlantic (~1.11 billion). A total of 1.3 billion broilers are produced in Georgia (14%), the top broiler-producing state. The poultry industry is vertically integrated into complexes, with each life stage of the chicken separated into different houses, and large integrators operate multiple complexes. Each complex typically encompasses parental breeder flocks ("breeders") whose eggs are sent to a single hatchery facility within the complex. Newly hatched broiler chicks are then disseminated to multiple broiler farms, and these are subsequently processed at a single processing plant belonging to that complex. Vertical integration is economically beneficial and allows for stricter biosecurity measures that provide greater control over pathogen spread for foodborne and avian pathogens. Vertical and horizontal transmission from breeders to broiler flocks is the largest *Salmonella* contributor to chickens at processing, making breeders the single most important target for *Salmonella* mitigation (10–12).

The U.S. Department of Agriculture–Food Safety Inspection Service (USDA-FSIS) regularly collects surveillance samples from processing establishments, including carcass and raw part rinses, and publicly reports these data, along with establishment performance standards. Given that current *Salmonella* regulation by USDA-FSIS has not led to a decrease in overall human salmonellosis, in October 2021, the agency introduced an initiative to reduce *Salmonella* in broilers (13). It is possible that this initiative may lead to the use of additional *Salmonella* controls during live production to reduce overall *Salmonella* load as birds arrive at the processing facility. Although there is no national monitoring system in place to survey *Salmonella* in broiler flocks, since 2016, with the support of the National Chicken Council, several integrators began monitoring *Salmonella* in their breeder flocks at 16 weeks (prior to egg production) and 42 weeks (after peak egg production). This monitoring is typically performed at state-supported, commercial, or academic laboratories and is not reported.

Vaccination of breeder flocks, and sometimes broiler flocks is an effective method of *Salmonella* control in live production (14). Commercially available live attenuated vaccines targeting serovar Typhimurium and killed vaccine against serovar Enteritidis are broadly used. An additional strategy is the use of autogenous vaccines that effectively reduce intestinal *Salmonella* in parental breeders, and in their subsequent broiler progeny (12, 15–18). These killed vaccines are developed for a specific broiler complex and are generated against one to five *Salmonella* serovars that have been collected from those premises. Development of effective autogenous vaccines that make the greatest positive impact to food safety rely on two attributes: (i) appropriate surveillance to identify and isolate serovars in both pre- and postharvest and (ii) recognition of greatest concern serovars. Both attributes require the identification of all *Salmonella* serovars present in a population, and this is not always achieved with conventional culture methods. As demonstrated by a previous study (19), some serovars with increased antimicrobial resistance may be hiding in the background of *Salmonella* populations and are only revealed following antimicrobial treatment, which effectively reduces all susceptible serovars and allows for the growth of resistant serovars.

Current surveillance relies on *Salmonella* isolation by enrichment and characterization of a few resulting colonies that grow on indicator agar (20). This is a serious limitation in *Salmonella* surveillance and source tracking since generally only the most abundant serovar(s) in a mixed population are detected, while the less abundant serovar(s) remain undetected (21, 22). Where clinically important serovars are undetected, traditional surveillance underestimates the presence of *Salmonella* serovars of the greatest food safety concern. High-throughput sequencing-based technologies have addressed this problem by discerning multiple serovars in a single sample and revealing serovars at orders of magnitude greater than logistically possible by picking

TABLE 1 Prevalence of *Salmonella* in broiler carcasses at processing, 2016 to 2020

	No (%) of samples[a]											
	Southeast		South Central		Atlantic		Midwest		Mountain & West		All regions	
Yr	Total	SP	Total	SP	Total	SP	Total	SP	Total	SP	Total	SP
2020	4,624	165 (3.57)	2,101	85 (4.05)	1,391	93 (6.69)	1,081	71 (6.57)	479	37 (7.72)	9,676	451 (4.66)
2019	4,439	170 (3.83)	1,982	72 (3.63)	1,372	83 (6.05)	1,016	63 (6.20)	461	29 (6.29)	9,270	417 (4.50)
2018	4,238	170 (4.01)	1,906	77 (4.04)	1,353	99 (7.32)	1,027	81 (7.89)	500	33 (6.60)	9,024	460 (5.10)
2017	4,289	230 (5.36)	1,896	115 (6.07)	1,336	68 (5.09)	1,056	61 (5.78)	491	36 (7.33)	9,068	510 (5.62)
2016	4,179	151 (3.61)	1,833	68 (3.71)	1,283	64 (4.99)	1,004	46 (4.58)	503	23 (4.57)	8,802	352 (4.00)
Total	21,769	886 (4.07)	9,718	417 (4.29)	6,735	407 (6.04)	5,184	322 (6.21)	2,434	158 (6.49)	45,840	2,190 (4.78)

[a]SP, *Salmonella* positive.

colonies off a plate (23, 24). *Salmonella* clustered regularly interspaced short palindromic repeat (CRISPR) spacer content is tractable with serovar identity, and these sequences have been employed effectively for molecular serotyping (25–29). CRISPR-SeroSeq (serotyping by sequencing the CRISPR loci) is an amplicon-based sequencing tool that uses *Salmonella* CRISPR identities to quantify the relative frequency of multiple serovars in a single sample, down to serovars comprising as little as 0.003% of the population (19, 22, 23, 30, 31).

Prior to this study, some poultry integrators reported to us that some serovars they find during live production (preharvest) do not align with those found during processing (postharvest). We initiated this study to determine whether this pattern occurred more broadly across national and regional surveillance data from processing. Using serovar population analyses by CRISPR-SeroSeq revealed that many preharvest samples contain multiple serovars, which explains the serovar diversity seen during processing. Although this study uses broiler production, the findings presented here are broadly applicable to other industries where *Salmonella* is a concern. In addition, this study highlights the importance of comprehensive surveillance monitoring in food production systems to identify and control pathogens prior to an outbreak.

RESULTS

Between 2016 and 2020, the percentage of *Salmonella*-positive broiler carcasses increased slightly from 4.00 to 4.66% across the United States (Table 1). For all five regions, the percentage of *Salmonella* positive carcasses peaked in 2017 or 2018. Conversely, this measure decreased significantly in raw, intact parts (referred to as "parts" in the present study) from 16.29 to 8.05% and peaked for most regions in 2016 (Table 2). During this time, the total number of carcass samples collected by USDA-FSIS increased by 10%, while the number of parts samples more than doubled. Significantly, for all regions across all 5 years, the percentage of *Salmonella*-positive samples was greater in parts (11.15%; 4,738/42,490) than in carcasses (4.78%; 2,190/45,840) (Tables 1 and 2).

The Southeast is the largest poultry-producing region in the United States, with more samples collected than any other region. Therefore, expectedly, it had the highest overall number of *Salmonella* isolated from carcasses and parts, with annual averages of 4.07% (886/21,769) and 11.50% (2,140/18,604) *Salmonella*-positive parts samples. Despite this, *Salmonella* incidence in 2020 was lowest in Southeast processing establishments, at 3.57% in carcasses and

TABLE 2 Prevalence of *Salmonella* in raw intact chicken parts at processing, 2016 to 2020

	No. (%) of samples[a]											
	Southeast		South Central		Atlantic		Midwest		Mountain & West		All regions	
Yr	Total	SP	Total	SP	Total	SP	Total	SP	Total	SP	Total	SP
2020	5,078	306 (6.03)	2,571	192 (7.47)	1,971	209 (10.60)	1,379	149 (10.88)	1,580	127 (8.04)	12,579	1,012 (8.05)
2019	4,229	345 (8.16)	2,022	121 (5.98)	1,466	183 (12.48)	928	101 (10.87)	1,221	92 (7.53)	9,872	1,001 (10.14)
2018	3,318	462 (13.92)	1,566	131 (8.37)	920	139 (15.11)	576	95 (16.49)	781	73 (9.35)	7,161	900 (12.57)
2017	3,236	540 (16.69)	1,570	170 (10.83)	827	126 (15.24)	570	74 (12.98)	646	91 (14.09)	6,850	842 (12.29)
2016	2,743	487 (17.75)	1,382	198 (14.33)	822	159 (19.34)	507	87 (17.16)	581	81 (13.94)	6,035	983 (16.29)
Total	18,604	2,140 (11.50)	9,111	812 (8.91)	6,006	816 (13.57)	3,960	506 (12.77)	4,809	464 (9.65)	42,490	4,738 (11.15)

[a]SP, *Salmonella* positive.

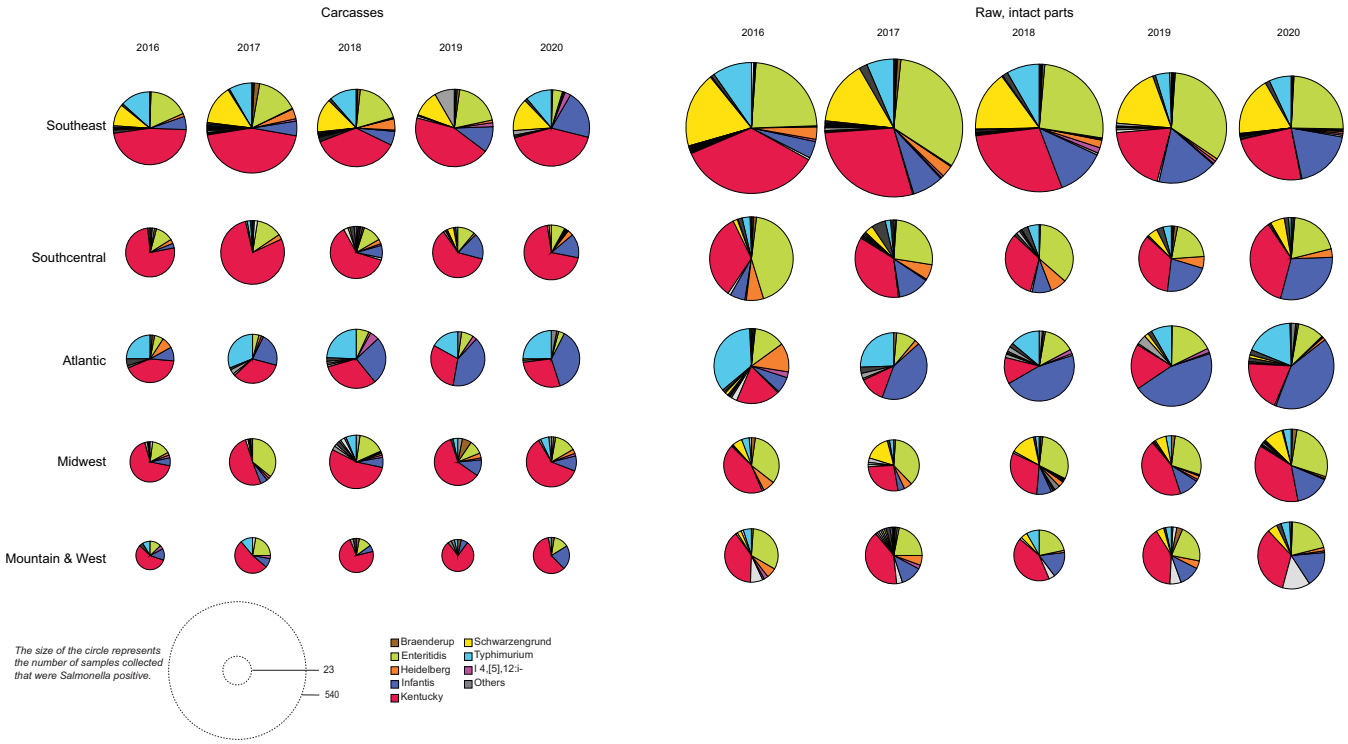


FIG 1 *Salmonella* serovar distribution in broiler carcasses and intact parts at processing across the United States, 2016 to 2020. Pie charts depict the prevalence of different serovars in broiler processing establishments in the United States. The size of the circle reflects the number of positive samples, with the smallest (23 samples) and largest (540 samples) numbers shown in the dotted circle guide at the bottom. The regions are defined as follows: Atlantic (CT, DE, MA, MD, ME, NH, NJ, NY, PA, RI, VT, VA, and WV), Southeast (AL, GA, FL, KY, MS, NC, SC, and TN), South Central (AR, LA, OK, and TX), Midwest (IL, IN, IA, KS, MI, MN, MO, NE, ND, OH, SD, and WI), and Mountain & West (AZ, CA, CO, ID, MT, NV, NM, OR, UT, WA, and WY). Regions are ordered based on broiler production per region, according to the USDA (U.S. Department of Agriculture–National Agricultural Statistics Service, 2021). Select serovars are highlighted as shown, and others are indicated in different shades of gray; full serovar information is provided in Tables S1 and S2 in the supplemental material.

6.03% in parts. Poultry production in the Atlantic and the South Central regions is comparable, and this is reflected in their similar *Salmonella* prevalence values. In carcasses, the Atlantic region had an average *Salmonella*-positive incidence of 6.04% (407/6,375), while the South Central region had an average of 4.29% (417/9,718) (Table 1). Both of these incidences were increased in parts, with averages of 13.57% (816/6,006) and 8.91% (812/9,111) in the Atlantic and South Central regions, respectively (Table 2). While the Southeast has the highest overall *Salmonella* prevalence, this is the only region where the number of *Salmonella* isolated has decreased each year in both carcasses and parts since 2017 (carcasses: 230 isolates in 2017 to 165 isolates in 2020; parts: 540 isolates in 2017 to 306 isolates in 2020), whereas for other regions the annual number of *Salmonella* isolated has somewhat increased. Importantly, the total number of samples per type and year has been relatively maintained in all regions.

The USDA-FSIS also reports the serovar information, and we analyzed these data as well. Between 2016 and 2020, the number of serovars found in parts (59 serovars) was greater than in carcasses (37 serovars) (see Tables S1 and S2 in the supplemental material), which fits the trend observed above with overall *Salmonella* incidence between carcasses and parts. The Southeast region had the greatest diversity, with an annual average of 22 serovars in parts and 14 serovars in carcasses. This was followed by the Atlantic and South Central regions, with an annual average of 14 and 13 serovars in raw, intact parts, and 8 and 9 serovars in carcasses, respectively. For most regions, the number of different serovars found per sample type peaked in 2016 and 2017 and has since decreased. For carcasses, the annual number of serovars peaked at 18 in the Southeast in 2017, while in parts the highest annual number of serovars was 26 in the Southeast in 2016.

In terms of serovar identity, there were also regional differences, as highlighted by the major serovars in Fig. 1. Serovar Typhimurium was more frequently isolated from both carcasses and parts in the Atlantic and Southeast regions (light blue; Fig. 1). Proportionally,

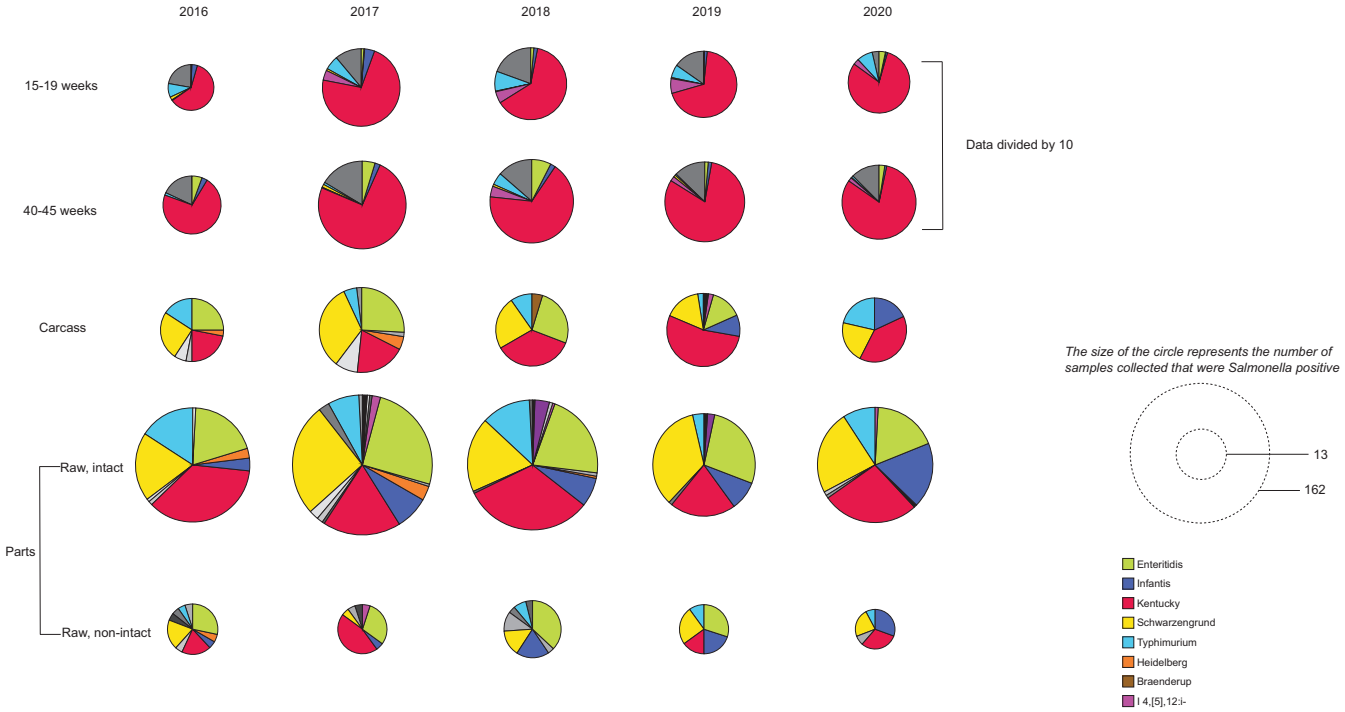


FIG 2 Serovar distribution for *Salmonella*-positive breeder, carcass, intact parts, and nonintact parts samples collected in Georgia, 2016 to 2020. Pie charts depict the prevalence of different serovars in breeder samples collected at 15 to 19 weeks and 40 to 45 weeks (top two rows) and samples from processing establishments in Georgia (bottom three rows). The size of the circle reflects the number of positive samples with the smallest (13 samples) and largest (162 samples) numbers shown in the dotted circle guide on the right. The values for the breeder samples are divided by 10. Select serovars are highlighted as indicated and others are shown in different shades of gray. For the samples from processing, the full serovar data are presented in Table S3 in the supplemental material.

serovar Typhimurium was greatest in the Atlantic region, comprising 24.08% (98/407) of carcass samples and 19.66% (160/814) of parts samples from 2016 to 2020. The relative proportion of serovar Typhimurium in the Southeast was lower, at 10.16% (90/886) and 7.25% (155/2,139) in carcass and parts samples, respectively. While serovar Infantis (dark blue) was isolated from both products in all regions each year, it was most prominent, relatively, in samples from the Atlantic. Here, it has rapidly increased from 6.92% (11/159) and 9.38% (6/64) in 2016 to 41.35% (86/208) and 37.63% (35/93) in 2020 in parts and carcasses, respectively. In the Southeast, serovar Schwarzengrund (yellow) was the third most isolated serovar from 2016 to 2020, with 16.83% (360/2,139) in parts and 12.87% (114/886) in carcasses. We also observed some trends that occurred across all regions. For instance, the relative proportion of serovar Kentucky (red) decreased from carcasses to parts, with an overall average of 49.95% (1,094/2,190) and 29.29% (1,387/4,735), respectively. In addition, serovar Enteritidis (green) increased between carcasses (12.83%; 281/2,190) and parts 25.51% (1,208/4,735) of parts and of carcasses.

Since our monitoring data from breeder flocks is relatively unbiased (a large number of different companies and complexes submit samples for *Salmonella* testing) and because Georgia represents the largest broiler-producing state, we next sought to compare the serovars that are isolated from breeders at 16 and 42 weeks with those found during slaughter. The number of isolates peaked in 2017 for carcasses (n = 58), raw, intact parts (n = 185), and breeders (young, n = 497; old, n = 635) (Fig. 2). For nonintact parts, the number of *Salmonella* isolated peaked in 2018 (27 isolates). Following these peaks in 2017 and 2018, the overall *Salmonella* incidence in all sample types has decreased. From 2016 to 2020, the average number of serovars isolated each year was higher in nonintact parts (13), compared to carcass rinses (6) and intact parts (7) (see Table S3). For all three processing sample types, the greatest diversity was found in 2017.

The serovar identity across the three sample types over time reveals some interesting patterns. Since 2016, serovar Kentucky has increased in prevalence, replacing serovar Enteritidis as

the most common serovar isolated from carcasses (Fig. 2). Relative to carcasses, serovar Kentucky is proportionally reduced in both intact and nonintact parts, as was observed in the national data (Fig. 1). Despite not being identified in carcasses until 2019, the incidence of serovar Infantis increased dramatically since 2016, and in 2020, accounted for 17.65% (30/170) of *Salmonella*-positive samples. Serovar Enteritidis was proportionally higher in parts from 2016 to 2020 than in carcasses, though it was not identified in nonintact parts in 2020.

Strikingly, from 2016 to 2020 serovar Kentucky was the most common serovar isolated from young and old breeder flocks and has proportionally increased each year to account for 80 and 81% of samples, respectively. Serovars Enteritidis and Typhimurium alternate in prevalence to account for the second and third most commonly isolated serovars between 2016 and 2020, although serovar Typhimurium, and its monophasic variant, I 1,4,[5],12:i:-, are more frequently associated with young breeders, whereas serovar Enteritidis is more often isolated from older breeders (Fig. 2).

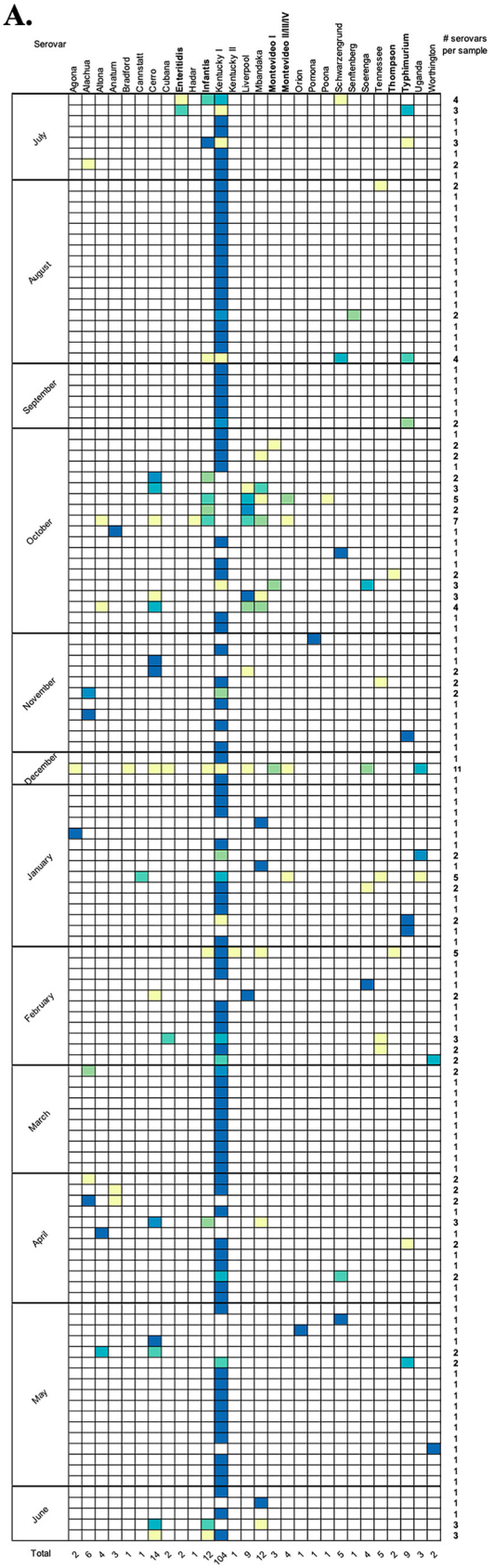
The discrepancy between high serovar Kentucky prevalence in breeder flocks but low prevalence at processing suggests that this serovar is effectively mitigated during slaughter. However, it does not explain the origin of other serovars such as Infantis, Enteritidis, and Schwarzengrund that are found at processing. One possible explanation is that multiple serovars exist in breeder flocks but the amount of serovar Kentucky is so high, that detecting the other serovars by traditional culture methodology (i.e., picking a small number of colonies) prevents the detection of less abundant serovars that might also be present. To determine whether this was the case, we sought to apply high-resolution serovar population analyses to samples collected from breeder flocks. CRISPR-SeroSeq was performed on 134 *Salmonella* positive samples that were collected at the Georgia Poultry Lab Network from July 2020 to June 2021. There was an average of 1.6 serovars per sample, with 32.09% (43/134) samples containing more than one serovar (range, 1 to 11), and a total of 26 serovars across the sample set (Fig. 3). Across the data set, the October 2020 samples were most diverse: we identified 13 different serovars (plus two different lineages of serovar Montevideo) and 57.89% (11/19) samples contained multiple serovars. The March 2021 samples were the least diverse, with only two serovars identified and a single instance of a multiserovar sample. Expectedly, serovar Kentucky was the most common serovar, followed by serovars Cerro and Mbandaka. Serovar Kentucky was present in 77.61% (104/134) samples, and as the majority serovar (darker blue) in 90.38% (94/104) of these samples. We identified five serovars of human importance, as denoted by the CDC Top 10 serovar list: Enteritidis, Infantis, Montevideo, Thompson, and Typhimurium. Notably, serovar Infantis was detected in 8.96% (12/134) samples, and it was present as a minority serovar in 91.67% (11/12) of these samples.

DISCUSSION

Salmonella contamination of poultry remains a significant and complex problem (32–36). The overall number of *Salmonella*-positive samples and the number of serovars identified in broiler processing plants is reflective of the size of production in the different regions. However, this is not reflected by the proportion of samples that are *Salmonella* positive. For example, in 2020, the two highest-producing regions (Southeast and South Central) had the lowest proportion of *Salmonella*-positive carcasses and parts. Further, the proportional decrease of *Salmonella*-positive carcasses (2017/2018-2020) and parts (2016 to 2020) across all five regions indicates that *Salmonella* control measures can be effective. This time frame is concurrent with the most recent performance standards from USDA-FSIS (37) and suggests that this approach has been useful at reducing the overall *Salmonella* prevalence in poultry.

Despite the success in reducing overall *Salmonella* incidence at processing, the number of human salmonellosis cases linked to poultry has remained relatively unchanged (13). This is likely due in part to the particular serovars that are present in poultry production, their propensity to colonize poultry, and their individual association with human illness. Further investigation is warranted to identify serovar-specific capabilities to persist in poultry production. When we began this study, our intention was to analyze and present the national data together; however, we noticed the region-specific trends and decided to present the

A.



B.

Serovar	Serovar prevalence across samples	Incidence of majority to minority (ratio)	Majority serovar in sample (%)	Minorityy serovar in sample (%)
Agona	2	1:1	50	50
Alachua	6	3:3	50	50
Altona	4	2:2	50	50
Anatum	3	1:2	33	67
Bradford	1	0:1	0	100
Cannstatt	1	0:1	0	100
Cerro	14	8:6	57	43
Cubana	2	0:2	0	100
Enteritidis	2	0:2	0	100
Hadar	1	0:1	0	100
Infantis	12	1:11	8	92
Kentucky I	104	94:10	90	10
Kentucky II	1	0:1	0	100
Liverpool	9	4:5	44	56
Mbandaka	12	3:9	25	75
Montevideo I	3	0:3	0	100
Montevideo II/III/IV	4	0:4	0	100
Orion	1	1:0	100	0
Pomona	1	0:1	0	100
Poona	5	3:2	60	40
Schwarzengrund	1	0:1	0	100
Senftenberg	4	2:2	50	50
Soerenga	5	0:5	0	100
Tennessee	2	0:2	0	100
Thompson	9	5:4	56	44
Typhimurium	3	2:1	67	33
Uganda	2	2:0	100	0
Worthington	1	1:0	100	0

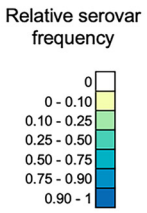


FIG 3 *Salmonella*-positive breeder monitoring samples often contain multiple serovars. CRISPR-SeroSeq was used to determine the relative abundance of *Salmonella* serovars within each sample. Each column is an individual sample that was derived from

(Continued on next page)

data by region. The growing proportion of serovar Infantis in the Atlantic region from 2016 to 2020 is particularly striking. While serovar Infantis has proportionally increased in all five regions in both carcasses and parts, in the Atlantic it has been the major serovar found in carcasses since 2019 and in parts from 2017. This may reflect climate or environmental conditions in the Atlantic that somehow promote colonization of poultry by serovar Infantis (or that suppress other serovars such as Kentucky) or that serovar Infantis has filled a vacated ecological niche (e.g., through vaccination targeting serovar Kentucky).

For several years, serovar Kentucky has been the most prevalent serovar isolated during poultry production in the United States, though in the U.S. serovar Kentucky does not have a high association with human illness (7, 38). The data presented here comparing *Salmonella* serovar incidence in breeder flocks with that at processing in Georgia shows that the high proportion of serovar Kentucky in flocks is reduced significantly during processing. This observation is in agreement with studies showing the competitive fitness of serovar Kentucky in colonizing chicken intestines (39) and also suggests that serovar Kentucky is susceptible to antimicrobial interventions used in the carcass chilling procedure. Further, in the national processing data, the proportion of serovar Kentucky is consistently lower in parts than on carcasses, suggesting that the additional antimicrobial intervention steps between chilling and parts cut up also effectively removes serovar Kentucky.

Across the United States, *Salmonella* incidence increases from carcasses to parts, despite the collection of similar numbers of samples. A similar increase has been noted in other studies comparing *Salmonella* prevalence in post-chill carcasses and parts (40–42). This might be due to cross-contamination of equipment used to generate parts (43). These findings suggest the need for additional interventions during processing of parts. The reduction of serovar Kentucky prevalence between carcasses and parts is accompanied by an increase of other serovars, specifically serovars Enteritidis and Infantis, which are both frequently associated with human salmonellosis (7, 8). There are a few possible, non-mutually exclusive, explanations for these observations. First, peroxyacetic acid is an effective antimicrobial commonly used in processing (44), and some serovars may tolerate this antimicrobial better than serovar Kentucky (45). Second, despite cleaning and sanitation, these patterns may reflect serovar differences in survival in processing environments, such as the ability to form strong biofilms (46–49). Third, some serovars, such as Enteritidis, can systemically infect chickens (50, 51), and separation of the carcass into parts may release internalized *Salmonella*. Significant further research is required to address the potential impact of these explanations on the serovars found in processing.

Salmonella serovars found at processing must have originated from live production, and the reduction of serovar Kentucky during processing is able to reveal the identity of additional serovars, some of which are often associated with human illness. Using CRISPR-SeroSeq, we determined that one third of samples contained more than one serovar. This type of approach overcomes the disadvantage of only detecting the most abundant serovar (or that which grows best), which occurs when only a small number of colonies are selected from a plate (52). CRISPR-SeroSeq identifies multiple serovars based on amplification of total genomic DNA in a sample, therefore allowing for a greater representation of *Salmonella* serovar diversity. This analysis explains some of the differences between the breeder samples and the processing plant samples. For example, it revealed that serovar Infantis is most often outnumbered by other serovars, including the serovars Kentucky, Liverpool, and Cerro, and that serovars Enteritidis and Typhimurium were minority serovars in 100 and 33% of instances where they were detected, respectively, though the former was only detected in two samples. The high number of samples containing serovar Cerro was surprising since this serovar is most

FIG 3 Legend (Continued)
the overnight tetrathionate enrichment culture from an environmental boot sock sample; these are arranged according to the date they were submitted, and the month is indicated. The individual serovars are shown on the left, and the heatmap shows the relative serovar abundance in each sample according to the key. Samples with more than one serovar are indicated in boldface (bottom row). The two serovar Kentucky lineages and four serovar Montevideo lineages are named as previously described (72, 73).

frequently associated with cattle (53). Potential explanations include the high number of cow-calf operations in Georgia (54), many of which are near broiler production farms, or introduction via feed that contains meat and bone meal or blended animal by-products (55, 56). Our data also suggest that there may be seasonal attributes that contribute to serovar diversity since the samples collected in October 2020 were more diverse than others and that the samples from October and November also had the lowest incidence of serovar Kentucky. The data analyzed here only represent a single year, and future analyses are required to determine whether these trends are significant. Continuing to apply high-resolution surveillance approaches can elucidate intraspecies population dynamics, since some serovars may prove to be consistently more dominant in populations, and there may be some environmental conditions which encourage competitive exclusion.

Targeted serovar-specific *Salmonella* reduction through autogenous vaccination has increased in use in the poultry industry, as integrators try to eliminate serovars of the greatest food safety concern and to also reduce the quantity of *Salmonella* on birds arriving at their processing facilities. Generation of an autogenous vaccine requires the serovar of concern to have been isolated within a complex previously, and this is subject to the limitations of *Salmonella* isolation during monitoring (i.e., the lower resolution of selecting and characterizing a small number of colonies). Population-based approaches, such as CRISPR-SeroSeq, aid in revealing the presence of serovars that may be less fit than others in live chickens but that can persist in the processing environment and potentially cause human illness (22, 23, 31). Such serovars would be ideal candidates to be targeted by autogenous vaccines. This approach, too, will improve *Salmonella* surveillance and food safety. Historically, successful, serovar-specific industry-wide interventions have reduced or eliminated the presence of those serovars (57). This often results in another serovar taking over (e.g., serovar Kentucky replacing serovar Enteritidis following efforts in the 1990s to eliminate the latter). The use of serovar population analyses followed by in-depth phenotypic characterization of different strains or serovars, particularly in preharvest poultry, may help to predict these shifts as they are occurring and allow vaccines or other interventions to be generated in a timelier manner.

There are a few caveats to our study: first, the samples analyzed here are not matched to each other, and we did not follow the same breeder flocks to broilers and then to processing. This may be why we did not identify many instances of serovar Enteritidis in our population analyses of *Salmonella*-positive breeder flocks. Further, USDA-FSIS does not sample carcasses and parts from the same processing establishment on the same day; thus, it is not possible to directly compare between the samples collected at processing as different flocks are slaughtered each day. Nonetheless, the large number of samples and the unbiased nature in which they are collected have allowed us to visualize broad, industry-wide trends. A second caveat is that although *Salmonella* quantity in broilers is linked to *Salmonella* found at processing (58), we did not include broilers in this study. This was because there is no broad *Salmonella* monitoring program for broilers beyond what integrators may individually perform and any on-farm sampling we would have done would be biased to one or a small number of integrators. Colonization of breeder flocks with *Salmonella* is a major contributor of *Salmonella* found in broilers, which is one reason why interventions such as vaccination are performed in breeders (11, 59). However, this does not discount the possibility of additional *Salmonella* contamination of broiler flocks from environmental sources such as litter, insects, feed, and rodents (60–65), and this was not captured in our study. A recent study demonstrated a high concordance of serovars on preintervention broilers (directly after kill) with those on broilers postintervention (66), so a future study centered on assessing *Salmonella* populations in broilers directly before processing could provide some useful information. A third caveat is the *Salmonella* isolation protocols performed by USDA-FSIS differ from those performed at the Georgia Poultry Laboratory Network (GPLN), since the choice of media used for enrichment and *Salmonella* isolation can impact which serovars are detected (22, 67–70), and this bias may explain some variability across sample types. We were surprised that although serovar Schwarzengrund was frequently identified at processing in the Southeast, including in Georgia, we did not often detect this serovar in

breeder samples by colony isolation and serotyping, nor by CRISPR-SeroSeq analysis of enriched samples from breeder flocks. Previous work has shown that serovar Schwarzengrund is preferentially isolated following enrichment in Rappaport-Vasilliadis (RV) broth compared to tetrathionate (TT) broth (22, 70). Unlike processing plant samples where the *Salmonella* is damaged and isolation requires a nonselective preenrichment step to allow the *Salmonella* to recover (20), isolation from farm samples was approved by the National Poultry Improvement Plan (NPIP) to be incubated directly into selective enrichment both. In the case of this study, all breeder samples were enriched in TT broth and not in RV broth, which may explain this discrepancy.

Salmonella contamination of poultry products remains a complex issue (35, 57), and this study highlights serovar differences regionally and during processing (i.e., between carcasses and parts), which adds to this complexity. Significantly, the population analyses performed here partially explains the serovar incongruity that occurs between pre- and postharvest by demonstrating that a third of all breeder samples contain more than one serovar and that when serovar Kentucky is present, it tends to account for a greater proportion of the *Salmonella* that is present. There are multiple sources which may contribute to *Salmonella* presence in a poultry environment, such as cross-contamination from workers, rodent activity, and contaminated feed products (71), so it is important to expand surveillance sampling to monitor these contamination routes, since this could lead to improved *Salmonella* control. Finally, although poultry is a considerable *Salmonella* reservoir, the contamination of other food animals, including cattle and swine, is also a significant food safety concern. These industries also face the pre- and postharvest *Salmonella* challenges that have been described here; the approaches and conclusions drawn here are relevant to those and other industries where *Salmonella* is a problem.

MATERIALS AND METHODS

Analysis of USDA-FSIS data. *Salmonella* data from January 2016 to December 2020 was downloaded from the USDA-FSIS website (https://www.fsis.usda.gov/science-data/data-sets-visualizations/laboratory-sampling-data). These data include establishment identity and location, date of isolation, sample type, and *Salmonella* serovar identity. For national analysis, regions were characterized as follows: Atlantic (Connecticut, Delaware, Maine, Maryland, Massachusetts, New Hampshire, New Jersey, New York, Pennsylvania, Rhode Island, Vermont, Virginia, and West Virginia), Southeast (Alabama, Florida, Georgia, Kentucky, Mississippi, North Carolina, South Carolina, and Tennessee), South Central (Arkansas, Louisiana, Oklahoma, Texas), Midwest (Illinois, Indiana, Iowa, Kansas, Michigan, Minnesota, Missouri, Nebraska, North Dakota, Ohio, South Dakota, Wisconsin), and "Mountain & West" (Arizona, California, Colorado, Idaho, Montana, Nevada, New Mexico, Oregon, Utah, Washington, Wyoming). Data for carcasses and parts were downloaded and analyzed separately. For the parts samples, the data were separated into "raw-intact chicken" and "raw-ground, comminuted or otherwise nonintact chicken" because these reflect different processing steps and different *Salmonella* risks/prevalence. For the national data, the nonintact category was not considered because in lower poultry production regions there were only a small number of positive samples, and these data reflected a single processor sampled multiple times or a small number of processors within that region. For the Georgia analysis, both the intact and nonintact parts data were considered.

***Salmonella* isolation and serotyping.** Several integrators participate in the routine surveillance program through the Georgia Poultry Laboratory Network (GPLN), where breeder flocks are tested for *Salmonella* at approximately 16 weeks (pullets; pre-egg production) and 40 weeks (post-peak egg production). To condense the sample collection into two subsets, samples from weeks 15 to 19 (young breeders) and 40 to 45 (old breeders) were grouped together for analysis. Hatching egg companies maintain breeder flocks and in accordance with NPIP regulations must test their flocks for *Salmonella* every 30 days. *Salmonella* data from hatching egg company samples that were received closest to 16 and 40 weeks from these companies were included in this study. The data includes eight different hatching egg companies and six different integrators. Samples are submitted to GPLN typically as boot socks in Whirl-Pak bags. Between 125 and 150 mL of tetrathionate (TT) enrichment broth was added to each boot sock sample, and these were incubated at 37°C for 20 to 24 h. The bags were gently mixed, and 100 μL of enrichment was transferred into a modified semisolid Rappaport-Vasiliadis (MSRV) agar plate, followed by incubation at 42°C. The plates were checked at 24 and 48 h and transferred onto two types of agar: brilliant green (BG) agar containing novobiocin and xylose lysine tergitol-4 (XLT-4). These were incubated at 37°C for 20 to 24 h, and four presumptive *Salmonella* colonies were selected. *Salmonella* was confirmed by biochemical identification using the Vitek system (BioMerieux) and then serotyped by conventional serum agglutination (BD Difco, Fisher Scientific, Atlanta, GA; Remel, Lenexa, KS; and SSI Diagnostics, Cedarlane, Burlington, NC) and using the Luminex xMap molecular assay (Luminex, Austin, TX).

Serovar population analyses by CRISPR-SeroSeq. We selected a subset of the *Salmonella*-positive samples submitted to GPLN from July 2020 to June 2021 to complete CRISPR-SeroSeq. While the other component of the GPLN surveillance study (described above) was focused on breeder samples collected at weeks 16 and 42, this subset included samples across a range of weeks in breeder production. Our

curated sample collection is once per week, shifting 1 day each week to reduce bias from companies who may regularly submit samples on the same day of the week. We divided the samples from each week into quadrants to have a representative data set for each month, using the first sample in each quadrant. The number of sample collection days differs per month, and the sample number is variable (higher sample numbers later in the week than earlier in the week), so our data set is not uniform across the months but contains at least one sampling day per month. The overnight TT enrichment cultures were briefly vortexed, and 1 mL of each was transferred into microcentrifuge tubes and centrifuged at 5,000 rpm for 10 min. The supernatant was removed, and the pellets were stored at −20°C. Genomic DNA was isolated from pellets using a Promega Genome Wizard kit (Madison, WI) according to the manufacturer's instructions and then resuspended in 200 μL of molecular-grade water and stored at −20°C. Genomic DNA was diluted 10-fold in molecular-grade water, and 2 μL was used as a template in the first PCR step for CRISPR-SeroSeq with primers targeting the conserved direct repeat sequences within *Salmonella* CRISPR arrays (23). PCR products were purified using the Ampure system (Beckman Coulter, Indianapolis, IN) according to the manufacturer's instructions. For the second PCR to add dual index sequences, 5 μL of the cleaned amplicon was used as a template, following the Illumina Nextera protocol (Illumina, San Diego, CA). PCR products were purified using Ampure and pooled in approximate equimolar ratios. Pooled libraries were multiplexed and sequenced on the Illumina NextSeq platform with 150 cycles, single-end reads. Each sequencing run contained two negative-control samples: a nontemplate water control from the first PCR and a nontemplate water control from the second PCR. A positive control containing *Salmonella* serovar Enteritidis genomic DNA with a known CRISPR profile was also included on each run. CRISPR-SeroSeq analyses were performed using a R script that scans sequence reads and uses BLAST to match sequence reads to a database of over 135 serovars, before writing the output directly to Excel. Serovars were called only if they contained multiple CRISPR spacers that were unique to that serovar.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

ACKNOWLEDGMENTS

This study was funded by a USDA-NIFA award to N.W.S. (2020-67017-30792).

We thank grateful Charles Hofacre and members of the Shariat Lab for providing a critical review and Christopher Noble, Colleen Sedney, and Amber Richards for their technical contributions.

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