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Trends In Antimicrobial Resistant Non-Typhoidal Salmonella Before And After The Food And Drug Administration's Guidance For Industry #213, United States 2013-2020

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Trends in antimicrobial resistant non-Typhoidal *Salmonella* before and after the Food and Drug Administration's Guidance For Industry #213, United States 2013-2020

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Abstract

Background*:* Previous research has shown a potential link between antibiotic use among food-producing animals and antimicrobial resistant enteric pathogens. In late 2016, the Food and Drug Administration completed the implementation of its Guidance for Industry #213, through which growth promotion was removed as an indication for use of medically-important antimicrobials among food-producing animals. This study aimed to describe trends in antimicrobial resistance among human *Salmonella* isolates from 2013-2020 in the United States before and after the implementation of this regulation.

Methods: All non-Typhoidal *Salmonella* isolates sent to the National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS) from 2013-2020 were included in this study (n=16,183). Thirteen antibiotics were tested by NARMS for the years included in this study. The Pearson's chi-squared test and the Fisher's exact test were used to evaluate differences in proportion of resistant isolates for thirteen antibiotics. Median-unbiased odds ratios were calculated for the change in proportion of isolates resistant to different antibiotic classes. Trends in resistance were assessed using the Cochran-Armitage trend test.

Results: A significant decrease was observed in the proportion of isolates resistant to ampicillin ($p<0.001$) and streptomycin ($p<0.001$), while an increase was observed for chloramphenicol ($p=0.002$), nalidixic acid ($p=0.001$) and SMX-TMP ($p<0.001$). Isolates from 2017-2020 had a lower likelihood of being resistant to at least one aminoglycoside (OR=0.69, 95% CI 0.62-0.76), penicillin (OR=0.80, 95% CI 0.72-0.88), or sulfonamide (OR=0.87, 95% CI 0.79-0.96) antibiotic, though a higher likelihood of being resistant to chloramphenicol (OR=1.28, 95% CI 1.09-1.50) or at least one fluoroquinolone (OR=1.97, 95% CI 1.72-2.27). Significant ($p<0.05$) decreasing trends over 2013-2020 were observed for aminoglycosides, penicilins, and

sulfonamides, while there was an increase in resistance over time to amphenicols, fluoroquinolones, and macrolides.

Conclusion: Though likelihood of resistance to some antibiotics has decreased since the implementation of GFI #213, there are still concerning trends in antibiotic resistance among *Salmonella.* As more data become available from isolates after implementation, further research is needed to better understand the impact of these regulatory changes.

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Lastly, I want to acknowledge the many people working to combat the growing threat of antimicrobial resistance, whether through the development of next-generation antibiotics, advocacy for better regulations, or any other work.

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Introduction

Non-Typhoidal *Salmonella enterica* infections are one of the leading causes of foodborne illness in the United States, causing an estimated 1.35 million infections per year, resulting in thousands of hospitalizations and hundreds of deaths[1]. While many patients recover from *Salmonella* infections without needing treatment, antibiotics are often prescribed for patients at higher risk of complications or experiencing severe outcomes. As is the case for many other pathogens, antimicrobial-resistant (AMR) *Salmonella* infections have been increasing in frequency during recent decades. Surveillance data has shown a 40% increase in annual incidence of non-Typhoidal Salmonella infections with clinically important antibiotic resistance during the 2015-2016 period, versus the period between 2004 and 2008[2]. Compared to antibiotic-susceptible infections, AMR *Salmonella* infections have been associated with worsened clinical outcomes (e.g., higher rates of hospitalization and longer hospital stays), higher rates of complications (e.g., bacteremia), and millions of additional dollars in medical expenditures[3]. Additionally, antimicrobial resistance among *Salmonella* poses a broader public health threat through the potential for horizontal transfer of antimicrobial resistance genes, whereby genetic elements coding for AMR are directly transferred from Salmonella to other bacterial organisms in the environment.

Food-producing animals are a primary source of non-Typhoidal *Salmonella* infections in humans[1]. Historically, the majority of medically important antibiotics in the U.S. have been sold for use in animal agriculture (rather than use in humans), both for therapeutic and non-therapeutic reasons[4]. Antibiotics are given to food-producing animals to treat diagnosed or suspected infection, or to prevent infection in high-risk settings. Prior to 2017, antibiotics could also legally be used for growth promotion, in which antibiotics are given over a long period of

time at sub-therapeutic doses to increase body mass of food-producing animals. For multiple reasons, agricultural usage of antibiotics can promote the development of AMR. Due to the nature of administering medication to food-producing animals (typically through feed or water), far more animals than necessary are exposed to the compound. Functionally, this produces the same effects as intentionally giving animals unwarranted antibiotics, an action that is inconsistent with good antibiotic stewardship. Growth promotion and some prophylactic use of antibiotics among food-producing animals include long-term, low-dose courses that exert selective pressure on bacterial populations in a manner that promotes resistance[5]. There is substantial overlap between the antibiotic classes used in food-producing animals and those used to treat human infections, though the exact compounds used in human medicine often vary from those used in animal agriculture. The FDA reports that prior to 2017, substantial amounts of medically-important antimicrobial compounds (antibiotics classified by the FDA as important for human therapy) were sold for both production and non-production uses in animal agriculture[6]. Tetracyclines (chlortetracycline, oxytetracycline, and tetracycline) by far compromised the greatest proportion of antibiotic sales, followed by penicillins (amoxicillin, ampicillin, cloxacillin, and penicillin), macrolides (gamithromycin, tildipirosin, tilmicosin, tulathromycin, tylosin, and tylvalosin), and aminoglycosides (dihydrostreptomycin, gentamicin, neomycin, spectinomycin).

Data have shown that a relationship between agricultural antibiotic use and antibiotic resistance among *Salmonella* is likely. Compared to reduced-antibiotic poultry meats, conventional poultry meats are more likely to harbor drug-resistant *Salmonella*[7]. Multiple pathogens, including *Salmonella*, have been shown to exhibit increased resistance to ampicillin, tetracycline, and nalidixic acid (antibiotics used to treat human infections) after being exposed to

sub-inhibitory concentrations of tilmicosin and florfenicol, two antibiotics commonly used in animal feed[8]. In addition to transmission directly through food, antibiotics used in agriculture have runoff effects for the surrounding environment. For example, higher concentrations of AMR *Salmonella*, with particularly high levels of sulfamethoxazole-resistant organisms, have been noted in the environment surrounding pig-rearing facilities[9]. Not all AMR in *Salmonella* can be attributed to food-producing animals; the diversity of resistance genes and patterns is greater among human bacterial isolates than with isolates obtained from retail meats and food-producing animals, suggesting alternate sources of resistance[10]. Even so, use of antibiotics in agriculture appears to influence the prevalence of antibiotic resistance among human infections, though the extent to which this occurs is controversial. A 2017 meta-analysis commissioned by the World Health Organization revealed that interventions (e.g., externally-imposed restrictions or voluntary reductions) restricting antimicrobial use in animal agriculture reduced prevalence of antibiotic resistance among bacterial pathogens isolated from food-producing animals by ~10-15% and from humans by ~24%[11].

In response to these concerns, the federal government has taken action to address the use of medically important antimicrobials in animal agriculture. The bulk of these changes began with Guidance for Industry #209 (GFI #209), issued by the Food and Drug Administration (FDA) in April 2012. The document outlined the FDA's concerns regarding antibiotic use in agriculture, as well as action that should be taken to mitigate the issues[12]. Guidance for Industry #213 (GFI #213) built upon GFI #209 by providing a timeline with specific steps to reach the goals outlined in GFI #209. The key action from this plan was to work with drug sponsors of medically important antimicrobials used in food-producing animals to shift these drugs to Veterinary Feed Directive (VFD) or prescription-only, and to remove growth promotion as an

indication for use[13]. Additionally, in 2015, while GFI #213 was in the process of being implemented, the FDA made changes to their VFD rule to require that VFDs only be given in the context of a legitimate veterinarian-patient-client relationship, further promoting judicious use of antibiotics[14]. In January 2017, the FDA announced that the implementation of GFI #213 had been completed in late 2016, effectively prohibiting the use of antibiotics for growth promotion purposes.

These regulatory changes appear to have had some effect on the agricultural use of antibiotics. According to the FDA, there was a substantial decrease (for all drug classes except for aminoglycosides) in the quantities of medically-important antibiotics sold for veterinary use in food-producing animals use beginning in 2017 compared to 2016[6]. Sales of all antibiotic classes decreased from 2016 to 2017 except for fluoroquinolones (sales of which continued to rise until 2019) and lincosamides (sales of which increased from 142,458 kg^2 in 2016 to 152,497 kg^2 in 2017, then briefly dropped to 125,514 kg^2 before increasing again). Most notably, sales of tetracyclines decreased from 5,861,118 kg^2 in 2016 to 3,535,701 kg^2 in 2017. Overall, sales of all medically-important antibiotics decreased from 8,356,340 kg^2 in 2016 to 5,559,215 in 2017. Furthermore, additional studies have evaluated the impact of these regulatory changes on farming practices. A survey of poultry farmers revealed a substantial reduction in antibiotic use in chicken hatcheries from 2013 to 2017[15]. Cattle farmers in Ohio interviewed after the implementation of GFI #213 largely stated that their antibiotic use had decreased as a result of the new regulations[16]. While it is unclear to what extent widespread changes in antibiotic usage during this time period can be attributed directly (e.g., through revocation of approval for certain antibiotic indications) or indirectly (e.g., through changing common perception of antibiotic use among the agricultural industry) to these federal regulatory changes, they do appear to have had

a substantial impact on farming practices. Nevertheless, concerns have been raised that these regulations are insufficient. There is great overlap between antimicrobials used for disease prevention and those used for growth promotion among food-producing animals; agricultural producers may therefore use antimicrobials for growth promotion under the guise of disease prevention, with no beneficial change in how the drug is used[17]. Additionally, these regulations do not address the unsanitary conditions in animal rearing facilities (e.g., overcrowding) that often underly the need for prophylactic antibiotics.

The National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS), a collaborative effort between the Centers for Disease Control (CDC), Food and Drug Administration (FDA), and United States Department of Agriculture (USDA) performs surveillance of AMR among enteric pathogens isolated around the country. Thus far, there has been limited research concerning the impact of GFI #213 on the prevalence of antimicrobial resistance within foodborne pathogens, particularly for human infections. The objective of this analysis is to describe trends in antimicrobial resistance of *Salmonella* infections in the U.S. from 2013-2020 and examine whether these trends have changed since the implementation of GFI #213 in late 2016. This information is important for assessing our progress in one aspect of turning the tide for antimicrobial resistance, both for *Salmonella* and more broadly.

Methods

Data Source

Human non-Typhoidal *Salmonella* isolate data were obtained from NARMS. State public health laboratories nationwide perform initial testing on clinical samples associated with human infections, and each twentieth isolate of *Salmonella* isolates are sent to the CDC for further testing as part of the NARMS program[18]. When isolates are selected, state officials enter metadata for each isolate through a CDC NARMS Database interface. From 2013 to 2020, the years included in this study, antibiotic susceptibility testing of *Salmonella* isolates was consistently performed by NARMS for the following antimicrobial agents[19]:

- Aminoglycosides: gentamicin and streptomycin
- Amphenicols: chloramphenicol
- Cephalosporins: cefoxitin and ceftriaxone
- Fluoroquinolones: ciprofloxacin and nalidixic acid
- Macrolides: azithromycin
- Penicillins: ampicillin and amoxicillin-clavulanic acid
- Sulfonamides: sulfamethoxazole-trimethoprim (SMX-TMP) and sulfisoxazole
- Tetracyclines: tetracycline

These results are then uploaded to the NARMS database[18]. NARMS categorizes isolates as susceptible, intermediate, or resistant to an antibiotic based on Clinical and Laboratory Standards Institute (CLSI) established breakpoints[19]. While additional antibiotics have been tested by NARMS at some point in the program's history, they have not been consistently tested during the years included in this analysis. In addition to antimicrobial susceptibility testing results, NARMS provides Department of Health and Human Services (DHHS) region, age group of the

patient (0-4, 5-9, 10-19, 20-29, 30-39, 40-49, 50-59, 60-69, 70-79, 80+), and specimen source for the isolate.

All non-typhoidal human *Salmonella* isolates submitted to NARMS from various specimen sources collected from 2013 to 2020 across the U.S. were used in this analysis. Cases of typhoid (caused by *S.* Typhi) and paratyphoid (*S.* Paratyphi A, tartrate negative *S.* Paratyphi B, and *S.* Paratyphi C) fever were excluded, as these infections are most commonly associated with travel to endemic areas. Isolates from 2017 to 2020 were used to assess outcomes after the implementation of GFI #213, which was completed by the beginning of 2017. Data from the previous four-year period were used as a comparison. All information used in this study was de-identified and open-access, thus institutional review board approval was not required.

Data Analysis

Isolates were divided into time categories based on their year of specimen collection as either before (2013-2016) or after (2017-2020) the completed implementation of GFI #213. To maintain consistency, given that no intermediate range of susceptibility has been identified for some antibiotics tested by NARMS, isolates considered to have "intermediate" susceptibility were grouped with susceptible isolates. Isolates were classified as being multidrug-resistant (MDR) if they were resistant to at least one antimicrobial from three or more classes (per CDC definition)[20]. Broader age groups (0-4, 20-59, 60+) were used to account for heightened risk of salmonellosis among children under 5 and severe outcomes in older adults. When grouping isolates into resistance by class, 2020 observations were removed for aminoglycosides, as susceptibility data for one of the two aminoglycoside antibiotics were missing for 2020.

The Pearson's chi-squared test was used to evaluate association between time period and characteristics of isolates (specimen source, DHHS region, and age group). The Bonferroni method was used to calculate adjusted p-values for post-hoc tests. The Pearson's chi-squared test (for all antibiotics except for azithromycin) was used to evaluate change in the proportion of antibiotics resistant to a given antibiotic after 2016. The Fisher's exact test was used in lieu of chi-squared for azithromycin, as very few isolates in this study exhibited azithromycin resistance. For this analysis only, based on the Bonferroni method for multiple comparisons, results with a p-value of under 0.004 (rather than 0.05) were considered significant.

Median unbiased odds ratios were determined for the likelihood of an isolate being resistant to at least one antibiotic in a given class and for the likelihood of an isolate being resistant to both antibiotics in that class (if NARMS tests multiple antibiotics in that class). Annual change in percentage of isolates resistant to each tested antimicrobial agent and class was calculated starting from the 2012-2013 difference to the 2019-2020 difference; the mean annual chance was calculated for the period before and after GFI #213. The Cochran-Armitage trend test was used to assess trends in proportion of resistance for the entire 2013-2020 time period, as well as separately for the 4-year periods before and after the implementation of GFI #213. P-values of under 0.05 were considered significant. All analyses were performed using RStudio Version 1.3.1093.

Results

Characteristics of Isolates

A total of 16,183 isolates were included in this study. Table 1 summarizes the characteristics of these isolates. Significant changes were observed for all three isolate characteristics assessed: specimen source, age group, and DHHS region. The proportion of isolates obtained from stool samples decreased from 85% in 2013-2016 to 82% in 2017-2020, while the percentage of isolates obtained from urine samples increased from 5.5% to 6.4%. The proportion of isolates obtained from blood or other specimen types remained relatively constant between each period. The percentage of isolates from the 60+ year age group increased from 23% to 28%, while the proportion of isolates from the 0-4 year (21% to 19%) and 5-59 year (56% to 52%) age groups decreased. Significant changes were also observed for the proportion of isolates obtained from certain DHHS regions. An increase was observed for Region 9 (8.9% to 14%), while significant decreases were observed for Region 1 (4.2% to 3.3%) and Region 3 (4.2% to 3.3%). The greatest proportions of isolates (during both time periods) were from Regions 4, 5, and 6.

Proportions of Antimicrobial-Resistant Isolates

Of all antibiotics tested, isolates were most likely to be resistant to tetracycline (13% of isolates in 2013-2016 and 12% of isolates in 2017-2020), and least likely to be resistant to azithromycin (fewer than 0.1% of samples in each time period). Changes in proportion of isolates resistant to individual antibiotics were observed for seven of the thirteen antibiotics tested by NARMS (Table 2). Ampicillin resistance decreased from 11% of isolates in the 2013 to 2016 time period to 9.1% in the 2017-2020 period ($p<0.001$) and streptomycin (13% to 9.1%,

p<0.001). Conversely, statistically significant increases were observed for three antibiotics: chloramphenicol (3.8% to 4.8%, p=0.002), nalidixic acid (3.9% to 7.4%, p<0.001), and SMX-TMP (1.7% to 2.6%, p<0.001). Though at the 0.05 significance level there was a change in ceftriaxone (2.9% to 2.4%, p=0.048) and sulfisoxazole (11% to 9.1%, p=0.009), these changes were no longer significant after Bonferroni correction. No significant changes were observed for other antibiotics or for the proportion of isolates resistant to at least one antibiotic.

The likelihood of isolates being resistant to one (OR=0.69, 95% CI 0.62-0.76) or both (OR=0.61, 95% CI 0.45-0.82) aminoglycosides was lower after 2016 (Table 3). While there was a significant decrease in the likelihood of resistance to one or more penicillins (OR=0.80, 95% CI 0.72-0.88) and sulfonamides (OR=0.87, 95% CI 0.79-0.96), the same pattern was not observed for resistance to both antibiotics within each of those classes. Conversely, isolates from 2017-2020 were more likely (OR=1.56, 95% CI 1.26-1.95) to be resistant to both sulfonamides. After 2016, isolates were more likely (OR=1.28, 95% CI 1.09-1.50) to be resistant to chloramphenicol. Similarly, likelihood of resistance to at least one fluoroquinolone increased (OR=1.97, 95% CI 1.72-2.27) for the later time period. There was no statistically significant change in odds of multidrug-resistance after 2016.

Trends in Resistance

2015 saw the highest percentage of isolates with resistance to one or more aminoglycosides, penicillins, sulfonamides, and tetracycline (Figure 1). Fluoroquinolone resistance peaked in 2019. Amphenicol and cephalosporin resistance remained relatively low and stable over time, reaching their maximums in 2019 and 2018, respectively. The mean annual change decreased after 2016 for aminoglycosides, penicillins, and sulfonamides; individually,

the mean decreased for streptomycin, ciprofloxacin, amoxicillin-clavulanic acid, ampicillin, and sulfisoxazole. Conversely, the mean increased for amphenicols (chloramphenicol), cephalosporins, fluoroquinolones, and tetracyclines (tetracycline); there was an individual increase for gentamicin, cefotixin, ceftriaxone, nalidixic acid, and SMX-TMP.

During the overall study period (2013-2020), there was a significant decreasing trend for resistance to aminoglycosides ($p<0.001$), penicilins ($p<0.001$) and sulfonamides ($p=0.001$), while there was an increase in resistance over time to amphenicols ($p=0.009$), fluoroquinolones ($p<0.001$), and macrolides $p=0.009$). There was no significant trend in cephalosporins and tetracyclines over time (Table 5). When split into individual antibiotics, there was some discordance with trends for certain antibiotic classes. A decreasing trend was observed for gentamicin ($p=0.005$), streptomycin ($p<0.001$), cefoxitin ($p=0.029$), ampicillin ($p<0.001$), and sulfisoxazole ($p=0.001$). An increasing trend was observed for ceftriaxone ($p=0.046$), nalidixic acid ($p<0.001$), and SMX-TMP ($p<0.001$). No significant trend was observed for ciprofloxacin, amoxicillin-clavulanic acid, and tetracycline. For some antimicrobials and classes there was discordance between the overall trends and the trends for each four-year period. Table 5 summarizes these results.

Discussion

Substantial changes in the likelihood of *Salmonella* resistance were observed for many antibiotics and classes of antibiotics after the completion of GFI #213's implementation in late 2016. It is unclear, however, to what extent these changes can be attributed to the regulation. Differences in likelihood of resistance between the two four-year periods may be partially attributable to the overall trends during 2013-2020 (which was observed for several antibiotics and classes of antibiotics). This window of time saw increased awareness of the threats associated with antimicrobial resistance and increased urgency towards combatting it, reflected by the CDC publishing its first Antibiotic Resistance Threats Report in 2013. Consumers of animal-based food products have been shown to be conscious of antibiotics when making purchases, which has driven demand for poultry products labeled as organic or raised without antibiotics[6]. Additionally, some changes in agricultural operations likely occurred before implementation was completed in late 2016.

Differences in mean annual change in resistance between the four-year periods, in addition to discordance between overall trends and four-year trends, suggest that for some antibiotics, a decrease or increase in proportion of resistant organisms either began or accelerated after 2016. For three out of seven antibiotic classes, and for six out of thirteen individual antibiotics, the mean annual change in resistance was less during the latter time period (either switched from positive to negative change or decreased by a greater amount per year). Differences in four-year trends before and after (at which time significant trends were only found for a few antibiotics and classes) GFI #213 suggest that changes in antibiotic resistance between years were not consistent throughout the study period. It is therefore possible that there were changes in antibiotic

resistance before and after the implementation of GFI #213 independent of overall trends in the past decade.

Based on the FDA Summary Report, it is clear that sales of medically important antimicrobials decreased substantially from 2016 to 2017. The results of this study suggest that for some antibiotics, these changes may be associated with lower rates of resistance in human *Salmonella* isolates, particularly for penicillins, aminoglycosides, and sulfonamides. However, there was a notable increase in fluoroquinolone and amphenicol resistance, and a lack of change for tetracycline (despite having the greatest volume of sales among antibiotic classes and the greatest drop in sales after 2016) and cephalosporin resistance. For several reasons, the proportion of resistance among human isolates may remain stable or increase despite reduction in agricultural antibiotic use. While some resistance mechanisms that are advantageous in the presence of antibiotics may present a fitness cost to *Salmonella*, this is not the case for all mechanisms; resistance may therefore remain prevalent in the population even with less exposure to antibiotics. Additionally, AMR *Salmonella* may be imported from abroad. In Australia, for example, fluoroquinolones have never been approved for use in food-producing animals, though a substantial proportion of isolates from Australian human *Salmonella* cases from 1979 to 2015 exhibited fluoroquinolone resistance[21]. The authors of this study note that many of the fluoroquinolone-resistant *Salmonella* isolates from Australian cases were associated with international travel.

It is also possible that GFI #213 has not sufficiently reduced agricultural antibiotic use. Further action may be needed to ameliorate antimicrobial resistance among foodborne bacteria associated with food-producing animals. The regulatory changes of GFI #213 may allow for too many “loopholes” as agricultural producers may increase their use of antibiotics for proclaimed

preventive reasons. The 2020 FDA report did in fact show a marked increase in sales of antimicrobials used for therapeutic (i.e. disease prevention and treatment) purposes in 2017, suggesting that producers may be using antibiotics for production purposes under a different label[6]. In 2018 the State of California passed a bill to further regulate preventive use of antibiotics, which can serve as a model for further regulations at the federal level[22].

These results also have implications for the treatment of clinical disease in humans. The Infectious Diseases Society of America recommends azithromycin or fluoroquinolones for patients with suspected bacterial gastroenteritis when certain risk factors are present[23]. While azithromycin resistance among Salmonella remains exceptionally uncommon (seen in less than 0.1% of isolates in this study), azithromycin-resistant Salmonella Newport has been implicated in a multidrug-resistant outbreak, and serves as a reminder of the importance of combatting azithromycin resistance in Salmonella[24]. The increase in the proportion of isolates resistant to fluoroquinolones is concerning, though very few isolates have shown resistance to azithromycin, providing a viable treatment option for many cases in which antibiotics are warranted. Ciprofloxacin is the most commonly used fluoroquinolone antibiotic for Salmonella; fortunately, among these isolates, those determined to be resistant were uncommon. However, this study did not account for intermediate susceptibility, which for ciprofloxacin has been associated with poor treatment outcomes[2]. Third generation cephalosporins (most commonly ceftriaxone) are often used for empiric treatment of severe infections[2]. Compared to most other classes of antibiotics, the proportion of isolates resistant to cephalosporins remained relatively low, there is still some concern posed by the overall increasing trend of ceftriaxone resistance seen in this analysis.

The distribution of specimen sources, age groups, and DHHS region all had significant changes between the two time periods. Though patients who test positive for *Salmonella* at a different site may also harbor the bacteria in their gastrointestinal tract, the site of collection may have some association with whether a patient's infection is related to foodborne transmission. Some gene clusters that code for virulence of *Salmonella* have been found to be strongly associated with resistance phenotypes[25]. Consequentially, it is possible that age may serve as a confounder, given that severe salmonellosis is more likely to occur in those under five years of age and elderly individuals. Additionally, the food intake of young children (and thus their foodborne pathogen exposures) typically differs from that of older individuals. There may be some regional differences (e.g., different population demographics, climate, or local farming practices) associated with the DHHS region for an isolate; however, due to the interconnected nature of the U.S. food system, it is not uncommon for outbreaks of *Salmonella* to affect areas around the country.

This study was substantially limited in that only four years of data were available after the implementation of GFI #213. Analysis of trends and changes in these trends will become more reliable as additional years of data become available. Ideally, with more years of data available, isolates from immediately before and after implementation was completed could be omitted to account for a delay in the impact of regulations on AMR. The dataset used presented many inherent challenges. Availability of specific dates of collection (rather than year of collection) would allow for a better understanding of how, and how quickly, these trends are changing, particularly through modelling approaches. With better availability of whole genome sequencing data (the consistency of which was lacking in this study, particularly for samples from earlier years), it may be possible to link human infection samples to food sources (samples

of which are collected by the FDA for NARMS) and assess specific resistance mechanisms. The samples tested by NARMS may not be entirely representative of all non-Typhoidal *Salmonella* infections in the U.S., as NARMS only tests a subset of reported salmonellosis cases. Additionally, many patients with *Salmonella* infections do not seek medical care (either due to a lack of symptom severity or a lack of access to medical care). Given a potential relationship between virulence and AMR among *Salmonella*, and the fact that those with severe illness are more likely to seek testing and treatment, there may be a higher proportion of AMR organisms among the isolates sent to NARMS compared to the broader population of human *Salmonella* isolates. An additional consideration is the ongoing COVID-19 pandemic, which caused many healthcare services to transition to telehealth; isolates from 2020 may be more likely (compared to other years) to originate from patients ill enough to require in-person hospital care.

As more data becomes available from the years after 2017, further research is needed to better understand the impacts of this regulation and similar changes on the prevalence of antibiotic resistance among human *Salmonella* infections. Similar studies should also be performed with other pathogens (e.g., *Campylobacter, E. coli*) typically associated with food-producing animals to better understand the impact of this regulation on antimicrobial resistance in other foodborne pathogens. Better data concerning use (rather than sales) of specific antimicrobial agents (which are grouped by antibiotic class in the FDA's Summary Report) would allow for further analysis of how antibiotic use correlates with antibiotic resistance among human *Salmonella* infections. Further research concerning this relationship, and how this relationship changes with legal interventions such as GFI #213 is crucial for mitigating a current and worsening public health threat and to lessen morbidity and mortality from antimicrobial-resistant foodborne illness.

Table 1. Characteristics of included *Salmonella* isolates.

The association between time period (either 2013-2016 or 2017-2020) with specimen source, age group, and Department of Health and Human Services (DHHS) region was assessed using Pearson's chi-squared tests. The Bonferroni adjusted p-values are reported for each specimen source, age group, and DHHS region.

Characteristic	Before (N=7,789)[1]	After (N=8,394)[1]	p-value[2]
Specimen Source			
Stool	6,593 (85%)	6,895 (82%)	<0.01
Urine	432 (5.5%)	537 (6.4%)	0.01
Blood	621 (8.0%)	785 (9.4%)	0.18
Other	143 (1.8%)	177 (2.1%)	1.00
Age Group			
0-4	1,667 (21%)	1,623 (19%)	<0.01
5-59	4,358 (56%)	4,396 (52%)	<0.01
60+	1,764 (23%)	2,375 (28%)	<0.01
DHHS Region[3]			
Region 1	331 (4.2%)	276 (3.3%)	0.02
Region 2	705 (9.1%)	723 (8.6%)	1.00
Region 3	901 (12%)	835 (9.9%)	0.02
Region 4	1,360 (17%)	1,344 (16%)	0.27
Region 5	1,259 (17%)	1,347 (16%)	0.34
Region 6	1,313 (17%)	1,476 (18%)	1.00
Region 7	475 (6.1%)	556 (6.6%)	1.00
Region 8	328 (4.2%)	340 (4.1%)	1.00
Region 9	693 (8.9%)	1,186 (14%)	<0.01
Region 10	324 (4.2%)	311 (3.7%)	1.00

[1] N (%)

[2] Bonferroni adjusted post-hoc p-values for Pearson's chi-squared test

[3] Department of Health and Human Services Regions: Region 1 (Connecticut, Maine, Massachusetts, New Hampshire, Rhode Island, and Vermont); Region 2 (New Jersey , New York); Region 3 (Delaware, Maryland, Pennsylvania, Virginia, West Virginia, Washington, D.C); Region 4 (Alabama, Florida, Georgia, Kentucky, Mississippi, North Carolina, South Carolina, and Tennessee); Region 5 (Illinois, Indiana, Michigan, Minnesota, Ohio, and Wisconsin); Region 6 (Arkansas, Louisiana, New Mexico, Oklahoma, and Texas); Region 7 (Iowa, Nebraska, Missouri, and Kansas); Region 8 (Colorado, Montana, North Dakota, South Dakota, Utah, and Wyoming); Region 9 (Arizona, California, Hawaii); Region 10 (Alaska, Idaho, Oregon, and Washington)

Table 2. Number and proportion of *Salmonella* isolates resistant to antimicrobial agents tested by NARMS before and after implementation of GFI #213 from 2013-2020.

Pearson's chi-squared tests were performed to determine changes in the proportion of isolates resistant to each antibiotic before and after the implementation of GFI #213 at the end of 2016. For azithromycin, considering the low number of resistant isolates, the Fisher's exact test was used in lieu of chi-squared. As fourteen different tests performed, results with a p-value of <0.004 (the threshold determined using Bonferroni correction for multiple comparisons) were considered significant after adjustment.

Antibiotic	**Before (N=7,789)**	**After (N=8,394)**	**p-value[1]**
Ampicillin	863 (11%)	762 (9.1%)	<0.001
Amoxicillin-Clavulanic Acid	214 (2.7%)	198 (2.4%)	0.129
Azithromycin	2 (<0.1%)	6 (<0.1%)	0.292
Cefoxitin	206 (2.6%)	191 (2.3%)	0.142
Ceftriaxone	224 (2.9%)	228 (3.4%)	0.048
Chloramphenicol	294 (3.8%)	402 (4.8%)	0.002
Ciprofloxacin	32 (0.4%)	39 (0.5%)	0.691
Gentamicin	128 (1.6%)	108 (1.3%)	0.068
Nalidixic Acid	300 (3.9%)	624 (7.4%)	<0.001
Sulfamethoxazole-Trimethoprim	133 (1.7%)	220 (2.6%)	<0.001
Sulfisoxazole	840 (11%)	800 (9.5%)	0.009
Streptomycin	1,010 (13%)	760 (9.1%)	<0.001
Tetracycline	975 (13%)	1,014 (12%)	0.410
Resistance to 1+ Antibiotic	1,605 (21%)	1,712 (20%)	0.755

Table 3. Resistance to different antibiotic classes before and after implementation of GFI #213

Median-unbiased odds ratios were calculated to determine if isolates from the 2017-2020 time period had a significantly higher likelihood of being resistant to at least one or (where applicable) both antibiotics from a given class when compared to isolates from 2013-2016.

Antibiotic Class	**Resistance to 1+ Agent**	**Resistance to Both Agents**
Aminoglycosides	0.69 (0.62 - 0.76)	0.61 (0.45 - 0.82)
Amphenicols	1.28 (1.09 - 1.50)	N/A
Cephalosporins	1.16 (0.98 – 1.39)	0.88 (0.72 – 1.08)
Fluoroquinolones	1.97 (1.72 – 2.27)	1.30 (0.80 – 2.16)
Macrolides	2.65 (0.59 – 20.06)	N/A
Penicillins	0.80 (0.72 – 0.88)	0.86 (0.70 – 1.04)
Sulfonamides	0.87 (0.79 – 0.96)	1.56 (1.26 – 1.95)
Tetracyclines	0.96 (0.87 – 1.05)	N/A
3+ Classes	0.90 (0.81 – 1.01)	N/A

Table 4. Mean annual change of percentage of resistant isolates by antibiotic and class before and after implementation of GF1 #213

The mean annual change in percentage of isolates resistant to each antibiotic and antibiotic class was calculated for each time period, beginning with the change from 2012 to 2013 and ending with the change from 2019 to 2020.

Antibiotic Class	**Antibiotic**	**Mean Annual Change 2013-2016**	**Mean Annual Change 2017-2020**
Aminoglycosides		+ 0.799	- 0.022
	Gentamicin	- 0.046	+ 0.218
	Streptomycin	+ 0.851	- 0.150
Amphenicols	Chloramphenicol	- 0.228	+ 0.454
Cephalosporins		- 0.023	+ 0.117
	Cefoxitin	+ 0.026	+ 0.052
	Ceftriaxone	- 0.035	+ 0.129
Fluoroquinolones		+ 0.426	+ 0.856
	Ciprofloxacin	+ 0.035	- 0.026
	Nalidixic Acid	+ 0.402	+ 0.856
Macrolides*			
Penicillins		+ 0.229	- 0.350
	Amoxicillin-Clavulanic Acid	- 0.048	- 0.143
	Ampicillin	+ 0.229	- 0.350
Sulfonamides		+ 0.117	- 0.294
	Sulfamethoxazole-Trimethoprim	+ 0.013	+ 0.251
	Sulfisoxazole	+ 0.168	- 0.294
Tetracyclines	Tetracycline	+ 0.074	+ 0.109

*Omitted due to less than 0.1% of isolates having resistance to azithromycin.

Table 5. Trends in antibiotic resistance by antibiotic and class

The Cochran-Armitage trend test was performed to determine the presence of increasing or decreasing trends in proportion of resistant isolates for each antibiotic and antibiotic class. The test was performed for the overall trend (2013-2020) as well as for each time period (2013-2016 and 2017-2020).

Antibiotic Class	**Antibiotic**	**Overall Trend**	**2013-2016 Trend**	**2017-2020 Trend**
Aminoglycosides		Decreasing	No trend	Decreasing
	Gentamicin	Decreasing	Decreasing	No trend
	Streptomycin	Decreasing	No trend	No trend
Amphenicols	Chloramphenicol	Increasing	Decreasing	No trend
Cephalosporins		No trend	No trend	No trend
	Cefoxitin	Decreasing	No trend	No trend
	Ceftriaxone	Increasing	No trend	No trend
Fluoroquinolones		Increasing	Increasing	Increasing
	Ciprofloxacin	No trend	No trend	No trend
	Nalidixic Acid	Increasing	Increasing	Increasing
Macrolides	Azithromycin	Increasing	Increasing	Increasing
Penicillins		Decreasing	No trend	No trend
	Amoxicillin-Clavulanic Acid	No trend	No trend	No trend
	Ampicillin	Decreasing	No trend	No trend
Sulfonamides		Decreasing	No trend	No trend
	Sulfamethoxazole-Trimethoprim	Increasing	No trend	No trend
	Sulfisoxazole	Decreasing	No trend	No trend
Tetracyclines	Tetracycline	No trend	No trend	No trend

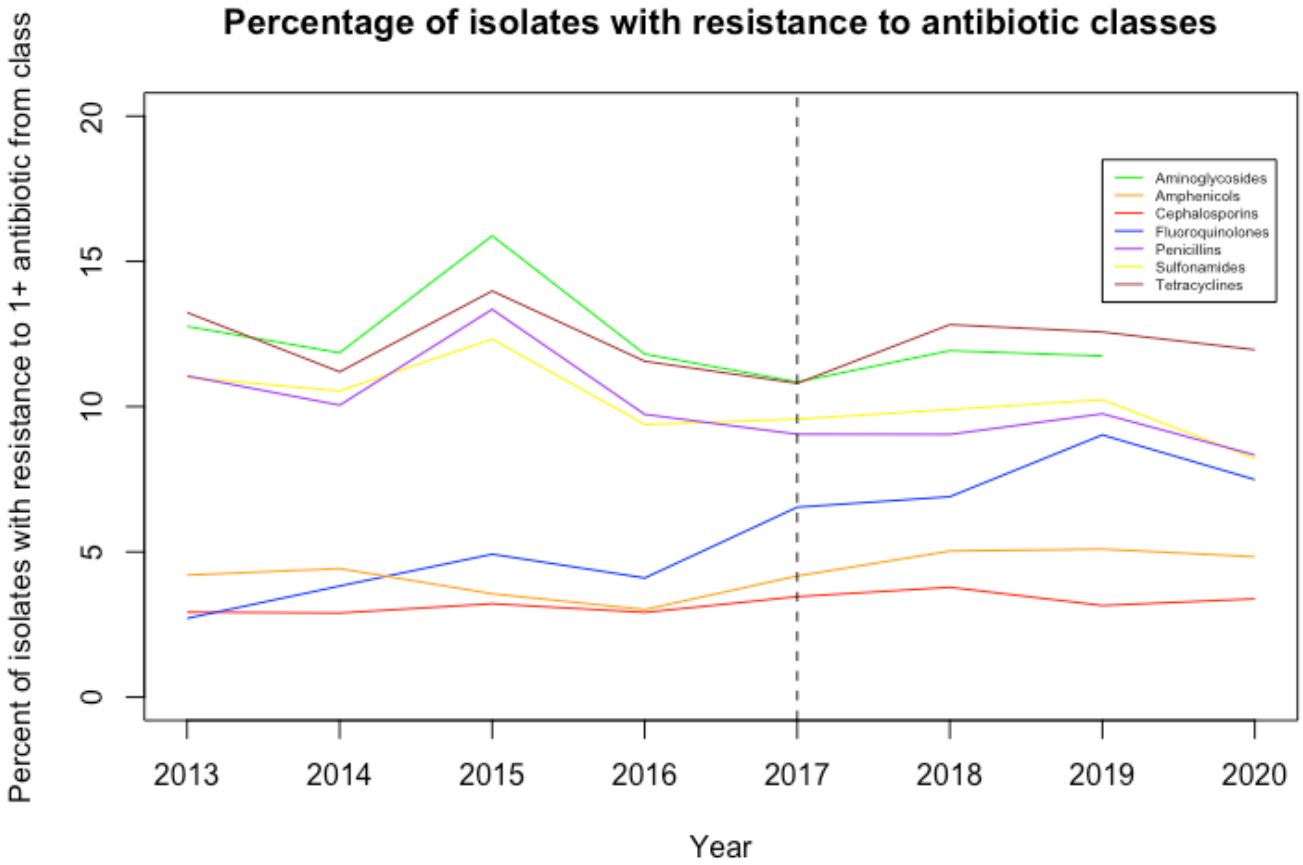

Figure 1. Percentage of isolates with resistance to different antibiotic classes over time. Maximum percentage of isolates with resistance to one or more aminoglycosides (green), penicillins (purple), sulfonamides (yellow), and tetracycline (brown) was seen in 2015. Fluoroquinolone (blue) resistance peaked in 2019. Amphenicol and cephalosporin resistance remained relatively low and stable over time, though reached their maximums in 2019 and 2018, respectively. Macrolide resistance was omitted due to less than 0.1% of isolates being resistant to azithromycin.

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