3rd-Generation Cephalosporin Resistance in Non-Typhoidal *Salmonella enterica* Isolates of Retail Meat from the U.S. National Antimicrobial Resistance Monitoring System (NARMS)

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Abstract

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Abstract

Non-typhoidal Salmonella is estimated to be the leading cause of death and hospitalization among foodborne pathogens in the United States. In 2012, the FDA implemented an Order of Prohibition that prohibited certain extra-label uses of third-generation cephalosporin antibiotics in major food-producing animals (FDA, 2012). The probability of resistance to ceftriaxone in Salmonella enterica isolates in retail meat from the National Antimicrobial Resistance Monitoring System (NARMS) was modeled as a function of time and location using a generalized additive mixed effects model. There was a statistically significant ($p<0.01$) association between ceftriaxone resistance and a fitted smooth function for time, and a decline in the probability of ceftriaxone resistance was observed following the 2012 Order of Prohibition. This observation is consistent with the hypothesis that the Order of Prohibition reduced selection pressure for extended-cephalosporin resistance in Salmonella enterica populations in food animals; however, the decline in resistance was largely attributable to changes in serovar prevalence over time. Subtype analyses of serovars Heidelberg and Typhimurium found a statistically significant ($p<0.05$) association between ceftriaxone resistance and a fitted smooth function for time for the Heidelberg serovar, where a decline in the probability of ceftriaxone resistance was observed following the 2012 Order of Prohibition, but the association between ceftriaxone resistance and time was not significant in serovar Typhimurium. Future analyses should examine genetic data available for later years of the NARMS retail meat dataset to examine whether genetic analysis is consistent with differences in genetic mechanisms of resistance by Salmonella serovar.

Background and Significance

Significance of Antimicrobial-Resistant Non-Typhoidal Salmonella

Foodborne non-typhoidal *Salmonella* infections represent a significant burden of disease in the United States. As of 2015 the Centers for Disease Control and Prevention (CDC) estimated that approximately 1.2 million illnesses and 450 deaths occurred in the United States each year due to infection with non-typhoidal *Salmonella* (CDC, 2015). The United States Foodborne Diseases Active Surveillance Network (FoodNet) reported that non-typhoidal *Salmonella* was the most commonly reported bacterial pathogen among all cases of laboratory-confirmed foodborne illness and the leading cause hospitalization and death from all pathogens from 2000-2008 (FoodNet, 2012). *Salmonella* infections caused the greatest total number of infections (7,719, for an incidence rate of 15.74 per 100,000) hospitalizations (2,104), and laboratory-confirmed deaths (32) of any bacterial pathogen reported in 2015 (FoodNet, 2017). Children under the age of 5 have a higher incidence of infection with *Salmonella* than any other age group (FoodNet, 2012).

Antimicrobial resistance among non-typhoidal *Salmonella* exacerbates the burden of non-typhoidal *Salmonella* infections in the United States. Infection with antibiotic-resistant *Salmonella* is associated with an increased risk of hospitalization, increased risk of invasive infection, and an increased duration of illness (Helms et al., 2006). According to National Antimicrobial Resistance Monitoring Systems (NARMS) surveillance of bacteria isolated from humans, antimicrobial resistance among *Salmonella* infections causing human disease have increased in the United States in recent decades (Crump et al., 2011). In 2013, the Centers for Disease Control and Prevention (CDC) identified drug-resistant non-typhoidal *Salmonella enterica* as one of the top 18 drug-resistant threats to the United States (CDC, 2013).

Antibiotic resistance is particularly significant in cases of invasive non-typhoidal salmonellosis. Up to 5% of individuals infected with non-typhoidal *Salmonella enterica* develop bacteremia, also referred to as invasive non-typhoidal salmonellosis [iNTS], a complication in which bacteria also infect the bloodstream, internal organs and/or joints of the patient (CFSAN, 2012). Ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole have traditionally been used to treat iNTS. However, current treatment recommendations for iNTS indicate fluoroquinolones or extended-spectrum cephalosporins (Crump et al., 2011). For the treatment of iNTS infections in children, cephalosporin antibiotics such as ceftriaxone are recommended over fluoroquinolone antibiotics (Iwamoto et al., 2013). Of the ceftriaxone-resistant *Salmonella enterica* isolates reported by NARMS between 1996-2013, 40% are from children (Iwamoto et al., 2013). As a result, cephalosporin resistance in *Salmonella enterica* represents a significant threat to children's health in the United States.

Third-Generation Cephalosporin Antibiotics: Use and Resistance

Third-generation cephalosporin antibiotics are a class of antibiotics with medical importance in both human and veterinary medicine. Cephalosporins are β-lactam antibiotics that were introduced in 1964 and are widely used in human medicine today (FDA, 2012). Extended-spectrum third-generation cephalosporins, including ceftriaxone, are valuable in treating serious infections in the hospital setting (FDA, 2012). The third-generation cephalosporin ceftiofur is also approved in the United States for animal diseases such as respiratory disease in swine, ruminants and horses and certain infections in cattle (Hornish and Kotarski, 2002).

Resistance to extended-spectrum cephalosporins in *Salmonella enterica* has been associated primarily with acquired genes encoding β-lactamases belonging to molecular classes A (extended-spectrum β-lactamases, or ESBLs) and C (cephalosporinases) (Miriagou et al.,

2004). By 2004, ESBL carriage was reported worldwide in numerous serovars of *Salmonella enterica* known to cause invasive infection in people, including serovars Typhimurium, Enteritidis, Saint Paul and Heidelberg (Miriagou et al., 2004). A study of U.S. *Salmonella enterica* enterica isolates, of animal origin, found extended-spectrum cephalosporin resistance among an extensive list of serovars, including serovars Typhimurium, Heidelberg, and Hadar (Gray et al., 2004).

Cephalosporin Use in Food Animals and Antimicrobial Resistance in Human Salmonellosis

Multiple studies indicate that antibiotic resistance, including resistance to cephalosporins, in *Salmonella enterica* in food animals is associated with foodborne non-typhoidal salmonellosis (Helke et al. 2017; Crump et al., 2011; Dutil at al., 2010; Spika et al., 1987; Angulo et al., 2000; Su et al., 2004). Iwamoto et al., (2013) evaluated the association between resistance to ceftriaxone in *Salmonella enterica* isolates from human infections, retail meat, and food animals in the NARMS data between 1996-2013. This analysis found a statistically significant relationship between the prevalence of ceftriaxone resistance from human isolates and ground beef isolates of the serovar Newport, human and cattle isolates of the serovar Typhimurium; human and chicken isolates of the serovar Heidelberg; and human and turkey isolates of the serovar Heidelberg.

In Canada, Dutil et al (2010) found that the annual incidence of *S.* Heidelberg resistance to ceftiofur in retail chicken was correlated with annual incidence of ceftiofur-resistant *Salmonella enterica* serovar Heidelberg in humans between 2003-2008 at ($r = 0.91$, $p<0.0001$). In addition, ceftiofur resistance in chickens appeared to be related to changes in use of antibiotics in chicken production over time. After a voluntary withdrawal of ceftiofur use in chicken hatcheries in 2005-2006, ceftiofur resistance was observed to decline from 62% in 2004 to 7% in

2006, but increased to 20% following the re-introduction of ceftiofur in 2007 (Dutil et al., 2010). Reports of reduced ceftiofur resistance in poultry following the voluntary withdrawal of ceftiofur in Canada may have been important in the FDA restrictions on cephalosporin use in the United States, and will be discussed further in the following section.

FDA Restriction on Extra-label Cephalosporin Use in Food Animals

In 2012 the U.S. Food and Drug Administration (FDA, 2012) prohibited certain extra-label uses of cephalosporin drugs in major food-producing animals in response to concerns that antibiotic use in food animals was contributing to antimicrobial-resistant infection in humans. These extra-label uses include the use of third-generation cephalosporin antibiotics for disease prevention (FDA, 2014). The restrictions were targeted towards extra-label uses of ceftiofur in poultry, including use as an *in ovo* prophylaxis against *E. coli* infections (Hofacre et al., 2013).

It is not clear to what extent the 2012 restriction on extra-label use of cephalosporin antibiotics affected the use of antibiotics, or when any changes occurred. The FDA first proposed a rule to limit cephalosporin use in food animals in 2008, which banned all extra-label use of cephalosporins in food animals. The rule was withdrawn following criticism that the order of prohibition was too extensive and banned uses of cephalosporin that did not represent a threat to public health. The revised Order of Prohibition implemented in 2012 allowed three changes that were banned by the 2008 Order: extralabel use of cephapirin (a cephalosporin antibiotic licensed in cattle), extralabel use of cephalosporins for unapproved indications, and extralabel use in food-producing minor species, such as rabbits (FDA 2014).

Unfortunately, changes in the in volume or type of cephalosporin use in food animals in the United States by sector are difficult to independently verify. The 2014 FDA Summary Report on Antimicrobials Sold or Distributed for Use in Food-Producing Animals indicated that

there was a 57% increase in cephalosporins sold for use in food-producing animals between 2009 and 2014, and cephalosporins sales increased every year between 2009 and 2014 (FDA, 2015). The FDA summary report does not differentiate by food animals sector, so it is unclear which species the antibiotics sold were used for; and data on cephalosporin use in the United States prior to 2009 is not available.

The restriction on cephalosporin use was the second case in which FDA specifically withdrew a previously approved antibiotic for use in food animals. In 2005, enrofloxacin was prohibited from use in poultry water due to concerns that enrofloxacin use was contributing to fluoroquinolone-resistant Campylobacter infection in humans. The enrofloxacin restriction was labelled a "public health success story" soon after it was enacted (Nelson et al. 2007), but Zawack et al. (2016) detected no statistically significant change in resistance of *Campylobacter jejuni* and *Campylobacter coli* to ciprofloxacin (another second-generation fluoroquinolone) in the NARMS retail meat and cecal sampling datasets after the withdrawal.

The United States has enacted other recent major regulatory changes in antibiotic use in food animals in recent years, such as the "Veterinary Feed Directive" Final Rule. The Veterinary Feed Directive (VFD) Final Rule, published by the FDA in June 2015, extended the use of veterinary feed directives to an increased number of medically important antimicrobials used in food animal production (FDA, 2017). It is important to consider other changes to the regulatory environment, given that cephalosporin resistance among bacterial isolates in the United States could potentially be confounded by changes in antibiotic use affecting multi-drug resistance.

National Antimicrobial Resistance Monitoring System (NARMS)

Surveillance data from the NARMS provides an opportunity to examine large-scale trends in resistance among *Salmonella enterica* isolates over time. NARMS is a partnership between the CDC, the US Food and Drug Administration (FDA), the US Department of Agriculture (USDA), and state and local health departments. NARMS tests and reports antibiotic resistance data on enteric bacteria from human infections, food animals cecal samples, and retail meat isolates. NARMS datasets are publicly available, and annual surveillance reports are published summarizing prevalence trends in antimicrobial resistance across all sectors. Medalla et al. (2013) used NARMS human isolate data to evaluate changes in ceftriaxone resistance among non-typhoidal *Salmonella enterica* isolates between 1996 and 2009, and concluded that the percentage of nontyphoidal *Salmonella enterica* isolates resistant to ceftriaxone increased from 0.2% to 3.4% (odds ratio=20, 95% confidence interval 6.3–64) during this time period.

The 2014 NARMS Integrated Report stated that the proportion of *Salmonella enterica* showing that from 2009 to 2014, ceftriaxone resistance declined from 3.4% to 2.4% in human isolates; from 38% to 18% in retail chicken meat isolates; and from 12.9% to 6% in chicken isolates from sampling at slaughter facilities. The report concluded "Although it is early, it appears that the use restrictions are having a benefit... The decline in ceftriaxone resistance following FDA's targeted prohibitions on extra-label cephalosporin use is similar to what others have observed following reductions in the use of ceftiofur in animal production, implying that the intervention is having the intended effect on some bacteria." (NARMS, 2016).

This statement refers in part to the reported effects of similar restrictions in the use of 3rd-generation cephalosporins in Canada and Norway. In May 2014, the Canadian poultry industry voluntarily eliminated preventive use of 3rd-generation cephalosporins. The Canadian Integrated

Program for Antimicrobial Resistance Surveillance (CIPARS) reports that between 2013 and 2014, and reported that an overall decreasing trend in *Salmonella enterica* resistance to ceftriaxone on the national level was "apparent" based on figures of cephalosporin resistance in *Salmonella enterica* isolates from humans and chicken isolates sampled at the farm, abattoir, and from retail meat over time (CIPAR 2014). In July 2010, the Danish pork industry instituted a voluntary ban on cephalosporin use in pig production. Agerso and Aarestrup (2013) report a statistically significant ($P<0.001$) decrease in the prevalence of extended-spectrum cephalosporinase (ESC)-producing *Escherichia coli* isolates from pigs at slaughter from 2010 (10.8% of isolates) to 2011 (3.7% of isolates). Both examples suggest that cephalosporin restrictions in food animals on the national level may generate reductions in cephalosporin resistance in enteric bacteria from food animals.

Salmonella enterica Serovars

The 2014 NARMS Integrated report emphasized individual differences between *Salmonella enterica* serovars related to ceftriaxone resistance patterns and disease severity. For example, the report described an overall decrease in resistance prevalence in Heidelberg isolates from retail chicken, in which ceftriaxone resistance peaked at 32% (14/44) in 2009, and had declined to 12.5% (3/24) in 2014. In contrast, *Salmonella enterica* serovar Dublin resistance levels were at 60% (6/10) of human isolates and 29% (9/31) of cattle isolates in 2014. (FDA, 2017). Neither *Salmonella enterica* serovars Heidelberg nor Dublin are in the top 5 leading causes of human infection in the United States, both are able to cause invasive disease; a 2008 study (Jones et al.) of *Salmonella enterica* infections reported to FoodNet from 1996-2006 investigated the relative risk of invasive disease, hospitalization and death among different *Salmonella enterica* serovars. The study found 13 serovars that were significantly more likely to

result in invasive disease than serovar Typhimurium, which is the leading cause of disease overall, with a 6% proportion of cases resulting in invasive infection. These serovars included Enteritidis (7% of cases resulting in invasive infection), Heidelberg (13%), Schwarzengrund (15%), Poona (17%), Choleraesuis (57%), and Dublin (64%).

Because cephalosporin antibiotics are recommended in the treatment of invasive *Salmonella enterica* infections, it will be important to examine trends in serovars that present a high risk for invasive disease, such as serovars Heidelberg and Dublin, or are commonly resistant to ceftriaxone. Iwamoto et al. (2013) report that the majority of the ceftriaxone-resistant isolates reported by NARMS surveillance for non-typhoidal salmonellosis in humans were one of three serovars: Newport (40%), Typhimurium (26%), or Heidelberg (12%).

Study Premise

This thesis examines changes in ceftriaxone resistance in *Salmonella enterica* isolates from NARMS retail meat samples from 2002 to 2015 both in aggregate and by serovar, to evaluate the hypothesis that a significant reduction in ceftriaxone resistance prevalence occurred after the implementation of the 2012 restrictions in cephalosporin use.

Ampicillin, tetracycline and chloramphenicol are included as comparison antibiotics to identify changes in ceftriaxone resistance associated with antibiotic regulation and use targeted to 3rd-generation cephalosporins, rather than broad changes in the regulatory environment or emergence of multi-drug resistant strains of *Salmonella*. A restriction on the use of 3rd-generation cephalosporins would be expected to reduce selection pressure on genes encoding resistance to 3rd-generation cephalosporins. This change would be reflected by changes in susceptibility to ceftriaxone in retail meats relative to other classes of medically important antibiotics.

Cephalosporin resistance genes can confer cross-resistance to ampicillin (Edirmanasinghe et al., 2017). Therefore, changes in use of cephalosporin antibiotics are expected to be highly associated with susceptibility to ampicillin. Ceftriaxone resistance does not confer cross-resistance to chloramphenicol and tetracycline, but resistance to ceftriaxone, chloramphenicol and tetracycline may be associated with ceftriaxone resistance on mobile genetic elements and within particular strains. For example *Salmonella enterica* serovar Typhimurium DT104 is a phage type of *Salmonella* that typically possesses resistance to ampicillin, chloramphenicol, streptomycin, sulfonamide, and tetracycline. *Salmonella enterica* serovar Typhimurium DT104 spread rapidly throughout the globe in both human and animal populations in the 1990's, and continues to be an important foodborne pathogen in the United States and worldwide (Leekitcharoenphon 2016).

Ceftiofur continues to be approved for therapeutic uses in cattle, pigs and poultry. Ampicillin is approved for therapeutic use in cattle. Tetracyclines (chlortetracyclines and oxytetracycline) were approved for growth promotion and prophylaxis in 2015, although both are now regulated under the Veterinary Feed Directive 2015 Final Rule. Chloramphenicol is no longer approved for use in animal populations, but the veterinary analog florfenicol is approved as a therapeutic in cattle and pigs (Subbiah et al. 2016).

Methods

Data Source

The data for this investigation was downloaded from the FDA NARMS public data web page (FDA, 2017) in October of 2015. The data consists of resistance profiles for bacterial isolates from the NARMS retail meat-sampling program. Retail meat samples - defined as a single retail chicken part, pork chop, or 25g aliquot of ground beef or turkey product - are

collected from a randomized list of grocery stores and sent from 14 participating state public health departments to FDA laboratories for processing. *Salmonella enterica* isolates are cultured by FoodNet sites on blood agar or MacConkey plates and sent to FDA laboratories to be identified by Vitek 2 Compact or other methods, classified by serovar according to the Kauffman-White scheme, and tested for resistance to a panel of antibiotics using minimum inhibitory concentration (MIC) according to Clinical & Laboratory Standards Institute (CLSI) guidelines. Detailed information on sample analysis and resistance testing can be found on the FDA NARMS Methods web page (FDA, 2016), while detailed information on the laboratory methods used to isolate bacteria and test for antibiotic resistance can be found in the NARMS Manual of Laboratory Methods (FDA, 2015).

Data containing antibiotic MICs for each sample are publicly available for multiple years for bacterial isolates from *Salmonella enterica* from retail meat from poultry, cattle and swine. This data includes serovar of bacteria, host species, MIC breakpoints for multiple antibiotics, and the month in which each sample was received by NARMS laboratories. This analysis relies on a version of the dataset downloaded from the publicly available datasets on the FDA website in 2016, in which interpretations based on CLSI breakpoints are presented in the dataset. Meat types represented in the dataset include ground beef, pork chops, ground turkey, and chicken breast.

Data Analysis

A generalized additive mixed-effects model was used to describe the prevalence of antibiotic resistance to cephalosporin drugs in Salmonella isolated from retail meat in the United States between the years of 2002 and 2015. The response variable presented here is the predicted probability of resistance to a given antibiotic controlling for time. The U.S. state from

which each isolate was obtained was included as a random effect. This approach will supplement NARMS analyses of ceftriaxone resistance over time by enables an estimate of statistical significance of changes in resistance over time, while controlling the possible confounding effect of location from which *Salmonella* isolates are obtained. The generalized additive model uses thin-plate splines to generate predictions of resistance which do not assume a simple functional form over all points in time, but rather allows the relationship between resistance and time to be fit for different regions of time in the data, and models correlation between timepoints. This model was developed using the mgcv package in R.

Prevalence of resistance is defined as the proportion of Salmonella isolates for which the minimum inhibitory concentration (MIC) of antibiotic is sufficient to categorize the isolate as "Resistant" or "Intermediate" rather than "Susceptible" as defined by the most recent NARMS guidelines for Salmonella testing, which are based on CLSI guidelines (NARMS, 2015).

Results

The NARMS retail meat dataset contains resistance phenotypes for 4,209 *Salmonella enterica* collected between January 2002 and June 2015. The total number of isolates per year varies by state (Table 1), and certain states contributed only a limited number of isolates in 2013 or later, such as Washington State (n=30). Table 2 displays the number of isolates by serovar; Typhimurium (n=775) and Heidelberg (n=682) are most frequently identified. Typhimurium is ranked as the second most frequent cause of *Salmonella enterica* infection in the United States from 2010-2014 by the FoodNet Annual Report 2015, while Heidelberg was ranked 6th.

Figure 1 displays the percentage of *Salmonella enterica* isolates from retail meat per year from 2002-2015 that are resistant to ceftriaxone, ampicillin, and chloramphenicol. Points represent percentages of resistant isolates per year. A smooth fit and 95% confidence interval is

indicated in red on each figure. The percentage of isolates resistant to ceftriaxone per year, including all states and serovars begins at 10% (n=153) in 2002, increases with some fluctuation over time and reaches a peak of 24% (n=487) in 2009, then declines to 4% (n=114) in 2015. However, the data in 2015 is incomplete and consists of January-June; excluding 2015, the resistance declines to 14% in 2014 (n=262). Table 3 presents the percentage of ceftriaxone-resistant isolates by serovar. Of the 10 most common serovars, *Salmonella enterica* serovar Typhimurium is the serovar with the highest percentage of isolates resistant to ceftriaxone across all years (48.8%), followed by *Salmonella enterica* serovar Kentucky (20.2%) and *Salmonella enterica* serovar Heidelberg (11.7%). The "All Other" category of serovars (12.4%) included serovars such as *Salmonella enterica* serovar Dublin, which had a high percentage of ceftriaxone resistance (80%) but low isolate numbers overall (n = 20).

As illustrated in Figure 1, the percentage of isolates resistant to ampicillin per year, including all states and serovars begins at 18% in 2002 (n=153), increases with some fluctuation over time and reaches a peak of 49% (n=487) in 2009, then declines to 23% in 2014 (n=262). The percentage of isolates resistant to tetracycline per year, including all states and serovars, begins at 46% in 2002 (n=153), increases with some fluctuation over time and reaches a peak of 70% (n=357) in 2009, then declines to 53% in 2014 (n=262). The percentage of isolates per year resistant to chloramphenicol remains below 8% over all years.

A generalized additive model with a response link, controlling for random effects of state, was applied to the outcome of resistance across the time period 2002-2015. Time (measured by the date of acquisition of the retail meat sample) was found to be a significant predictor of the predicted probability of resistance to a given antibiotic at $p<.01$ for all four antibiotics.

The predicted probability of resistance over time for each antibiotic is presented in Figures 2, 3, 4, and 5. When controlling for the effects of location, the predicted probability of resistance to ceftriaxone, ampicillin and tetracycline increased from 2007 to 2011, and declined from 2011 to 2014, with non-overlapping confidence intervals for all years except for the probability of resistance to tetracycline between 2007 and 2011. The predicted probability of chloramphenicol resistance does not change across the study period. The probability of resistance in 2007, 2011 and 2014 to all antibiotics across serotypes is presented in Table 5.

However, when the dataset is restricted to *Salmonella* isolates with 200 or more observations in the dataset (Typhimurium, Heidelberg, Kentucky, Enteritidis, Hadar, and Saintpaul, accounting for approximately 70% of isolates in the retail meat dataset overall) and *Salmonella* serovar is introduced as a random effect in this dataset, it is significant at $p < 0.01$ as a predictor of resistance of all four antibiotics. Time is no longer a statistically significant predictor of resistance at $p<0.01$ when *Salmonella* serovar is included in the model, indicating that changes in predicted probability of resistance over time may be attributed to changing serovar prevalence over time. Individual subtype analyses of serovars Typhimurium and Heidelberg are presented below.

There are a total of 682 Heidelberg isolates in the database across all years and states. There is no clear difference in prevalence of serovar Heidelberg over time relative to other common serotypes (Figure 10), but the prevalence of ceftriaxone resistance in serovar Heidelberg did appear to vary over time to a greater degree than serovar Typhimurium or other serovars (Figure 11). A generalized additive model, controlling for random effects for state, demonstrated that the time of collection of an isolate was significantly associated with the predicted probability of resistance to a given antibiotic at $P<.05$ to ceftriaxone and ampicillin,

but there was not a significant association between time and resistance to tetracycline or chloramphenicol (Figure 10). The predicted probability of resistance to ceftriaxone and ampicillin in 2007, 2011 and 2014 are presented in Table 5. The predicted probability of resistance to both ceftriaxone and ampicillin is observed to increase between 2007 and 2011 and decline between 2011 and 2014 with non-overlapping confidence intervals for all years except for ceftriaxone resistance between 2007 and 2011. Trends in predicted probability of resistance over time are illustrated in Figures 7 and 8.

There are a total of 736 Serovar Typhimurium isolates in the database across all years and states. *Salmonella enterica* serovar Typhimurium resistance to ceftriaxone, ampicillin, tetracycline and chloramphenicol across all years is presented in Figure 9. A generalized additive model, controlling for random effects for state, was applied to generate the predicted probability for resistance in *Salmonella enterica* serovar Typhimurium isolates as a function of time. Time is not a significant predictor of the predicted probability of resistance to a given antibiotic at $p<.05$ for ceftriaxone or ampicillin, although it was a significant predictor of tetracycline and chloramphenicol resistance. Figure 10 presents the percentage of *Salmonella enterica* serovar Typhimurium isolates per year relative to other serovars. *Salmonella enterica* serovar Typhimurium was higher in 2012 (30% of all isolates) than in either 2008 (15%) or 2014 (17%).

Discussion

The predicted probability of ceftriaxone resistance among *Salmonella enterica* isolates in NARMS retail meat declined following the introduction of the 2012 Order of Prohibition restricting cephalosporin use in food animals from 0.19 (95%CI 0.15,0.22) in 2011 to 0.11 (95%CI: 0.09,0.13) in 2014 in a model controlling for effects of state. A decline in predicted

probability to ampicillin and tetracycline was also observed between 2011 and 2014 with non-overlapping confidence intervals between those years. Chloramphenicol was not observed to decline. These observations suggest that the 2012 Order of Prohibition on certain extra-label uses of cephalosporin antibiotics in food animals was associated with a decline in *Salmonella enterica* resistance to extended-spectrum cephalosporins and other antibiotics linked to ceftriaxone resistance by cross-resistance (ampicillin) and mobile genetic elements (tetracycline).

This trend initially appears to be consistent with the hypothesis that the Order of Prohibition on extra-label cephalosporin use reduced selection pressure for cephalosporin resistance among *Salmonella enterica* in food animals, and led to a corresponding decline in the prevalence of extended-spectrum cephalosporin resistance among *Salmonella enterica* isolates in retail meat. However, there are several important caveats. The *Salmonella enterica* serovar of isolates was found to be significantly ($p<0.01$) associated with the prevalence of ceftriaxone resistance in *Salmonella enterica* isolates in retail meat. When the dataset is restricted to serovars with more than 200 isolates across all years and serovar was introduced as a random effect to the GAM model of predicted probability of ceftriaxone resistance over time, the isolates' date of collection (the time metric) is no longer significantly associated with resistance to ceftriaxone at $p<0.01$.

In other words, the variation in resistance to ceftriaxone over time in the entire dataset of *Salmonella enterica* retail meat isolates is driven by changes in serovar prevalence in the dataset. *Salmonella enterica* isolates in the NARMS retail meat database are observed to vary widely by serovar. For example, *Salmonella enterica* serovar Typhimurium is approximately 48.8% resistant to ceftriaxone across all years, while other common serovars are less than 20% resistant (Table 3). The prevalence of *Salmonella enterica* serovar Typhimurium is high relative to other

isolates between 2009 and 2012, then declines to below other common serotypes by the end of the study period (Figure 10). When the analysis is restricted to *Salmonella enterica* serovar Typhimurium isolates (including Typhimurium var 5-), ceftriaxone and ampicillin resistance in *Salmonella enterica* serovar Typhimurium are not significantly associated with time at $p<0.05$ after including random effects for the location (US state) in which the isolate was collected. These observations support the hypothesis that changes in ceftriaxone resistance observed after the 2012 restriction were a result of changes in prevalence of *Salmonella enterica* serovar.

It is possible that decreased selection pressure to cephalosporin antibiotics led to a decrease in the relative fitness of highly resistant serotypes such as *Salmonella enterica* serovar Typhimurium, generating a decline in prevalence of those serovars. This explanation, however, requires two major assumptions: that genes carrying resistance to ceftriaxone have a deleterious effect on *Salmonella enterica* fitness, and that the changes in serotype prevalence observed in the NARMS dataset cannot be attributed to random fluctuation. Neither of these assumptions are met for *Salmonella enterica* in the NARMS retail meat dataset. Antibiotic resistance typically confers a fitness cost in *Salmonella* bacteria, but studies of *Salmonella enterica* serovar Typhimurium indicate that *Salmonella* has evolved compensatory mechanisms to reduce the fitness cost of resistance to beta –lactam antibiotics, such as cephalosporins (Zhang et al. 2006).

Studies by Gupta et al. (2003) in ceftiofur resistance in serovar Newport in the NARMS dataset, and Molbak et al (1998) in serotype Typhimurium demonstrate that large-scale changes in the prevalence of *Salmonella* resistance to cephalosporin antibiotics across geographic regions can be affected by the prevalence of specific *Salmonella* strains. Gupta et al. (2003) found that between 1998 and 2001, the prevalence of resistance to ceftriaxone in the national NARMS dataset of human *Salmonella* infections increased from .05% to 2.4%. This time period

corresponded to the emergence of a strain of *Salmonella enterica* serovar Newport called MDRAmpC in both humans and cattle. In 1998, *Salmonella* isolates were primarily serovar Typhimurium, but in 2001, 85% of isolates were the Newport-MDRAmpC strains. The strain was resistant to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, tetracycline, amoxicillin/clavulanic acid, cephalothin, cefoxitin, and ceftiofur, and displayed decreased susceptibility to ceftriaxone. The authors conclude that the increase in ceftriaxone resistance prevalence in *Salmonella* isolates recorded nationwide "largely driven" by the growth of Newport-MDRAmpC.

In a foodborne outbreak *Salmonella enterica* serovar Typhimurium in Denmark, a strain of quinolone-resistant *Salmonella enterica* serovar Typhimurium appeared in five human patients and was traced to two herds of swine. There was no evidence of recent quinolone use in either herd and the authors state that selection pressure from quinolone antibiotics was unlikely, indicating that resistant strains of *Salmonella* may emerge in food animal populations in the absence of immediate selection pressure for the relevant antibiotic (Molbak et al, 1999). In sum, it is feasible that fluctuations in drug-resistant strains of *Salmonella enterica* in the dataset generated a decline in ceftriaxone resistance after 2012 that was unrelated to changing selection pressures by ceftriaxone antibiotics.

While the decline in prevalence of ceftriaxone resistance among *Salmonella enterica* in the retail meat dataset overall is not clearly associated with the 2012 Order of Prohibition, there may have been serovar-specific trends. A subtype analysis of *Salmonella enterica* serovar Heidelberg found a statistically significant (<0.05) change in resistance to ceftriaxone and ampicillin. The probability of resistance to both antibiotics declined between 2011 and 2014 with non-overlapping confidence intervals between those years (Table 5). No statistically

significant ($p<0.05$) change over time in the resistance to tetracycline or chloramphenicol was observed in serovar Heidelberg. This is consistent with the hypothesis that a change in selection pressure occurred in or near 2012 that specifically affected genes conferring resistance to cephalosporin antibiotics and antibiotics that are related to cephalosporin resistance by cross-resistance, such as ampicillin.

It is not clear why *Salmonella enterica* serovar Heidelberg would be particularly susceptible to changes in cephalosporin use relative to serovar Typhimurium. Both isolates are predominantly found in poultry in the NARMS retail meat dataset (Table 4), although Typhimurium was isolated almost exclusively from turkey and Heidelberg was isolated in both chicken and turkey isolates. Prior evidence that *Salmonella enterica* has evolved compensatory mechanisms to balance the fitness costs of resistance genes focused on serovar Typhimurium (Zhang et al. 2006), while genes encoding resistance to cephalosporin antibiotics in serovar Heidelberg may impose greater fitness costs and be more significantly deleterious in the absence of selection pressure from antibiotic use.

The lack of data regarding isolate genotypes in the NARMS retail meat dataset of *Salmonella enterica* isolates for the majority of the study period is a considerable limitation to this analysis. It is possible that a significant proportion of serovar Typhimurium isolates that were identified to carry resistance to ceftriaxone were multi-drug resistant strains of Typhimurium, such as DT104. In a multi-drug resistant strain such as DT104, it would be difficult to isolate an association between resistance to a single antibiotic and changes in use of that antibiotic, especially where other changes in antibiotic use are not known. Further research would be necessary to examine whether resistance to ceftriaxone in *Salmonella enterica* serovar

Typhimurium is the NARMS retail meat dataset is primarily mediated through multi-drug resistant strain types.

An important additional limitation to this analysis is that it is not clear whether and to what extent the 2012 Order of Prohibition was associated with changes in 3rd-generation cephalosporin use in food animals in or near 2012. The Order of Prohibition on extended-spectrum cephalosporin use was first introduced in 2008, and was withdrawn following criticism of the extent of the restrictions (FDA 2014). It has been suggested that there was a decline in 3rd-generation cephalosporin use in the poultry industry from the time the Order of Prohibition was first introduced in 2008 (Schmidt, 2012). Both *Salmonella enterica* serovar Heidelberg and *Salmonella enterica* serovar Typhimurium were most frequently isolated from poultry (Table 4), may have been affected by any early changes in antibiotic use in the poultry industry. It is interesting to note that the probability of resistance to ceftriaxone in serovar Heidelberg peaked at 0.2 (95%CI: 0.16,0.24) in 2010 and declined for the rest of the study period. This trend is consistent with changes in ceftriaxone use prior to the implementation of the Order of Prohibition in 2012. The uncertainty in this analysis regarding when changes in antibiotic use in food animals would be expected in response to the 2012 Order of Prohibition highlights the need for data on antibiotic use in agriculture to fully evaluate the impact of policy and antibiotic use changes.

Conclusion

3rd-generation cephalosporins are recommended for the treatment of invasive *Salmonella enterica* infections, particularly in pediatric cases (Iwamoto et al, 2013). It is important to evaluate the efficacy of regulating the use of medically important antibiotics in food animal

production, since regulations of antibiotic use in food animals may constitute a valuable tool to preserve the efficacy of antibiotics in both human and animal medicine.

This analysis indicates that extended-spectrum cephalosporin resistance among *Salmonella enterica* isolates in retail meat declined following the 2012 Order of Prohibition on cephalosporin use in food animals, and that changes over time were statistically significant at $p<0.05$ after controlling for the location from which the isolates were obtained. The decline in ceftriaxone resistance was largely attributable to changes in *Salmonella enterica* serovar over time, and it is not possible to determine whether changes in serovar prevalence are causally associated with the restrictions on extended-spectrum cephalosporin use in food animals. Variation by serovar in cephalosporin resistance may affect how changes in antibiotic regulation affect specific serovars. A decline in resistance to extended-spectrum cephalosporin antibiotics was observed within the *Salmonella enterica* serovar Heidelberg serovar, but not serovar Typhimurium, after 2012.

Two major limitations of this analysis were the lack of analysis of the genetic basis for ceftriaxone resistance within each serovar of *Salmonella* in the dataset, as well as a lack of data regarding antibiotic use over the study period. Next steps for this analysis will include examining NARMS data on genes encoding beta-lactam resistance among *Salmonella* isolates in retail meat from 2011-2015, the years for which genetic testing results are available on-line. In a broader scale, increased surveillance of both antibiotic use and resistance prevalence among bacterial isolates in food animals will be useful in examining the relationship between antimicrobial policy, antimicrobial use, and trends in antimicrobial resistance among pathogens.

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Tables

Table 1. Total number of *Salmonella enterica* isolates from retail poultry meat identified over selected years by U.S. state.

	2002	03	04	05	06	07	08	09	10	11	12	13	14	15	Total
Colorado	0	0	12	26	41	81	96	63	70	73	53	48	36	25	624
Maryland	37	37	45	44	47	59	60	58	27	35	29	34	11	10	533
Connecticut	25	47	38	37	30	0	78	50	50	37	37	48	39	0	516
Pennsylvania	0	33	30	30	31	22	48	80	65	58	43	33	19	19	511
New York	44	17	66	35	31	29	16	37	25	21	35	20	8	7	391
California	11	25	34	53	42	41	24	31	22	20	14	24	32	14	387
Louisiana	30	16	23	27	47	35	55	20	27	20	18	11	21	2	352
New Mexico	0	12	28	39	22	23	33	49	27	30	23	24	12	5	327
Georgia	0	0	9	29	26	25	35	29	25	30	21	21	15	3	268
Missouri	0	0	0	0	0	0	38	51	36	13	50	31	25	16	260
Oregon	6	25	39	33	21	5	8	19	26	20	22	15	7	2	248
Tennessee	0	0	0	0	0	0	0	0	0	0	0	11	13	9	33
Washington	0	0	0	0	0	0	0	0	0	0	0	10	18	2	30
Minnesota	0	0	0	0	0	0	0	0	0	0	0	23	6	0	29

Table 2. Total number of NARMS Retail Isolates by year and serovar, listed by the 10 most common serovars.

Serovar	2002	03	04	05	06	07	08	09	10	11	12	13	14	15	Total
Typhimurium*	15	26	53	32	24	32	74	124	90	81	104	73	44	3	**775**
Heidelberg	35	48	71	75	69	55	88	55	38	40	23	48	30	7	**682**
Kentucky	13	24	43	62	60	25	31	46	23	50	65	47	35	26	**550**
Saintpaul	17	26	24	25	19	39	32	80	50	36	9	22	12	9	**400**
Hadar	11	13	19	22	26	57	72	23	22	32	8	15	13	3	**336**
Enteritidis	14	5	3	12	17	13	33	27	29	22	30	27	27	14	**273**
Schwarzengrund	1	3	21	8	11	7	6	9	13	9	8	14	7	4	**121**
IIIa 18:z4,z23:-	0	2	2	17	6	0	16	19	25	14	16	0	0	0	**117**
Reading	7	13	16	10	8	8	6	0	0	1	5	5	18	15	**112**
All Other	40	52	72	90	98	84	133	104	110	72	77	102	76	33	**1143**
***Including *Typhimurium* var 5-**															

Table 3. Total number of NARMS Retail Isolates by serovar and resistance phenotype to ceftriaxone.

Serovar	Number of Isolates				% Resistant
	Resistant*	Intermediate	Susceptible	Total	
Typhimurium**	378	0	397	775	**48.8%**
Heidelberg	80	0	602	682	**11.7%**
Kentucky	111	0	439	550	**20.2%**
Saintpaul	11	1	388	400	**3.0%**
Hadar	3	0	333	336	**0.9%**
Enteritidis	4	0	269	273	**1.5%**
Schwarzengrund	5	0	116	121	**4.1%**
IIIa 18:z4,z23:-	1	0	116	117	**0.9%**
Reading	6	0	106	112	**5.4%**
All Other	12	0	85	97	**12.4%**
*Includes "Intermediate" *Salmonella saintpaul* isolate.					
**Includes *Salmonella typhimurium* var 5-					

Table 4. Total number of NARMS Retail Isolates by serovar and meat type.

	Chicken Breast	Ground Beef	Ground Turkey	Pork Chop
Typhimurium*	686	14	36	39
Heidelberg	304	2	361	15
Kentucky	518	6	25	1
Saintpaul	6	6	382	6
Hadar	33	0	294	9
Enteritidis	254	4	15	0
Schwarzengrund	27	1	92	1
IIIa 18:z4,z23:-	3	0	114	0
Reading	0	0	110	2
All Other	311	136	567	129
Total	**2142**	**169**	**1996**	**202**
*Includes *Salmonella typhimurium* var 5-				

Table 5. Predicted probability of resistance to ceftriaxone, ampicillin, tetracycline, and cephalosporin in *Salmonella enterica* isolates across all serotypes and in *Salmonella enterica* serovar Heidelberg.

	Predicted Probability of Resistance										
	All Serotypes					***Salmonella enterica* serovar Heidelberg**					
Antibiotic	**2007**		**2011**		**2014**	**2007**		**2011**		**2014**
Ceftriaxone	0.09(0.07,0.11)	↑	0.19(0.15,0.22)	↓	0.11(0.09,0.13)	0.12(0.09,0.15)	↑	0.18(0.14,0.22)	↓	0.08(0.05,0.1)
Ampicillin	0.27(0.24,0.3)	↑	0.41(0.37,0.45)	↓	0.23(0.2,0.26)	0.36(0.3,0.41)	↑	0.59(0.52,0.65)	↓	0.27(0.21,0.33)
Tetracycline	0.52(0.48,0.55)	↑	0.55(0.52,0.58)	↓	0.48(0.44,0.51)	No statistically significant changes in resistance over time observed at $p<0.05$.				
Chloramphenicol	0.03(0.03,0.04)	=	0.03(0.02,0.03)	=	0.03(0.03,0.04)					
Arrows indicate the direction of change between years.										

Figures

Figure 1. The percent of isolates per year resistant to ceftriaxone, ampicillin, tetracycline and chloramphenicol are presented across all serovars and states. A smooth was applied using a generalized additive model, and a 95% confidence interval for the smooth is indicated in red.

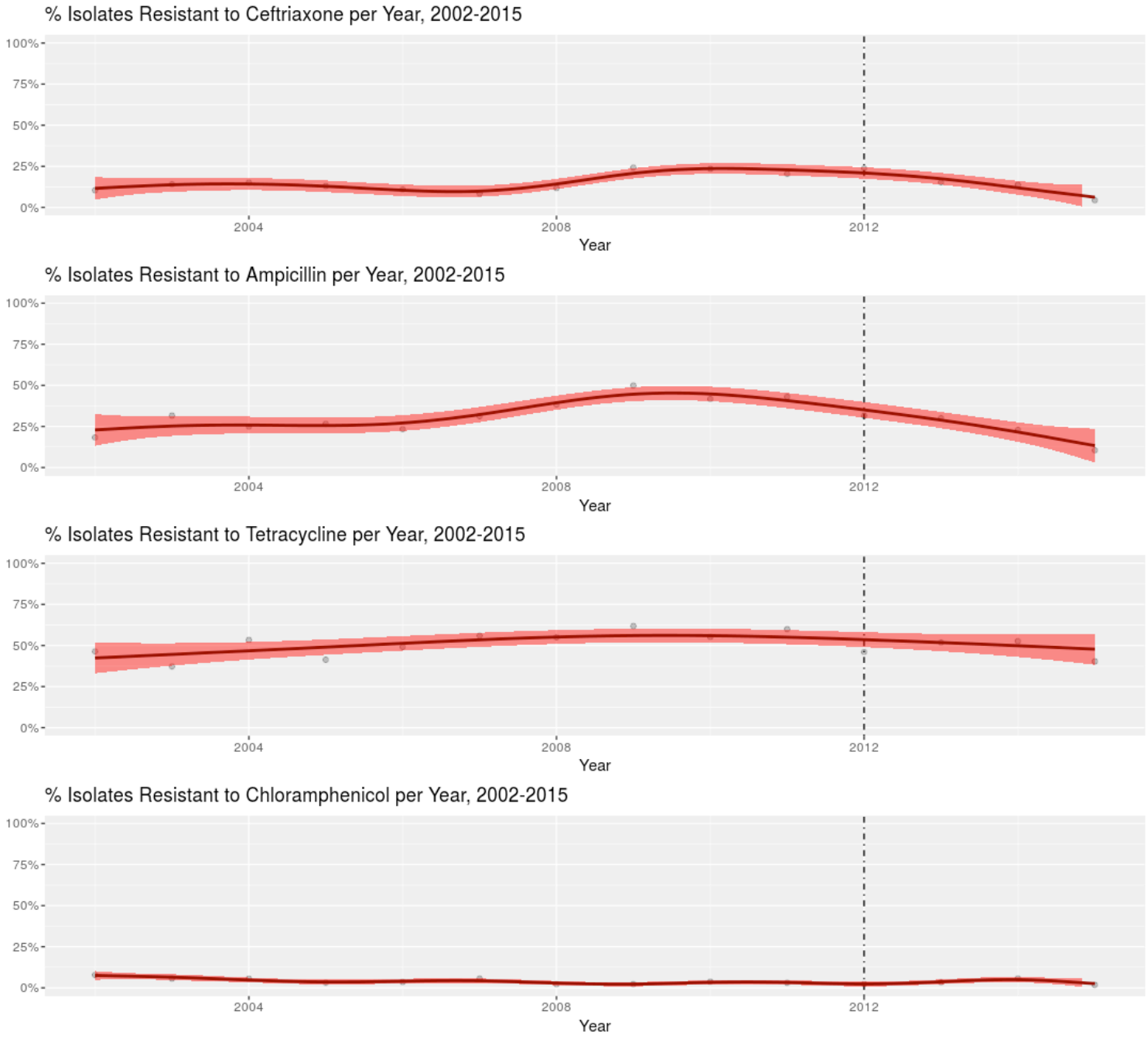

Figure 2. Predicted probability of resistance to ceftriaxone over time, based on a generalized additive model of resistance in all *Salmonella enterica* serovars to ceftriaxone from 2002-2015. The state from which isolates originated is included as a random effect.

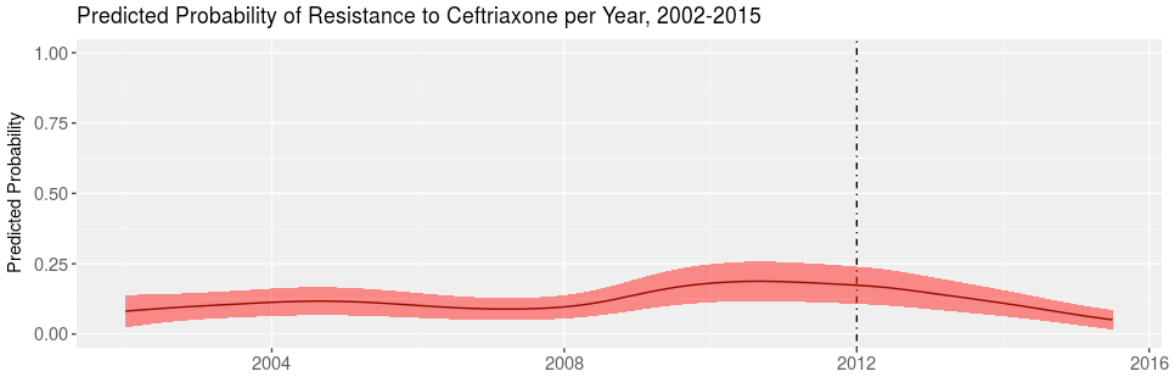

Figure 3. Predicted probability of resistance to ampicillin over time, based on a generalized additive model of resistance in all *Salmonella enterica* serovars to ampicillin from 2002-2015. The state from which isolates originated is included as a random effect.

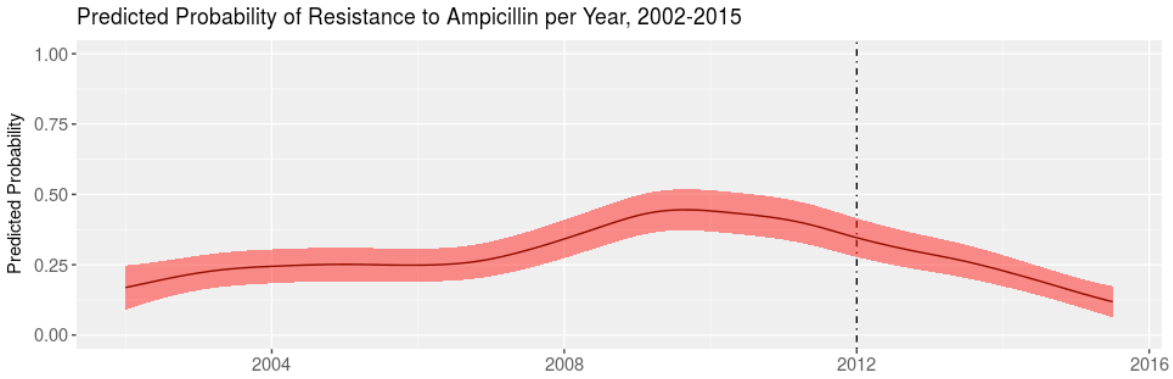

Figure 4. Predicted probability of resistance to tetracycline over time, based on a generalized additive model of resistance in all *Salmonella enterica* serovars to tetracycline from 2002-2015. The state from which isolates originated is included as a random effect.

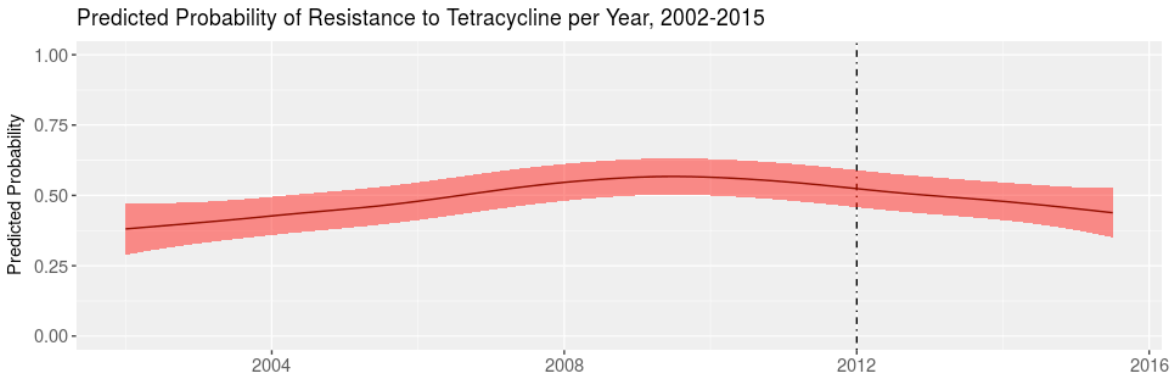

Figure 5. Predicted probability of resistance to chloramphenicol over time, based on a generalized additive models of resistance in all *Salmonella enterica* serovars to chloramphenicol below from 2002-2015. The state from which isolates originated is included as a random effect.

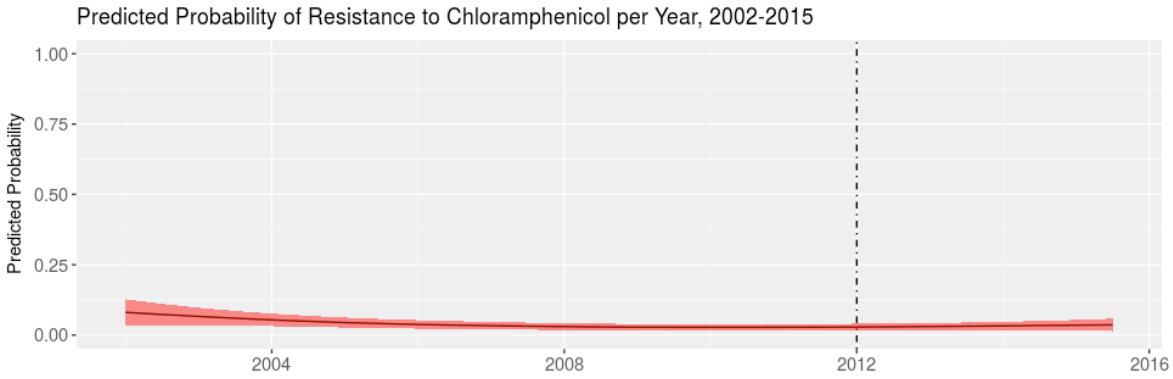

Figure 6. The percent of *Salmonella enterica* serovar Heidelberg isolates per year resistant to ceftriaxone, ampicillin, tetracycline and chloramphenicol are presented across all serovars and states. A smooth was applied using a generalized additive model, and a 95% confidence interval for the smooth is indicated in red.

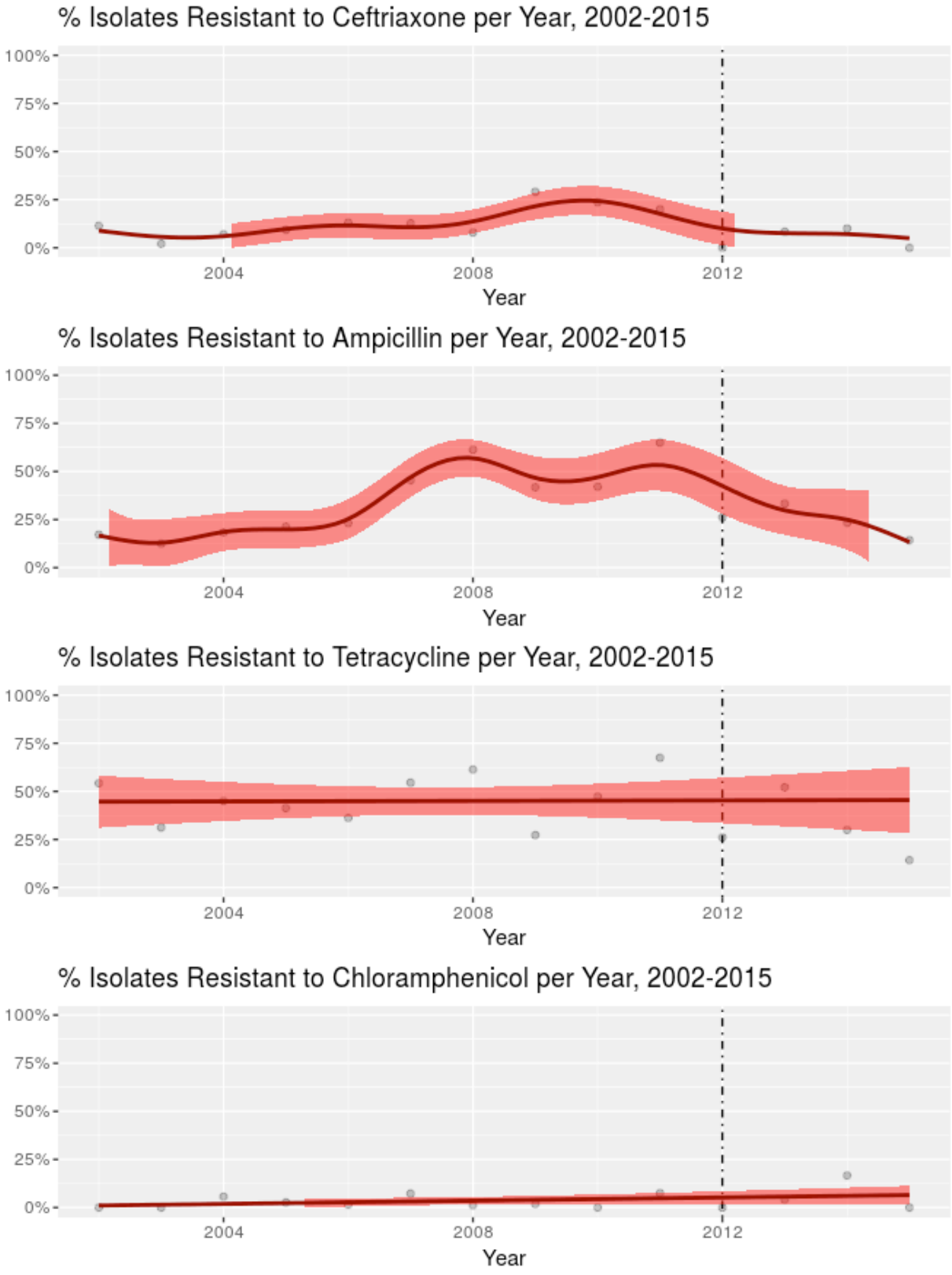

Figure 7. Predicted probability of resistance to ceftriaxone over time, based on a generalized additive model of resistance in isolates of *Salmonella enterica* serovar Heidelberg to ceftriaxone from 2002-2015. The state from which isolates originated is included as a random effect.

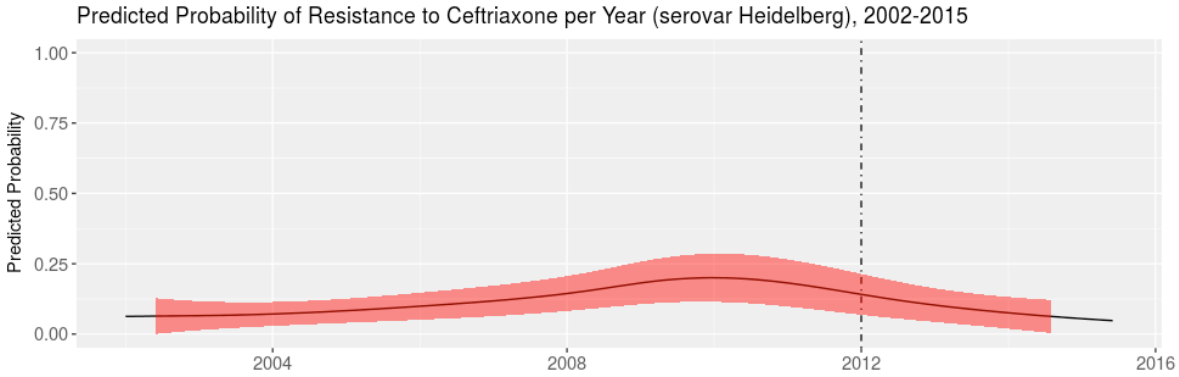

Figure 8. Predicted probability of resistance to ampicillin over time, based on a generalized additive model of resistance in isolates of *Salmonella enterica* serovar Heidelberg to tetracycline from 2002-2015. The state from which isolates originated is included as a random effect.

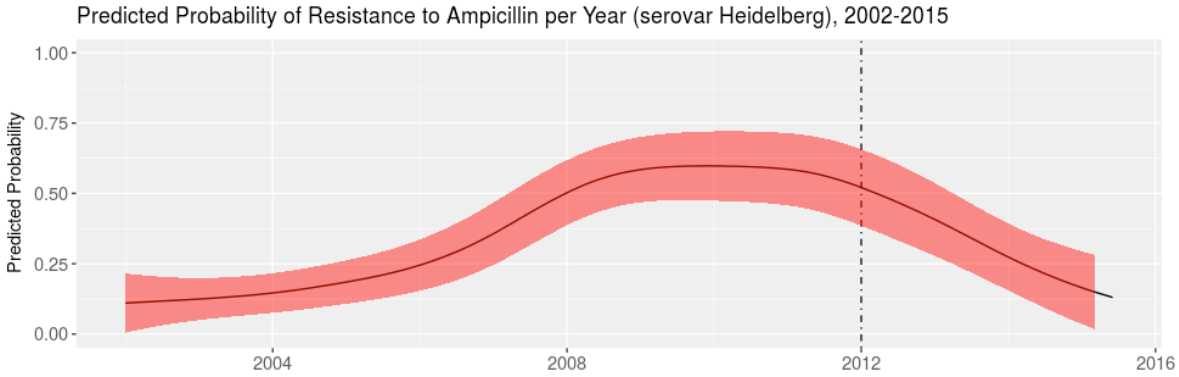

Figure 9. The percent of *Salmonella enterica typhimurium* isolates per year resistant to ceftriaxone, ampicillin, tetracycline and chloramphenicol are presented across all serovars and states. A smooth was applied using a generalized additive model, and a 95% confidence interval for the smooth is indicated in red.

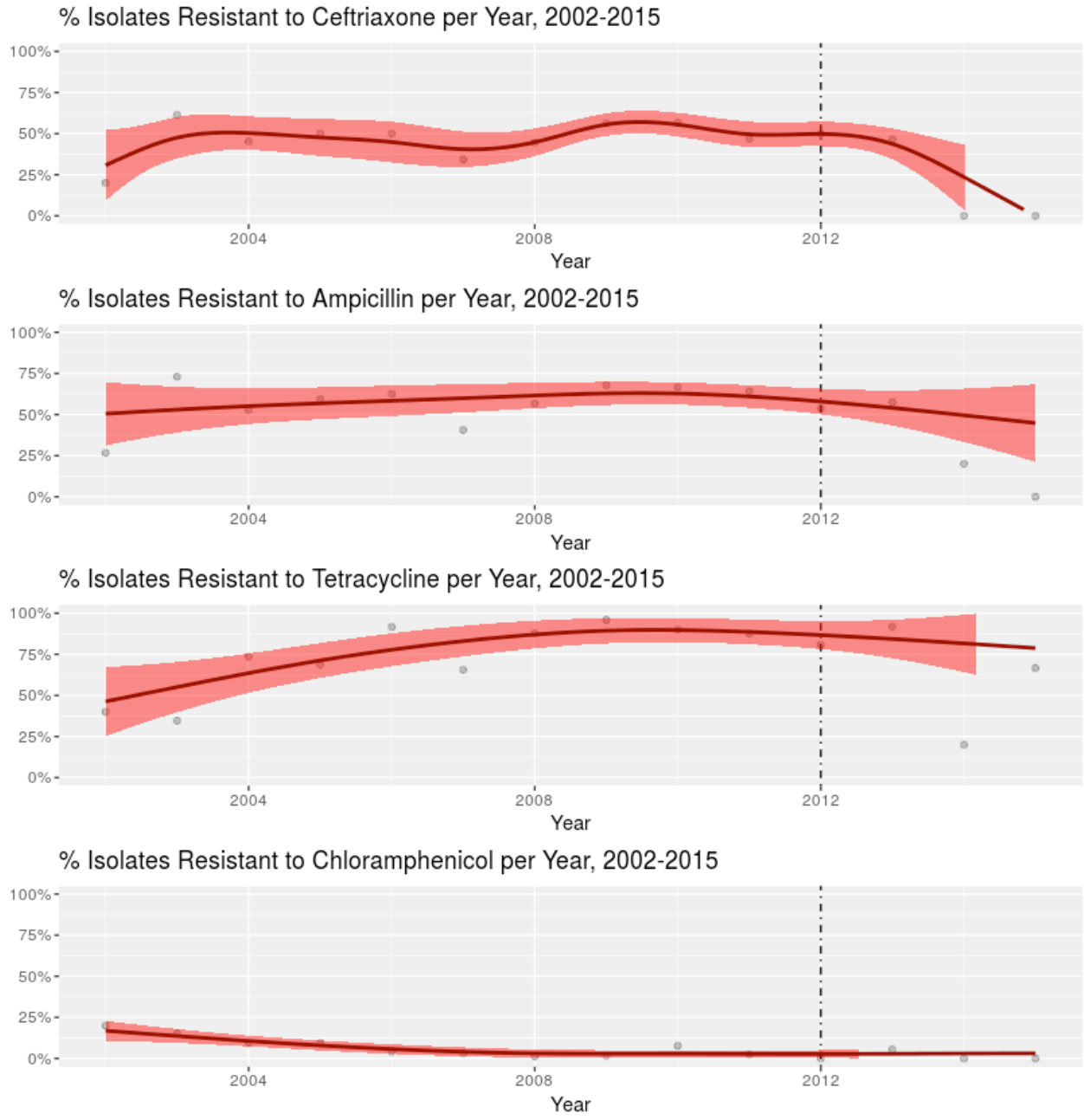

Figure 10. Number of *Salmonella enterica* isolates by serovars contained in the NARMS retail meat dataset, 2002-2015.

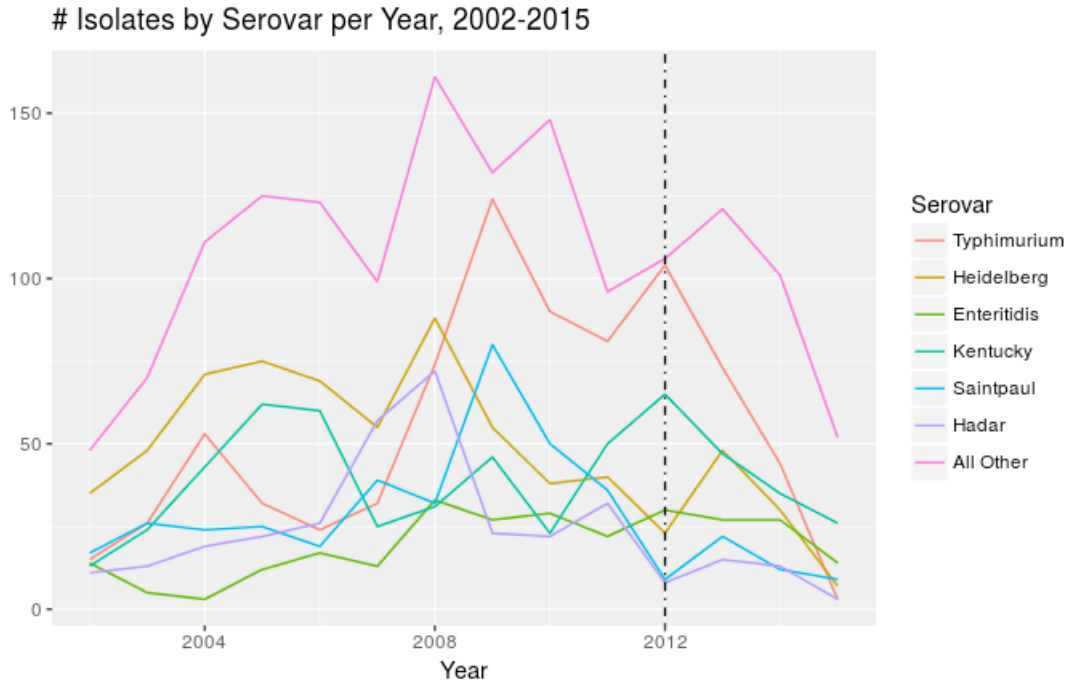

Figure 11. Resistance to ceftriaxone in all *Salmonella enterica* serovar Heidelberg, serovar Typhimurium, and all other serovars contained in the NARMS retail meat dataset, 2002-2015. A GAM smooth with 95% confidence interval is applied to resistance prevalence per year.