A COMPREHENSIVE ANALYSIS OF THE UNITED STATES' NATIONAL ANTIMICROBIAL RESISTANCE MONITERING SYSTEM

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Kelson Folkvard Braaten Zawack

May 2017

A COMPREHENSIVE ANALYSIS OF THE UNITED STATES' NATIONAL ANTIMICROBIAL RESISTANCE MONITERING SYSTEM

Kelson Folkvard Braaten Zawack, Ph. D.

Cornell University 2017

Surveillance is a key component of controlling antimicrobial resistance. In the United States this function is carried out by the National Antimicrobial Monitoring System. This effort combines the United States Department of Agriculture Food Safety Inspection Service collecting samples from slaughter facilities, the Food and Drug Administration collection samples from retail and the Center for Disease Control collecting samples from human medicine. In order to better understand single and multi-drug resistance as well as how their monitoring could be improved a comprehensive analysis of this surveillance data was undertaken.

After an introductory chapter, the second chapter presents an analysis of single drug resistance measured both in terms of the amount of antibiotic required to prevent bacterial growth, minimum inhibitory concentration, and the proportion of isolates exceeding a given resistance cutoff. The effects of measuring resistance in these two different ways are compared along with the consequences of resistance cutoff choices. An analysis of variability is then carried out and used in an assessment of power and sample sizes. The examination of trends in single drug resistance reveals that these trends depend very much on the host, bacteria, and antimicrobial context regardless of whether one considers minimum inhibitory concentration or resistance proportion and that the resistance cutoff chosen has a dramatic impact on the nature of the trend

observed. Measurements of single drug resistance are overdispersed which means large sample sizes are required to detect changes in resistance.

The third chapter focuses on identifying multi-drug resistance associations by constructing contingency tables of resistance counts and modeling then with log-linear models. This approach uncovers associations that are in some cases so extreme they cannot be tested for using asymptotic or exact conditional methods and instead require a Bayesian approach. Interrogation into the nature of these interactions reveals a spectrum of interactions including a hierarchy among the β-lactams.

The fourth chapter explores the variability of interactions discovered in chapter three. As was the case with single drug resistance, multi-drug resistance also displays more variability than expected. This increased variability or overdispersion is likely due to unaccounted factors like antimicrobial use, husbandry practices and food handling hygiene procedures.

BIOGRAPHICAL SKETCH

Kelson was born in Fort Wort, Texas. He graduated from Northfield High School in Northfield Minnesota in 2004 and Carleton College in 2008. He worked in Singapore for three years before starting at Cornell in 2011.

ACKNOWLEDGMENTS

Advisers: Dr. James Booth and Dr. Yrjö Gröhn

Special Committee: Dr. Martin Wells and Dr. Jacob Bien

Collaborating Principal Investigator: Dr. Cristina Lanzas

Collaborating Researchers: Dr. William Love, Dr. Min Li

Support: This work was inspired by the National Institute for Mathematical and Biological Synthesis (NIMBIOS) working group "Modeling Antimicrobial Resistance (AMR) Intervention". NIMBioS is sponsored by the National Science Foundation through NSF Award #DBI-1300426, with additional support from The University of Tennessee, Knoxville.

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

Overview

A key component of controlling antimicrobial resistance is surveillance. National surveillance systems are essential because they keep track of the resistance levels being experienced at various places in a country across time. They are not an experiment so they cannot conclusively elucidate underlying mechanisms, but they can shed light on potential mechanisms. More importantly by recording resistance levels they can alert regulators to problems and provide a historical record of how the problem arose. In monitoring antimicrobial resistance, there are two levels. Single drug resistance monitoring is concerned with of the resistance of microbes to individual antimicrobials and how this resistance changes in different situations like geographic location, host environment, or time. Multi-drug resistance monitoring is concerned with simultaneous resistance to multiple drugs, which drug resistances are coupled together, and how these associations change with changing environments. In the United States, antimicrobial resistance surveillance is carried out by the National Antimicrobial Monitoring System (NARMS). What follows is a detail analysis of single drug, and multi drug resistance in the United States as captured by NARMS.

The National Antimicrobial Monitoring System (NARMS)

NARMS consists of 3 components. The United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) collects isolates from animal slaughter facilities, the Food and Drug Administration (FDA) collects isolates

from retailers, and the Center for Disease Control and Prevention (CDC) collects isolates from humans (NARMS, 2016a). The program focuses on *Salmonella*, *Campylobacter*, *Escherichia*, and *Enterococcus*. The CDC also monitors *Shigella*, and *Vibrio*.

The slaughter samples collected by FSIS are collected as part of its Pathogen Reduction/Hazard Analysis and Critical Control Point (PR/HACCP) food safety monitoring of slaughter facilities (NARMS, 2016a). Since food safety, not antimicrobial resistance surveillance is the primary purpose of PR/HACCP the slaughter component of the surveillance system does not have a single design, but it's design changes as regulators adapt PR/HACCP procedures to improve the efficacy and efficiency of food safety monitoring. This component of NARMS began in 1997. At this time FSIS selected slaughterhouses for sampling based on a two-tiered system. In the first tier, facilities were sampled at random with the goal of visiting each facility once a year. In the second tier, facilities with compliance violations were subject to additional sampling. Most samples were collected through the first tier, but no record is available of whether an isolate was sampled as part of tier one or tier two. No information is available on the location of the facility, either. At the beginning only *Salmonella* was sampled. For chickens, samples were isolated from carcass rinses, and ground products. For turkey, samples were isolated from carcass swabs and ground products. Swine samples were isolated from carcass swabs and cattle samples were isolated from ground products. *Campylobacter* sampling began a year later in 1998 from chicken carcass rinses submitted to the eastern lab. In June 2006 FSIS changed the way it selected slaughterhouses to focus on those with higher amounts of

Salmonella and *Salmonella* serotypes of public health concern. In 2011 FSIS stopped sampling cows and bulls due to low bacterial yield. They also began testing for *Campylobacter* from young chicken and turkey carcasses at all 3 laboratories. A year later in 2012 sampling of market hogs, steers and heifers was discontinued again because they contained so few bacteria. In 2013 FSIS began continuous sampling of *Salmonella* from comminuted chicken and turkey. They also began a cecal sampling program. In this program facilities are randomly selected based on facility size, animal class, and slaughter volume. For chickens and turkeys, samples are taken from groups of 5 young birds. For swine, samples are taken from individual market swine and sows. For cattle, samples are taken from individual dairy cows, beef cows, steers, and heifers. In 2014 FSIS stopped testing ground beef unless a facility had failed its last inspection but began testing all raw beef samples collected for shiga-toxing producing *Escherichia coli* providing a small non-risk based sample set.

The retail sample collection program run by FDA is essentially its own study and thus the design has been fairly constant over time (NARMS, 2016a). Retail meet sample collection began in 2002 with sites participating in the Foodborne Diseases Active Surveillance Network. These include Connecticut, Georgia, Maryland, Minnesota, Oregon and Tennessee. New York, California, Colorado and New Mexico joined the program over the course of the next 2 years. Pennsylvania joined in 2008, Missouri, Louisiana, and Washington joined in 2012 and Iowa, Kansas, South Dakota and Texas joined in 2016. All sites select retailers to visit by identifying all zip codes in a 50-mile radius, identifying all retailers in those zip codes using the Chain Store Guide, dividing the zip codes into quadrants, and randomly selecting a subset of the

stores in each quadrant. Quadrants are randomly ordered across the twelve months. Each month forty meat samples are chosen. Ten of the samples are chicken breasts with bone in and skin on. Since 2011 wings, legs or thighs in that order of preference will be taken if no breasts are available. Ten of the samples are ground turkey. Ten of the samples are 80% lean ground beef. The final ten samples are pork chops. *Salmonella* are isolated from all sites and all meat types. All sites isolate *Campylobacter* form chicken and turkeys expect for Pennsylvania during its first year in the program. Beef and Swine stopped being sampled for *Campylobacter* in 2008 due to low incidence. George, Oregon, Maryland and Tennessee also sample for *Escherichia* and *Enterococcus*.

The human samples program run by CDC is also essentially its own study and has a fairly constant design (NARMS, 2016a). The program began in 1996 with sampling of non-Typhi *Salmonella* and *E. coli* 0157 from the food net sites Connecticut, Georgia, Maryland, Minnesota, Oregon, Tennessee, New York, California, Colorado, New Mexico, Pennsylvania, Missouri, Louisiana, and Washington. In 1997 *Campylobacter* testing began and expanded to California, Connecticut, Colorado, Georgia, Maryland, Minnesota, New Mexico, New York, Oregon, and Tennessee by 2003. In 1999 *Salmonella* serotype Typhi and Shigella testing began. In 2003 *Salmonella*, *Shigella* and *E. coli* O157 testing was expanded to all states. In 2009 sampling of *Vibrio* species other than *V. cholera* was added.

Antimicrobial Resistance Testing

NARMS determines antimicrobial resistance using the broth microdilution method (NARMS, 2015; NARMS 2016b). Specifically, colonies are isolated onto

microbe specific media. A colony is picked from those isolated from each source and grown in broth spiked with serial dilutions of the antibiotic being tested for. Growth is determined spectrophotometrically by comparing the amount of light reflected by an inoculated sample to that reflected by a control sample with an increase in light reflection indicating bacterial growth. The lowest dilution that prohibits grown is termed the minimum inhibitory concentration (MIC). MIC values can be converted to a susceptible/resistance value using a relevant resistance cutoff (FDA, 2014).

Previous Work

The results of the NARMS program is summarized each year in a number of government reports, most notably the annual NARMS integrated report (FDA, 2014). This report focus on the percentage of isolates exceeding the chosen resistance cutoffs. For single drug resistance, it covers a number of specific microbe/antibiotic case studies chosen either because of the importance of the microbe as a pathogen and the importance of the antibiotic in treating infections of the given pathogen or because of the usefulness of the particular microbe/antibiotic combination as a general indicator of trends in antimicrobial resistance. At the level of multidrug resistance, the integrated report discusses the number of isolates that are resistance to 3 or more drugs making no distinction between the three drugs to which the microbe is specifically resistant. The report also focuses on the extended spectrum beta-lactamase (ESBL) resistance pattern and the ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (ACSSuT) resistance pattern. ESBL isolates are determined by taking isolates resistant to beta-lactam antibiotics like ceftriaxone and cefoxitin, sequencing

their genomes, and determining if they possess known ESBL genes. ACSSuT resistance is determined based on the phenotypic data.

In hopes of more fully characterizing multidrug resistance several authors have developed methods that try to move beyond the known multidrug patterns and learn new association patterns directly from surveillance data. One of these relies on an ad hoc strategy for assembling additive Bayesian models (Lugwig, 2013). A second makes use of the graphical lasso (Love, 2016).

Current Work

The work that follows builds on this research in order to provide a more comprehensive understanding of the NARMS dataset on both the single drug and the multidrug resistance fronts.

The next chapter builds out the single drug front by analyzing all microbe, antibiotic, and host combinations. For each combination resistance at slaughter and retail stages was compared in order to understand how resistance changes as meat products move through the supply chain. A comparison of trends in resistance as defined by the NARMS susceptibility/resistance cutoffs and resistance as defined by continuous MIC values was carried out as well as an analysis of how choice of resistance cutoff effects these trends. The amount and causes of variability in the dataset was formally analyzed. Finally, this analysis of variability to used to understand the power to detect changes in antibiotic resistance over time provided by the NARMS dataset.

The following chapter builds out the multidrug front by applying log-linear models of contingency tables to understand the structure of multidrug associations. In

particular, by using a log-linear model strategy this chapter moves beyond the 2-way interactions detected in the additive Bayesian models and the graphical lasso to detect higher order interactions. Since many of these interactions turn out to be difficult to detect with standard asymptotic methods and exact conditional methods a Bayesian test is developed. Finally, the potential biological significance of the detected interactions is examined.

The final chapter examines how the variability observed in the second chapter effects the interactions inferred in the third. First, the amount of variation expected for a multinomial random variable is compared to the amount observed in the multidrug contingency tables. Since multidimensional variability is difficult to make sense of, an analysis of the variability in the interaction terms is carried out. A power analysis is carried out using the assumption of asymptotic normality for the interaction parameters and compared to a more exact power analysis using a Dirichlet multinomial simulation.

REFERENCES

Print References

Love, W. J., Zawack, K. A., Booth, J. G., Grohn, Y. T., Lanzas, C., 2016. Markov Networks of Collateral Resistance: National Antimicrobial Resistance Monitoring System Surveillance Results from *Escherichia coli* Isolates, 2004-2012. PLoS. Comput. Biol. 12(11), e1005160. doi:10.1371/journal.pcbi.1005160.

Ludwig, A., Berthiaume, P., Boerlin, P., Gow, S., Léger, D., Lewis F.I., 2013. Identifying associations in *Escherichia coli* antimicrobial resistance patterns using additive Bayesian networks. Prev. Vet. Med. 110, 64-75. http://dx.doi.org/10.1016/j.prevetmed.2013.02.005.

The National Antimicrobial Resistance Monitoring System., 2015. Retail Meat Surveillance Laboratory Protocol.

The National Antimicrobial Resistance Monitoring System., 2016a. Methods.

The National Antimicrobial Resistance Monitoring System., 2016b. Manual of Laboratory Methods 3rd Edition.

U.S. Food and Drug Administration., 2014. NARMS Integrated Report: 2014

CHAPTER 2

MONITORING ANTIMICROBIAL RESISTANCE IN THE FOOD SUPPLY CHAIN AND ITS IMPLICATIONS FOR FDA POLICY INITITIVES

Introduction

Antimicrobial resistance (AMR) is one of the most serious health threats to both animals and humans. The mitigation of AMR in animal agriculture is therefore critical for both the agri-food industry and for public health (Oliver, 2011; Marshall 2011). Globally and nationally, there is much attention on developing approaches to mitigate AMR (WHO 2011; The White House 2015; FDA 2012; FDA 2013a). Overuse of antimicrobials contributes to the emergence and proliferation of resistant bacterial strains. Historically, antimicrobials have been administered to livestock and poultry to address both animal health as well as production purposes. In order to slow down the development and proliferation of AMR in food animal agriculture, the Food and Drug Administration (FDA) has initiated a risk mitigation strategy to limit use of medically important antimicrobials to therapeutic uses under veterinary oversight by working with drug companies to change product labels (FDA 2012; FDA 2013a). A key piece of evidence in evaluating the efficacy of this policy is detecting the change in AMR before and after the policy has been implemented. To do so, it is necessary to understand the baseline variation of AMR over time and at different stages of the food supply chain. The most comprehensive information on AMR in United States agriculture is the newly public data from the National Antimicrobial Resistance Monitoring System (NARMS). NARMS longitudinally monitors

resistance of non-typhoidal *Salmonella*, *Campylobacter* spp., *Escherichia coli*, and *Enterococcus* spp. to a variety of antibiotics (FDA, 2015a). Resistance of each isolate is reported as a minimum inhibitory concentration (MIC) which can then be transformed into a susceptible/resistance value based on whether this MIC exceeds a given resistance threshold. Where defined, NARMS uses the clinical breakpoints published by the Clinical and Laboratory Standards Institute (CLSI) to interpret MIC values as susceptible or resistant, and uses epidemiological cut-offs where clinical breakpoints are not defined (FDA, 2015b). The clinical breakpoints defined by CLSI are determined by the probability of therapeutic failure in humans and are intended to guide clinical decision making (Martínez, 2014). Epidemiological cutoffs represent the level of resistance which demarcates the boundary between the wild type population and resistant mutants. They are determined as the value that separates the main part of the MIC distribution from the upper tail (Martínez, 2014).

The historical trends of AMR have been preliminarily explored in the annually published NARMS reports. These reports focused on resistance proportions and modeled slaughter and retail separately. Dichotomizing MIC values however risks a loss of information (Naggara, 2011; Fedorov 2009). Dichotomization of MIC results also cannot detect shifts from low to high resistance levels, which provide an early warning for increasing resistance in a population. Clinical breakpoints may also shift over time to reflect changes in AMR interpretation, reporting, and methods, resulting in changes in reported resistance proportions not related to changes in population (Hombach, 2013; Hamada, 2015). Pulsed-field gel electrophoresis (PFGE) profiles of *Campylobacter* and *Salmonella* from broiler flocks found identical clones from

primary production through slaughter to retail products (Lienau, 2007; Nógrády, 2008), indicating that there may be a connection between resistance levels at various stages of the food supply chain.

The objectives of this study were to evaluate the baseline trend and variations in the NARMS data and provide useful information to improve data collection, analysis and synthesis in the national AMR monitoring system. To achieve these, we extended the annual NARMS reports by examining both percent resistance and mean MIC values. Moreover, we considered each of these values simultaneously at the slaughter and retail stage. We examined the structure of the variation in resistance within\between years, and across geographic regions. Using this baseline, we estimated how much data must be collected to be able to determine if the change in FDA policy did indeed have an effect. Finally, using this information we examined the impact of a previous change in FDA antimicrobial policy, removing approval of enrofloxacin for use in poultry water in 2005.

Materials and Methods

The dataset

The NARMS data were obtained from the FDA website. The data files for Retail Meats, HACCP 1997-2005, HACCP 2006-2013 and Cecal were combined and stored in an SQLite database. MIC values were $\log_2$ transformed, which reduced skewness (Wagner, 2003). The MICs of isolates susceptible to the lowest antibiotic concentration tested were taken to be this lowest concentration and the $\log_2$ transformed MICs of isolates resistant to the highest concentration tested were incremented by 1.

Although we examined many different combinations of microbes, hosts and antibiotics, we focus here on chicken as the host and examined resistance of *Campylobacter jejuni* to tetracycline, *Campylobacter coli* to erythromycin, *Salmonella enterica* serovar Typhimurium to ampicillin, and *Escherichia coli* to streptomycin. In accordance with NARMS guidelines resistance for all *Campylobacter* and *E. coli*-streptomycin was determined using epidemiological cutoffs and resistance for *S.* Typhimurium-Ampicillin was determined based on the CLSI breakpoint (USDA 2014). Chicken data had the most consistent data across all time points and stages. Analyzed drugs were chosen not only for their significance in human medicine, but also for their importance in veterinary medicine and extent of use in food production (FDA, 2003). Bacteria were selected for their significance as pathogens, number of observations, and MIC distribution patterns. The time frame of 2004 to 2012 was chosen because both slaughter and retail data for chicken were available during this time period (retail data were first available in 2004 and 2012 was the last year the HACCP slaughter data were available), which made the stage effect analysis possible. Sample per year and stage ranged from 21 to 2232.

MIC distributions over time and across stages

In order to obtain a baseline understanding of AMR changes over time and across different stages of the food supply chain (i.e., slaughter and retail), we began by first exploring the resistance data for each microbe/host/antibiotic combination. Line graphs were used to visualize resistance data and boxplots were used to visualize the distribution of the MICs.

Generalized linear modeling of resistance

To quantitatively assess trends in AMR, we constructed models of resistance both as a binary variable using logistic regression and as a continuous variable using linear regression, using $\log_2$ (MIC). To quantitatively assess the sources of variation we constructed a linear mixed effects model. All models were implemented using the Python Statsmodels package (Seabold, 2010). The significance of all coefficients was assessed using a likelihood ratio test.

Modeling resistance prevalence – logistic regression

Logistic regression was carried out by modeling the log odds of resistance versus non-resistance as a function of stage and year. We chose 2004 retail data as the baseline level. The model is shown in equation 1

$$\ln\left(\frac{p_{ij}}{1-p_{ij}}\right) = a + \beta_i + \beta_{Slaughter} Slaughter + e_{ij} \qquad (1)$$

where α and β are coefficients, i indexes over the years, j indexes over slaughter and retail stages, and *Slaughter* is an indicator variable designating whether the sample came from slaughter or retail. The e term is the error.

To determine the robustness of this analysis to the choice of resistance threshold *C. coli*-erythromycin was taken as an example and the regression was carried out using each MIC as a cutoff. The stability of the model was evaluated based on the similarity of the resulting coefficients.

Modeling MIC distribution – linear regression

As a means of both sidestepping the choice of a breakpoint, and adding an additional viewpoint on AMR, MIC was treated as a continuous variable and the mean

$\log_2$ MIC was modeled as a linear function of stage and year. The linear model is shown in equation 2.

$$log_2 MIC_{ij} = a + \beta_{slaughter} Slaughter + \beta_i + e_{ij} \qquad (2)$$

Modeling sources of variation – linear mixed effects model

To study the sources of variation in resistance, $\log_2$ MIC at each stage was modeled separately as a function of a fixed intercept for state and a random intercept for year according to equation 3.

$$log_2 MIC_{ik} = \mu + Year_i + State_k + e_{ik} \qquad (3)$$

Here *i* indexes over years and *k* indexes over the states.

Power analysis

To formally evaluate the effectiveness of the FDA policy change in a manner consistent with the exploratory and regression analyses, we propose a model with a constant base level of resistance around which the yearly levels vary, a period of change, and then a new resistance level is established (Figure 1). Under this model a hypothesis test of mean resistance level before and after the policy change can assess the change in resistance. It is assumed that there is a different amount of variation within years and between years so the test is carried out on average yearly resistance. This test can be done with either percent resistance or MIC values (SAS, 2004). A power analysis was performed to benchmark the efficacy of this test. Calculating power requires knowledge of the standard deviation, sample size, and magnitude of the effect to be detected. For the t-test of mean $\log_2$ MIC sensible standard deviations were selected by calculating the empirical distribution for the standard deviation of the

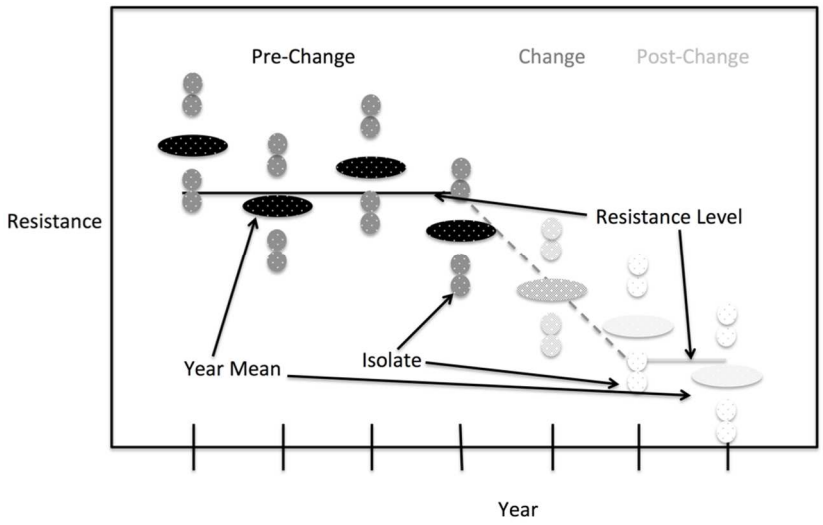


Figure 1: Model of Antimicrobial Resistance

The model of antimicrobial resistance used in evaluating policy changes. It is assumed there is a constant base level of resistance around which the yearly levels vary, a period of change, and then a new resistance level is established.

mean $\log_2$ MIC per year. The number of isolates per year was assumed to be 200, a number generally exceeded in historical data. The current 9 years of data were used as the pre-policy change sample size. Post-policy change sample sizes were assumed to be between 1 and 10 years. The desired detectable effect was taken to be between 0 and 1 $\log_2$ MIC. Power curves were then plotted according to equation 4.

$$\text{power} = P\left(t < t_{critical\_val\_low}\right) + P\left(t > t_{critical\_val\_up}\right) \qquad (4)$$

where $t = \sqrt{nw_2}\left(\frac{\overline{x_2} - \overline{x_2}}{s}\right)$ with n-2 degrees of freedom and non-centrality parameter δ where $\delta = \sqrt{nw_1w_2}\left(\frac{\mu_{diff}}{\sigma}\right)$. $t_{critical\_val\_low}$ is the $\frac{\alpha}{2}$ quantile of the central t distribution

with n-2 degrees of freedom, $t_{critical\_val\_up}$ is the $1-\frac{\alpha}{2}$ quantile of the central t distribution with n-2 degrees of freedom, n is the total number of observations, and the w's the fraction of observations in each sample.

Resistant/Susceptible counts are binomial quantities and so their standard deviations are functions of the proportions. However, because the data come from a mix of conditions like geographical location, season, and production quality these proportions are over-dispersed with respect to binomial variation, and the standard deviation will also be a function of this over-dispersion parameter. Reasonable proportions and over-dispersion parameters were obtained by plotting the empirical distribution of each quantity. Isolates per year was set to 200, pre-policy change sample size was 9 years, post-change sample size was between 1 and 10 years, and effect sizes were chosen so as to be detectable with such sample sizes. The power curves were then plotted according to equation 5.

$$\text{power} = \Phi\left(rc_{low} - \frac{p_2 - p_1}{se}\right) + 1 - \Phi\left(rc_{up} - \frac{p_2 - p_1}{se}\right) \qquad (5)$$

where $r = \sqrt{\frac{p(1-p)\left(\frac{1}{k_1}-\frac{1}{k_2}\right)}{\frac{p_1(1-p_1)}{k_1}+\frac{p_2(1-p_2)}{k_2}}}$, $se = \sqrt{\phi\frac{p_1(1-p_1)}{nk_1} + \frac{p_2(1-p_2)}{nk_2}}$, $p = \frac{k_1}{k}p_1 + \frac{k_2}{k}p_2$, Φ is the normal PDF, c a critical value, k the number of years in a sample and ϕ the over-dispersion.

The role of isolate count was determined by calculating power as in equation 4, but replacing the single standard deviation parameter s with the combination of between year and within year standard deviations in equation 6.

$$s = \sqrt{{s_{between}}^2 + \frac{{s_{within}}^2}{n}} \qquad (6)$$

where n is the number of isolates per year. The resulting power was plotted for n between 1 and 2000 assuming $s_{between}$ is .6, s_{within} is 3, the pre-policy change sample size was 9 years, post-change sample size was 5, and the effect size was a 1 fold change in MIC.

In assessing power .8 was chosen as the desirable threshold.

Assessing the effect of the change in enrofloxacin policy

In 2005 the FDA withdrew of approval of enrofloxacin in poultry water. Due to this policy change having much the same form as the current policy change it serves as a useful case study. Since enrofloxacin is metabolized to ciprofloxacin the analysis focused on the latter antibiotic. We evaluated its resistance using the exploratory analysis, generalized linear models and t-test as in the baseline study. Since the policy change occurred in 2005 we extended to time period under consideration back to 2002.

Results

Analysis of MIC distributions over time and across stages

Preliminary analysis of the raw data revealed that the trend in AMR was largely dependent on the bacteria-drug combination. In most cases like *C. jejuni*-ciprofloxacin, *C. jejuni*-tetracycline, *C. coli*-erythromycin, *S.* typhimurium-ampicillin, average $\log_2$ MIC increased slightly over time (Figures 2-4). In other cases like *E. coli*-streptomycin there was a marked decrease in resistance during the study period (Figure 5). The relationship between slaughter and retail was also case dependent with slaughter sometime higher than retail and retail sometimes higher than slaughter. The distribution of MICs was generally highly skewed, in many cases the median, first

quartile, and even the minimum all coincided. The percent resistance followed the same trend as the $\log_2$ MIC, but with much higher variability.

Logistic modeling

The logistic model (Table 1) confirmed the results of the exploratory analysis. Both the coefficients themselves and the significant coefficients were dependent on the specific drug-bacteria combination. Although the intercept and at least one of the year coefficients was significant in all cases, which particular year coefficient was significant varied widely. For *E.coli*-Streptomycin all year coefficients were significant. In *C. coli*-Erythromycin only the coefficient for 2011 was significant. The coefficient for slaughter was significant for *E. coli*-Streptomycin and *S.* Typhimurium-Ampicillin. It was not significant for *C. coli*-Erythromycin or *C. jejuni*-Tetracycline, not because there was no difference between slaughter and retail, but because slaughter was sometimes higher than retail and sometimes lower.

The distribution of MIC (Figure 6) for *C. coli*-erythromycin showed that at both slaughter and retail there was one narrow peak above the $\log_2$ MIC cutoff of 5 and a broad peak between -1 and 2. The broad peak between -1 and 2 is likely to represent the MIC distribution for the wild-type isolates, while the isolates with $\log_2$ MIC above 5 are likely to represent the non-wild type isolates. The sensitivity analysis (Table2) showed that the model was highly sensitive to the choice of cutoff. For all the years, the coefficients in the logistic models vary to a large degree with the choice of cutoff. Many of the coefficients are negative in some models and positive in others. The identity of the significant coefficients also changes between models. This

suggests that while estimating the proportion of bacteria with resistance above a threshold is important it does not tell the whole story.

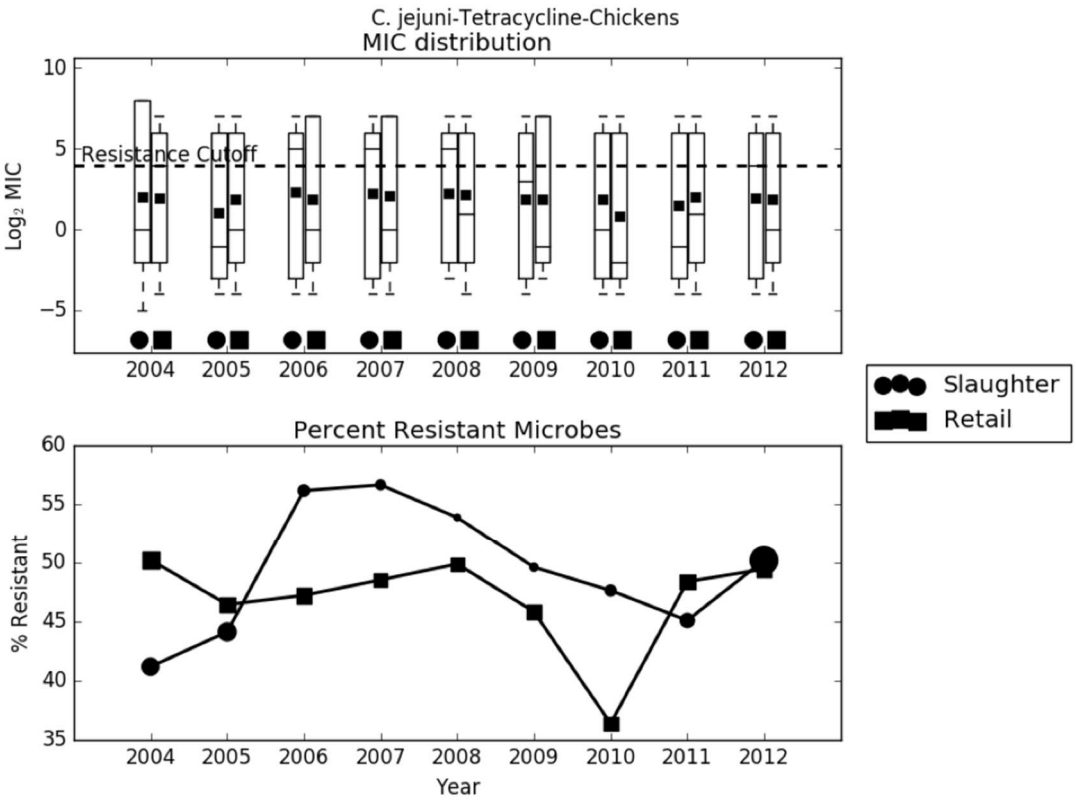
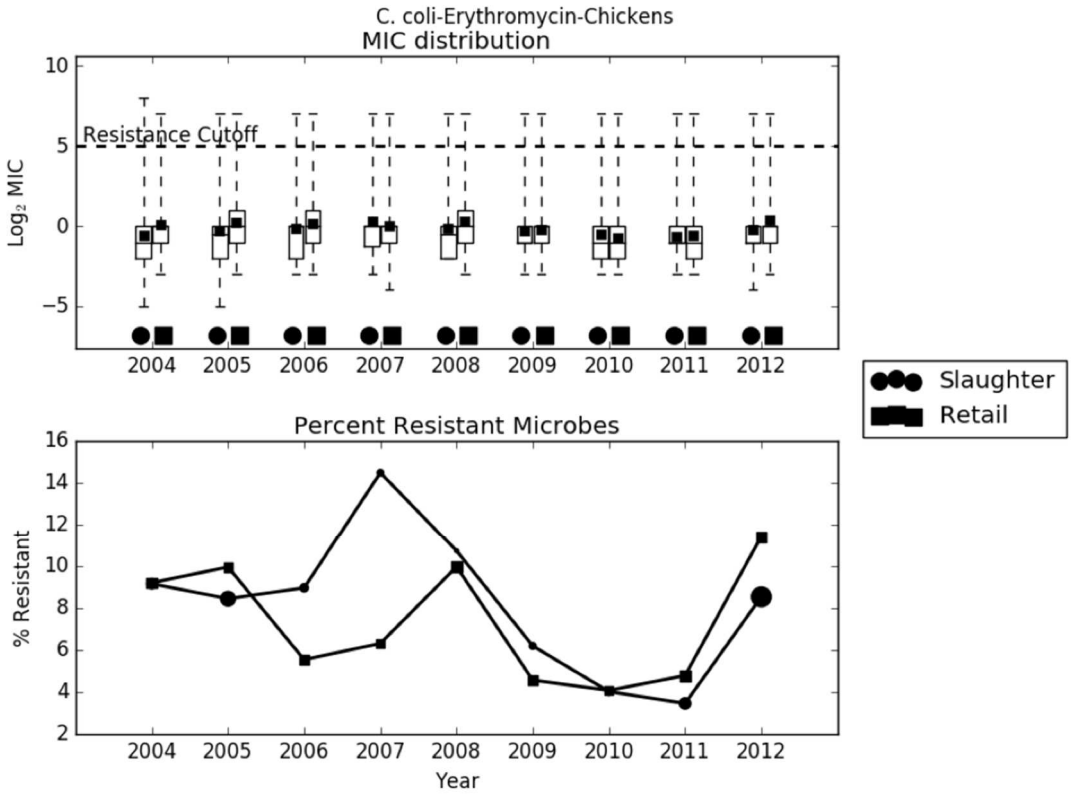


Figure 2: Exploratory analysis of *Campylobacter jejuni* resistance to tetracycline. A) Boxplots of $\log_2$ minimum inhibitory concentration (MIC). The lower whisker is the minimum observed MIC in a given year, the lower edge of the box the first quartile, the line the median, the square the mean, the top of the box the third quartile, and the upper whisker the maximum. The dashed line indicates the breakpoint between resistant and susceptible isolates. B) Line graph of percent isolates with MIC values above the resistance breakpoint. Sizes of points are proportional to the number of observations in the given year and stage. Sample sizes ranged from 78 to 1,348.



A)

Figure 3: Exploratory analysis of *Campylobacter coli* resistance to erythromycin. A) Boxplots of $\log_2$ minimum inhibitory concentration (MIC). The dashed line indicates the breakpoint between resistant and susceptible isolates. B) Line graph of percent isolates with MIC values above the resistance breakpoint. Sizes of points are proportional to the number of observations in the given year and stage. Samples sizes ranged from 76 to 693.

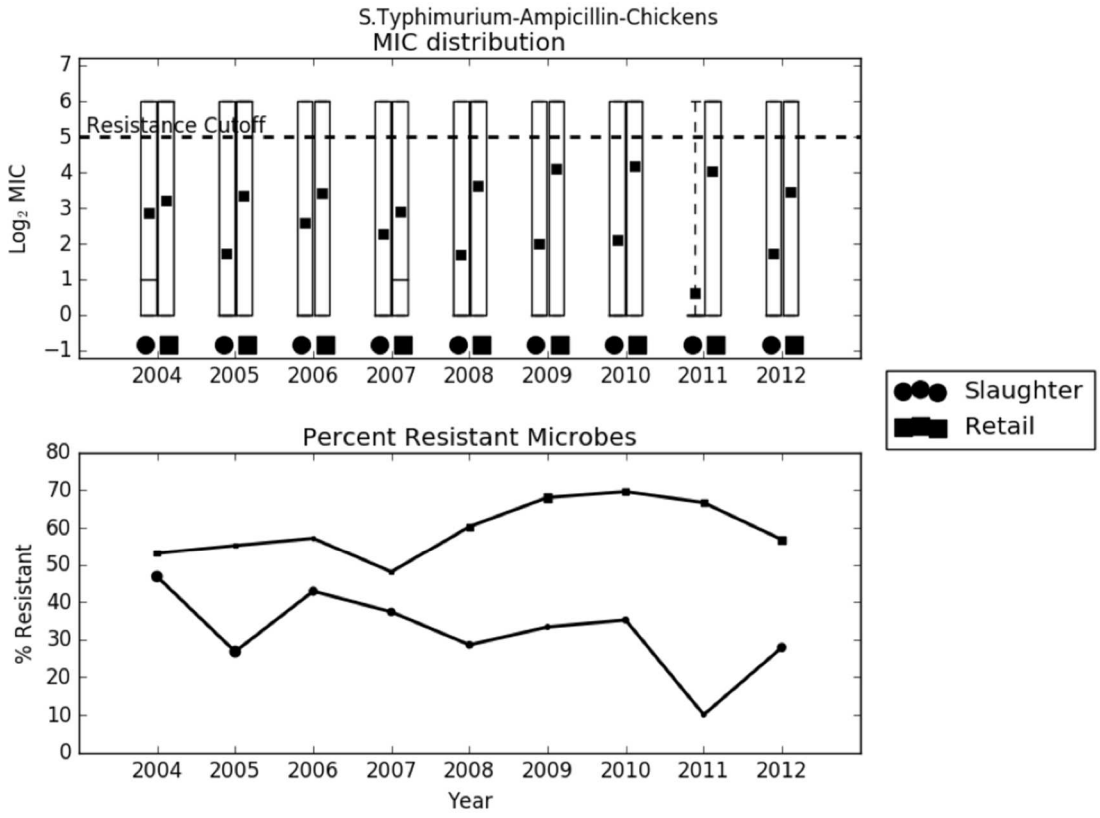


Figure 4: Exploratory analysis of *Salmonella* Typhimurium resistance to Ampicillin. A) Boxplots of $\log_2$ minimum inhibitory concentration (MIC). The dashed line indicates the breakpoint between resistant and susceptible isolates. B) Line graph of percent isolates with MIC values above the resistance breakpoint. Sizes of points are proportional to the number of observations in the given year and stage. Samples sizes ranged from 21 to 104.

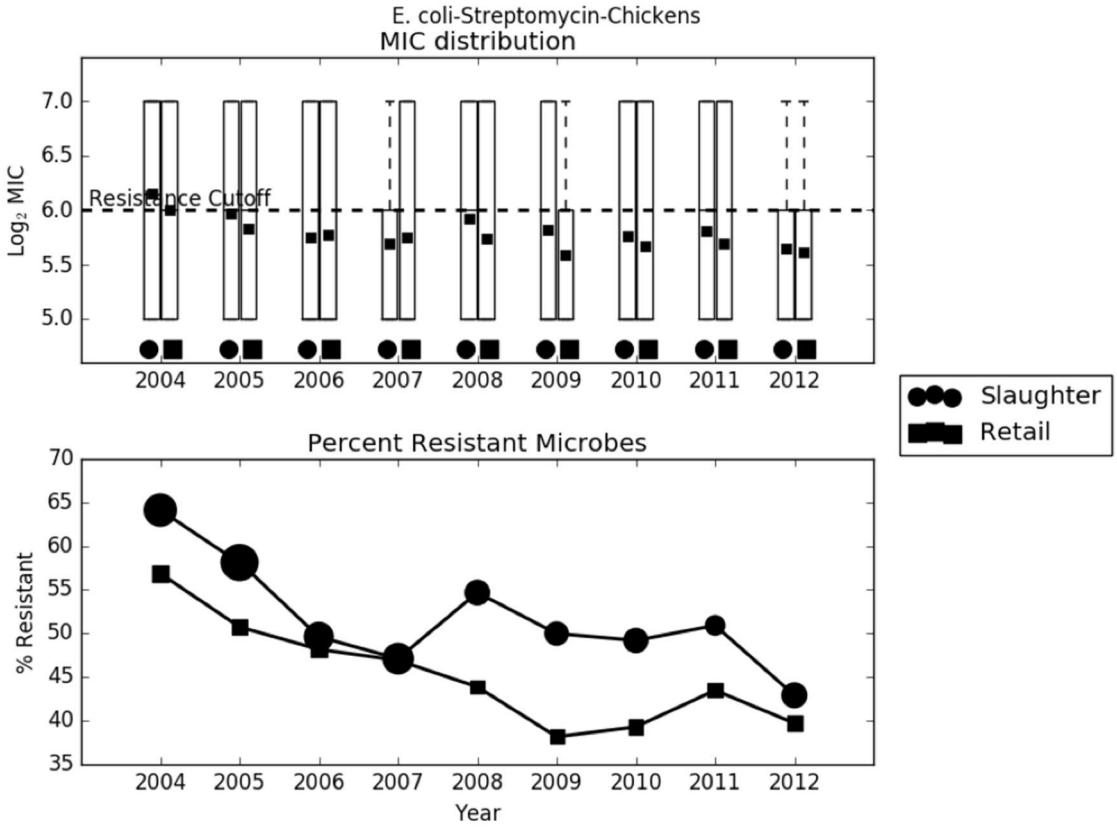


Figure 5: Exploratory analysis of *Escherichia coli* resistance to streptomycin. A) Boxplots of $\log_2$ minimum inhibitory concentration (MIC). The dashed line indicates the breakpoint between resistant and susceptible isolates. B) Line graph of percent isolates with MIC values above the resistance breakpoint. Sizes of points are proportional to the number of observations in the given year and stage. Samples sizes ranged from 299 to 2232.

Table 1: Values of coefficients for the logistic regression of resistance on year and stage.

	C. coli -	*C. jejuni* -	*E. Coli* -	*S.* Typhimurium -
α	-2.29*	-0.19*	0.31*	0.81*
β_{2005}	-0.04	-0.03	-0.25*	-0.67*
β_{2006}	-0.28	0.19	-0.54*	-0.06
β_{2007}	0.00	0.23*	-0.65*	-0.37
β_{2008}	0.10	0.21	-0.43*	-0.49*
β_{2009}	-0.64	0.05	-0.63*	-0.13
β_{2010}	-0.88	-0.21	-0.64*	-0.12
β_{2011}	-0.87*	0.05	-0.55*	-0.51*
β_{2012}	0.00	0.16*	-0.82*	-0.59*
$\beta_{Slaughter}$	0.00	0.03	0.26*	-1.13*

* significant at α=.05

Linear modeling

To obtain a more complete picture a linear model of mean $\log_2$ MIC was also fit (Table 3). This model confirmed that in general both the year and slaughter coefficients were significant but that the identity of the significant coefficients as well as their values depended on the bacteria-drug combination.

Mixed effects model

The standard deviation of the year random effect ranges from 0 to 2 and is generally below 1 (Figure 7). Without the state fixed effect the residual standard deviation ranges from 0 to 4.25 and except for cases of very low total standard deviation is almost always greater than the amount of variation explained by year. The addition of

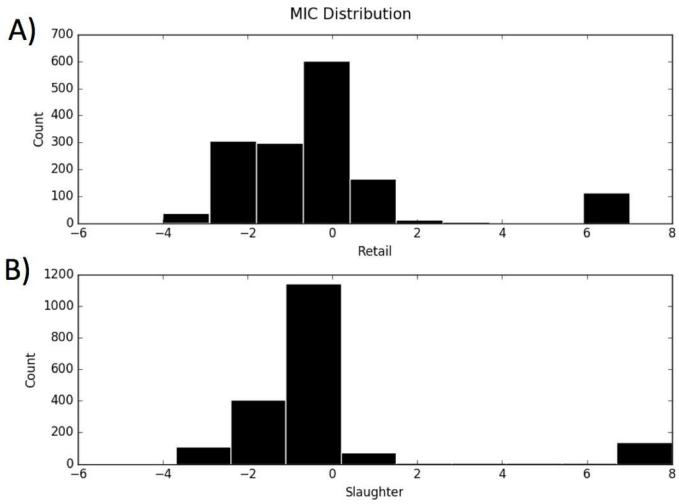


Figure 6: Distribution of $\log_2$ MIC for *Campylobacter coli* resistance to erythromycin. A) Distribution at retail. B) Distribution at slaughter. Counts are summed over the full range of years, 2004 to 2012.

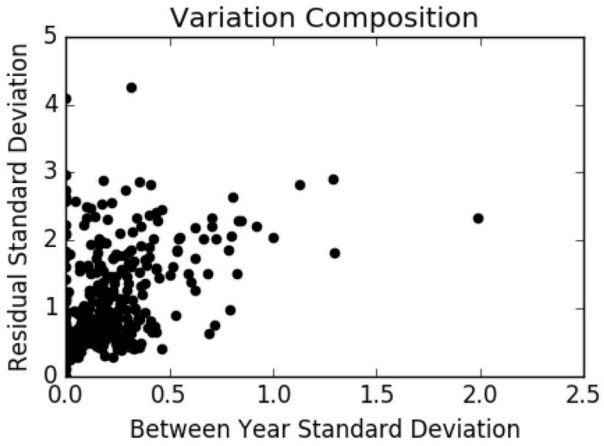


Figure 7: Variation Composition.

Scatterplot of between year standard deviation vs. residual standard deviation across all bacteria, drug, stage combinations.

Table 2: Sensitivity of logistic regression of resistance to choice of breakpoint. Data are for *Campylobacter coli* resistance to erythromycin. Column labels specify the $\log_2$ MIC breakpoint used to distinguish resistant and susceptible. The final column, 5, represents the clinically relevant breakpoint.

	Breakpoint $\log_2$ MIC			
	-1	0	1	5
α	0.93*	0.06	-1.46*	-2.29*
β_{2005}	0.20	0.51*	0.19	-0.04
β_{2006}	0.29	0.78*	0.53*	-0.28
β_{2007}	0.40*	0.71*	0.23	0.00
β_{2008}	0.72*	0.57*	0.40	0.10
β_{2009}	0.69*	0.23	-0.10	-0.64
β_{2010}	-0.01	-0.22	-0.71*	-0.88
β_{2011}	0.17	-0.16	-0.82*	-0.87*
β_{2012}	0.57*	0.41*	0.21	0.00
$\beta_{Slaughter}$	-0.30*	-0.54*	-0.61*	0.00

* significant at α=.05

Table 3: Values of coefficients for the linear regression of $\log_2$ MIC on year and stage.

	C. coli -	*C. jejuni* -	*E. Coli* -	*S.* Typhimurium -
α	-0.11	1.89*	5.58*	3.52*
β_{2005}	0.10	-0.69*	-0.06*	-0.71*
β_{2006}	0.23	-0.07	-0.13*	-0.11
β_{2007}	0.27	0.02	-0.16*	-0.47
β_{2008}	0.35	0.08	-0.10*	-0.62*
β_{2009}	-0.04	-0.26	-0.15*	-0.18
β_{2010}	-0.44*	-0.85*	-0.16*	-0.19
β_{2011}	-0.36*	-0.30	-0.13*	-0.63*
β_{2012}	0.23	-0.17	-0.20*	-0.69*
$\beta_{Slaughter}$	-0.30*	0.05	0.06*	-1.33*

* significant at α=.05

the state fixed effect does little to change this, decreasing the residual standard deviation by less than .25.

Power analysis

As the first step of the power analysis we determined that across all bacteria, drug, stage combinations the standard deviation of the $\log_2$ MIC values decreased sharply from 0 with most values below .6 and almost all below 1 (Figure 8). As a result power curves were plotted for standard deviation of .6 and 1. These curves indicate that at a standard deviation of .6 it would be possible to detect a 1 $\log_2$ MIC change in 5 years, and at a standard deviation of 1 a 1.5 unit $\log_2$ MIC change could be detected in 7 years.

In analyzing the power of the proportion test it was determined that the vast majority of average resistance proportions across all bacteria, drug, stage combinations were below .25 with most were below .5 and the majority of over-dispersion factors were below 2 (Figure 9). Assuming an over dispersion of 2, in 6 years it would be possible to detect a 6% decrease in resistance if initial resistance was 25% and it would be possible to detect an 8% decrease in 5 years if the initial resistance was increased to 50%.

The number of isolates sampled each year plays an important role in the power. Power increases dramatically between 1 and 100 isolates per year. It increases slowly between 100 and 500 isolates. Beyond 500 isolates the power sharply plateaus.

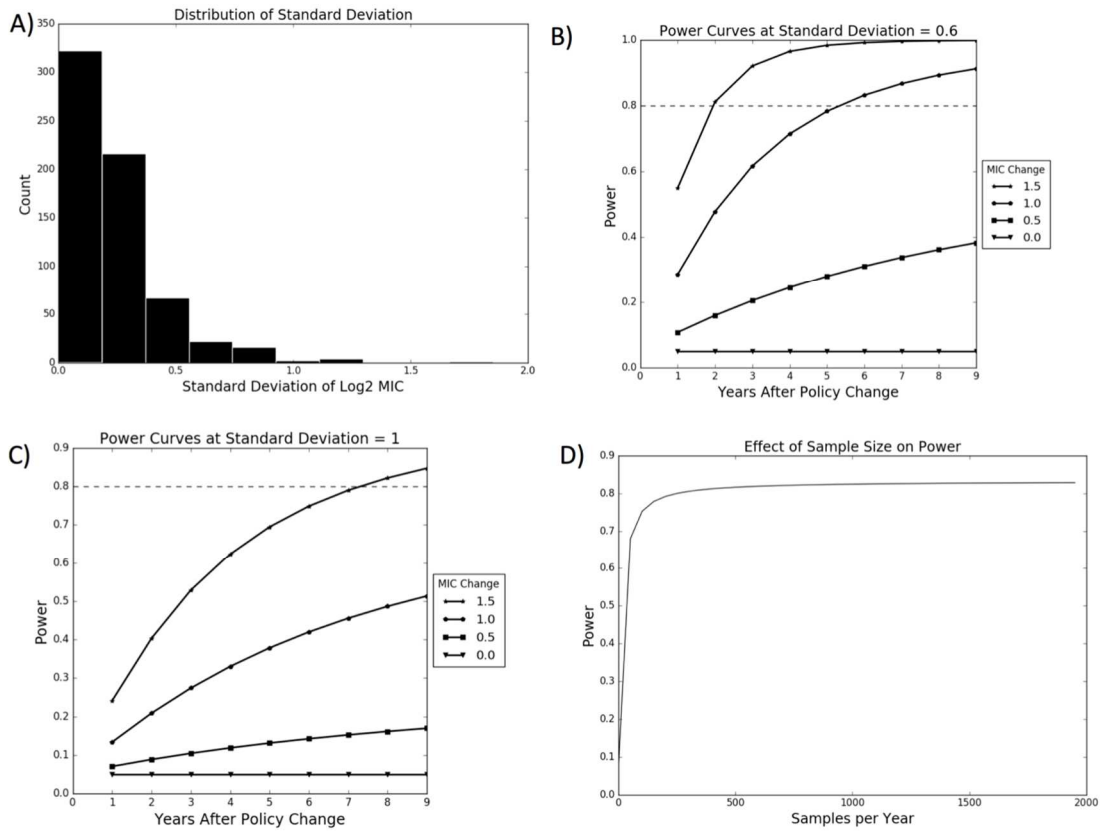


Figure 8: Power analysis for testing changes in mean $\log_2$ MIC.

A) Distribution of empirical standard deviation across all bacteria, drug, stage combinations. B) Calculation of power assuming a standard deviation of .6. C) Calculation of power assuming a standard deviation of 1. D) Power of a test comparing 9 years before a policy change with 5 years after the change as a function of the number of isolates sampled each year. Dashed lines indicate a power of .8, the standard minimum power desired for a hypothesis test

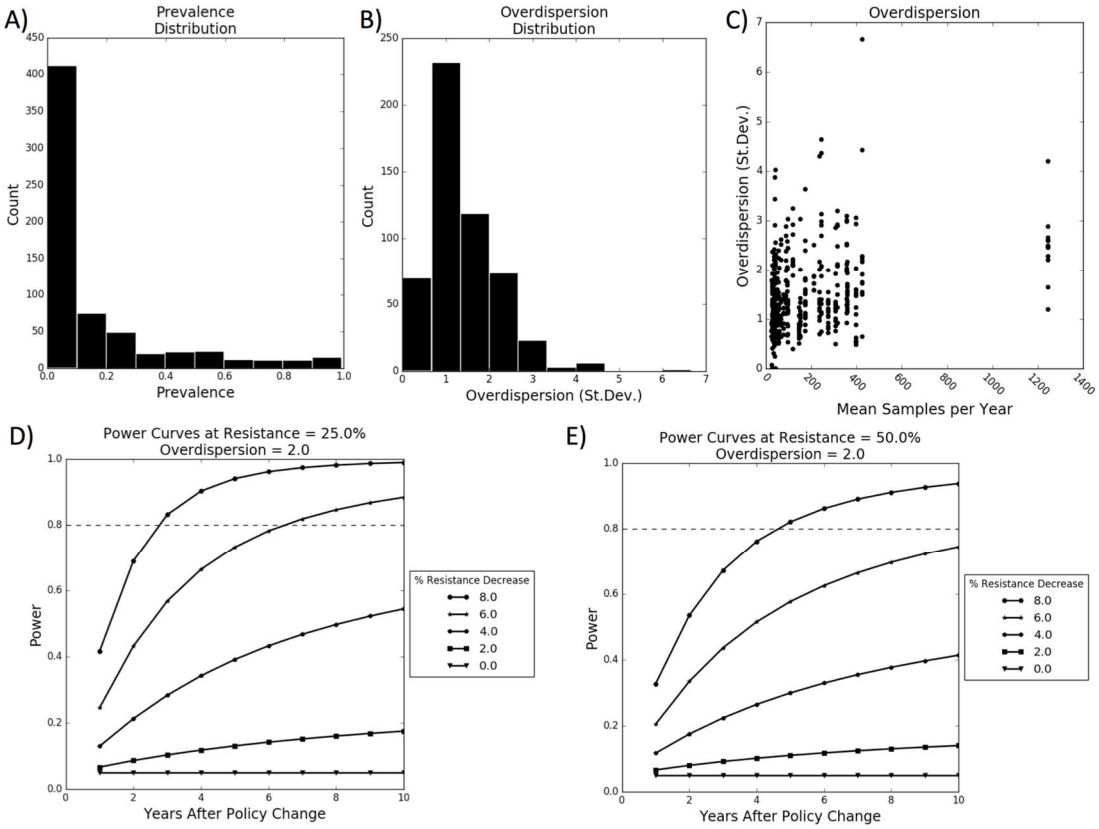


Figure 9: Power analysis for testing changes in percent resistance.

A) Distribution of empirical percent resistance across all bacteria, drug, stage combinations. B) Distribution of empirical over-dispersion. C) Plot of over-dispersion vs. sample size D) Calculation of power assuming an initial resistance of 25% and an over-dispersion of 2. E) Calculation of power assuming an initial resistance of 50% and an over-dispersion of 2. Dashed lines indicate a power of .8, the standard minimum power desired for a hypothesis test.

The above estimates assume that only one hypothesis test is being carried out but in practice it would be necessary to perform one test for each bacteria – drug combination. In practice results would need to be adjusted to account for multiple testing to protect against false positives.

Analyzing the effects of a change in enrofloxacin policy

The exploratory analysis of the trend in *C. jejuni* resistance to ciprofloxacin shows that since the policy change in 2005 both mean $\log_2$ MIC and percent resistance has remained essentially constant if not slightly increasing at both slaughter and retail (Figure 10). Both generalized linear models also confirm this fact (Table 4). The t-test of the difference in mean $\log_2$ MIC before and after the policy change is not significant at retail (effect=.028, p=.9) or slaughter (effect=.44, p=.13). Removing approval for enrofloxacin in poultry did not decrease resistance to fluoroquinalones.

Discussion

Antimicrobial resistance is a growing threat with serious implications not only for human and veterinary health but also the food supply, animal agriculture and the economy. To counteract this threat the FDA has proposed tightening restrictions on the use of antimicrobials. This change however will undoubtedly have consequences for food production and pricing. With this in mind it is essential to understand historical baseline resistance trends and to insure it will be possible to assess the effects of the policy change on future levels of resistance.

The exploratory analysis undertaken here demonstrates that over the past decade percent resistance and mean $\log_2$ MIC at both slaughter and retail have

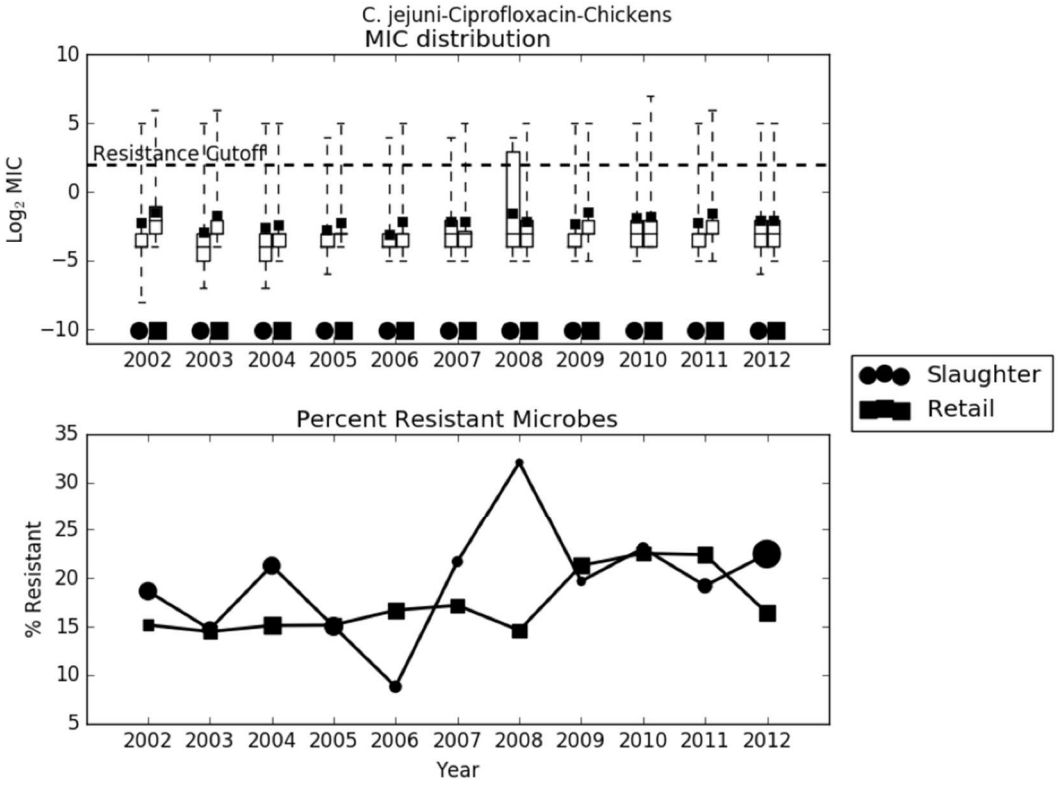


Figure 10: Exploratory assessment of the 2005 tightening of enrofloxacin policy on *Campylobacter jejuni* resistance to ciprofloxacin. A) Boxplots of $\log_2$ Mean Inhibitory Concentration. The dashed line indicates the breakpoint between resistant and susceptible isolates. B) Line graph of percent isolates with MIC values above the resistance breakpoint. Sizes of points are proportional to the number of observations in the given year and stage. Data from 2002 to 2003 are included in this analysis as it is an assessment of a 2005 policy change. Ciprofloxacin is studied because it is the metabolic product of enrofloxacin. Sample sized ranged from 78 to 1348.

Table 4: Generalized linear modeling assessment of the 2005 tightening of enrofloxacin policy on *Campylobacter jejuni* resistance to ciprofloxacin.

	C. jejuni-Ciprofloxacin	
	Logistic	Linear
α	-1.64*	-1.68*
β_{2003}	-0.20	-0.41*
β_{2004}	0.06	-0.56*
β_{2005}	-0.17	-0.55*
β_{2006}	-0.23	-0.62*
β_{2007}	0.12	-0.26
β_{2008}	0.09	-0.26
β_{2009}	0.28	0.13
β_{2010}	0.36*	0.05
β_{2011}	0.24	0.06
β_{2012}	0.21	0.00
$\beta_{Slaughter}$	0.14*	-0.44*

* significant at α=.05

fluctuated up and down in a bacteria-drug specific manner. The logistic and linear models confirm this observation as both year and stage coefficients were significant in likelihood ratio tests. While logistic and linear models reveled the same overall trends, each model provides a distinctly useful lens on AMR. Logistic regression provides a straightforward characterization of situations where there is a known epidemiological cutoff or clinical breakpoint between resistance and susceptibility. Logistic regression however is highly sensitive to the choice of breakpoint making it opaque to interpret when the resistance threshold is difficult to determine (Jaspers 2014). In this case linear regression of $\log_2$ MIC provides a more straightforward characterization of resistance patterns. Additionally, because linear regression explains the mean resistance level, it can in general provide a more holistic lens on AMR.

Mixed effects models show that there are different levels of variation between and within years. Surprisingly however there is more variation within years than between years and accounting for state does little to resolve this variation. This indicates that most of the AMR variation is due to the structure of resistance in the population.

Our analysis showed that by performing a hypothesis test comparing the level of resistance before and after a change it is possible to detect a change in resistance level as small as 1 $\log_2$ MIC in 5 years or a 6% change in resistance in as little as 6 years. Moreover, the current 200 isolates per year level of data collection provides more than sufficient power and could in fact be reduced to 100 samples per year without significant reduction in power. Although it might be interesting to test the change in slope of the trend line before and after a policy change, our exploratory analysis showed that resistance patterns do not often have clear trends and so we chose to focus on a comparison test of resistance levels. Even though it would take 6 years to detect a change in AMR after the new level had been reached, which may itself take several years, this is a fairly short period of time on a policy making scale. As a result, even though it may be difficult to predict the outcome of the current change in FDA policy it will not be difficult to assess changes on AMR. An implicit assumption of the proposed approach is that changes on the AMR levels after the policy implementation can be attributable largely to the policy. A combined analysis of the antimicrobial use and resistance would provide a greater level of evidence (EFSA, 2006). However, in United States, antimicrobial use data are limited to sales of active compounds

aggregated by drug class intended for use in all food-producing animals since 2009 (FDA, 2013b).

Analyzing the change on resistance before and after implementation of enrofloxacin policy reveals the complexities of managing and evaluating AMR. To the extent that there was a trend AMR increased following the policy change but this was not significant even for $\log_2$ MIC which provides a more sensitive test. Previous studies aiming to evaluate the enrofloxacin ban reported unchanged resistance levels for ciprofloxacin in *Campylobacter* isolates recovered from chicken and chicken carcasses (Nannapaneni, 2009; Price 2007). These previous surveys sampled a small number of isolates during short periods of time (2004 to 2006) and narrow geographical locations. Based on our analysis, AMR changes during short time spans (e.g. 2 years) are unlikely to show changes on AMR levels even if the policy was effective. A more comprehensive study analyzed changes on the proportion of resistant isolates to ciprofloxacin for *Campylobacter* in the NARMS retail meat samples from 2002 to 2007, and found no changes (Zhao, 2010).

REFERENCES

Print References

European Food Safety Authority., 2006. ECDC/EFSA/EMA first joint report on the integrated analysis of the consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals.

Fedorov. V., Mannino. F., Zhang. R., 2009. Consequences of dichotomization. Pharm. Stat. 8(1), 50-61. doi: 10.1002/pst.331.

Chemother. 68(9), 2092-2098. doi: 10.1093/jac/dkt136. PubMed PMID: 23633681.

Hamada, Y., Sutherland, C.A., Nicolau, D.P., 2015. Impact of Revised Cefepime CLSI Breakpoints on *Escherichia coli* and *Klebsiella pneumoniae* susceptibility and potential impact if applied to *Pseudomonas aeruginosa*. J. of Clin. Microb. 53(5), 1712-1714. doi: 10.1128/jcm.03652-14.

Hombach. M., Mouttet. B., Bloemberg. G.V., 2013. Consequences of revised CLSI and EUCAST guidelines for antibiotic susceptibility patterns of ESBL- and AmpC beta-lactamase-producing clinical Enterobacteriaceae isolates. J Antimicrob.

Jaspers, S., Aerts, M., Verbeke, G., Beloeil, P.A., 2014. Estimation of the wild-type minimum inhibitory concentration value distribution. Stat. Med. 33, 289-303.

Lienau. J.A., Ellerbroek, L., Klein. G., 2007. Tracing flock-related *Campylobacter* clones from broiler farms through slaughter to retail products by pulsed-field gel electrophoresis. J. Food. Prot. 70, 536–542.

Marshall, B.M., Levy, S.B., 2011. Food animals and antimicrobials: impacts on human health. Clin. Micobiol. Rev. 24, 718-733.

Martínez, J.L., Coque, T.M., Baquero, F., 2014. What is a resistance gene? Ranking risk in resistomes. Nat. Rev. Microbio. 13, 116–123.

Naggara, O., Raymond, J., Guilbert, F., Roy, D., Weill, A., Altman, D.G., 2011. Analysis by categorizing or dichotomizing continuous variables is inadvisable: an example from the natural history of unruptured aneurysms. AJNR. Am. J. Neuroradiol. 2011, 32(3), 437-40. doi: 10.3174/ajnr.A2425. PubMed PMID: 21330400.

Nannapaneni, R., Hanning, I., Wiggins, K.C., Story, R.P., Ricke, S.C., Johnson, M.G., 2009. Ciprofloxacin-resistant *Campylobacter* persists in raw retail chicken after the fluoroquinolone ban. Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess. 26, 1348-1353.

Nógrády, N., Kardos, G., Bistyák, A., Turcsányi, I., Mészáros, J., Galántai, Z., Juhász, Á., Samu, P., Kaszanyitzky, J.É., Pászti, J., Kiss, I., 2008. Prevalence and characterization of *Salmonella infantis* isolates originating from different points of the broiler chicken–human food chain in Hungary. Int. J. Food Microbiol. 127, 162-167.

Oliver, S.P., Murinda, S.E., Jayarao, B.M., 2011. Impact of antibiotic use in adult dairy cows on antimicrobial resistance of veterinary and human pathogens: a comprehensive review. Foodborne Pathog. Dis. 8, 337-355.

Price, L.B., Leila, G.L., Vailes, R., Silbergeld, E., 2007. The Persistence of Fluoroquinolone-Resistant *Campylobacter* in Poultry Production. Environ. Health. Perspect. 115, 1035-1039.

SAS Institute Inc., 2004. SAS/STAT 9.1 User's Guide, p 3409-3592.

Seabold, S. Perktold, J., 2010. Statsmodels: Econometric and statistical modeling with python. Proc. of the 9th Python in Sci. Conf.

United States Department of Agriculture., 2014. NARMS -National Antimicrobial Resistance Monitoring System Animal Isolates.

U.S. Food and Drug Administration., 2003. Guidance for Industry #152: Evaluating the safety of antimicrobial new animal drugs with regard to their microbiological effects on bacteria of human health concern.

U.S. Food and Drug Administration., 2012. Guidance for Industry #209: The judicious use of medically important antimicrobial drugs in food-producing animals.

U.S. Food and Drug Administration., 2013a. Guidance for Industry #213: New antimicrobial drugs and new animal drug combination products administered in or on medicated feed for drinking water of food-producing animals: Recommendations for drug sponsors for voluntarily aligning product use conditions with GFI #209.

U.S. Food and Drug Administration., 2013b. Animal Drug User Fee Act.

U.S. Food and Drug Administration., 2015a. NARMS now: integrated data.

U.S. Food and Drug Administration., 2015b. NARMS Methods.

Wagner, B.A., Salman, M.D., Dargatz, D.A., Morley, P.S., Wittum, T.E., Keefe, T.J., 2003. Factor analysis of minimum-inhibitory concentrations for *Escherichia coli* isolated from feedlot cattle to model relationships among antimicrobial-resistance outcomes. Prev. Vet. Med. 57, 127-139.

World Health Organization., 2014. Global Action Plan on Antimicrobial Resistance.

The White House., 2015. National action plan for combating antibiotic-resistant bacteria.

Zhao, S., Young, S.R., Tong, E., Abbott, J.W., Womack. N., Friedman, S.L., McDermott PF. 2010. Antimicrobial Resistance of *Campylobacter* Isolates from Retail Meat in the United States between 2002 and 2007. Appl. Environ. Microbiol. 76, 7949-7956.

Web References

[Dataset] National Antimicrobial Monitoring system. http://www.fda.gov/AnimalVeterinary/SafetyHealth/AntimicrobialResistance/NationalAntimicrobialResistanceMonitoringSystem/ucm416741.htm. 10-17-2015

CHAPTER 3

INFERRING THE INTERACTION STRUCTURE OF RESISTANCE TO ANTIMICROBIALS

Introduction

Antimicrobial resistance is a serious threat to human and animal health garnering much attention domestically and internationally. According to the CDC, in the US more than 2 million people a year contract an antibiotic resistant infection. In the EU more than 25,000 people a year die due to antibiotic resistant bacteria, based on data from the European Commission Directorate-Generals on Health and Food Safety. In 2011 The EU issued an action plan on antibiotic resistance, and the US followed suit in 2015. Within the domain of antibiotic resistance an especially concerning problem is that of multi-drug resistance as it may increase the chances that there will be no therapeutic agent available to treat a given infection. It is therefore essential to understand not only resistance, but the dynamics of multi-drug resistance.

To gain a more complete picture of multi-drug resistance it is important to interrogate the associations between the various drug resistances. Associations can have clinical as well as mechanistic consequences. In the clinical setting they could be used in recommending courses of treatment for an infection that is not responding to a particular antibiotic. They could also be used in designing antimicrobial cocktails. Beyond the clinic, associations between resistances may shed light on underlying mechanisms. A group of drugs that are always susceptible together or always resistant together may indicate that the resistance is due to a common underlying biological

mechanism. More complex associations may indicate linkage between resistance genes or linkages in the selection pressures occurring because drugs are often present in the same environment.

Inferring associations between resistances can be accomplished by comparing the probability of resistance under various combinations of the resistance status of other drugs. If, as shown in Table 1a, a microbe is resistant to drug X 10 out the 200 or 5% of the time when it is susceptible to drug Y, but 100 out of the 200 or 50% of the time when it is resistant to drug Y, then we can conclude there is a 2-way association between drugs X and Y. Such associations can be visualized using a network or graph with drugs as nodes and edges between drugs that share a 2-way association. By looking at combinations of resistance and susceptibility for multiple microbes we can infer associations between more than two variables. This is the case in Table 1 if Table 1a is taken to be data for microbes susceptible to drug Z and Table 1b is taken to be microbes resistant to drug Z. In Table 1a, among isolates that are susceptible to drug Z, a microbe is resistant to drug X 5% of the time when it is susceptible to drug Y and 50% of the time when it is resistant to drug Y. In Table 1b, among isolates resistant to drug Z, a microbe is resistant to drug X 185 out of 200 or 92.5% of the time when it is susceptible to drug Y and 4 out of 4 or 100% of the time when it is resistant to drug Y. Since drug Z modifies the relationship between drugs X and Y we conclude there is a 3-way association between drugs X, Y, and Z. When there is an association involving more than 2 drugs it is called an interaction.

Previous studies have examined the 2-way association structure of multidrug resistance. Additive Bayesian models were applied to examine 2-way associations in

resistance of *Escherichia coli* isolated from pig pens in Canada (Lugwig et al., 2013). Graphical models have been used to examine the dependence structure of *E. coli* isolated from chicken at slaughter and retail stages in the US (Love et al., 2016). Both studies determined that multidrug resistance is highly structured with dependences between antibiotics in the same drug class as well as between classes. In particular Love et al. (2016) identified two subnetworks, one for the β-lactams and a second covering a mixture of drug classes. These studies also showed that these dependences are supported by known biological mechanisms. An example is associations between drugs that are known to be degraded by β-lactamases. Another example is associations between drugs for which resistance genes are known to be carried on the same plasmid.

Pairwise associations capture much of the clinically relevant information as they reveal the major connections between the drugs. However, they are less helpful in shedding light on underlying mechanism as they do not reveal more complex relationships (interactions) involving multiple drugs. With this in mind this paper examines the interaction structure of antibiotic resistance. It does so by making use of log-linear models for contingency tables, exact conditional testing, and Bayesian inference.

Materials and Methods

The dataset

One of the most comprehensive sources of data on antimicrobial resistance in the United States is that collected by the National Antimicrobial Monitoring System (NARMS). Since 1996, NARMS, a collaboration among the United States

Department of Agriculture, Food and Drug Administration and Center for Disease Control and Prevention, has been monitoring antimicrobial resistance in slaughter houses, retail meat, and human enteric bacteria. It monitors antibiotic resistance of *Escherichia, Enterococcus, Campylobacter* and *Salmonella* isolated from beef, chicken, turkey and pork at slaughter and retail. Resistance is also monitored in human enteric *Campylobacter* and *Salmonella*. NARMS reports resistance as minimum inhibitory concentrations along with guidelines for setting resistance thresholds.

For the purposes of this study the publically available data sheets were downloaded from the NARMS website. Susceptibility status was determined using the NARMS guidelines. The dataset consisted of slaughter and retail data from the years 2011 through 2013 as these were the three most recent years for which there were data at both stages. Chicken was chosen as the host and *E. coli* as the bacteria since this maximized sample size. In order to appear in the analysis a drug had to be present in more than 80% of all samples, and a sample had to be tested for each such drug. All analyses were carried out in Python (Van Rossum, 1995). Regressions were done using the Statsmodels package (Seabold, 2010). Hypothesis tests were done using Scipy (Perez, 2011).

Log linear model

One standard way to infer interactions like that in Table 1 is using log-linear models for contingency tables (Agresti, 2002, pp. 314). In these models the expected count in each cell of the table is modeled as a function of the resistance and

Table 1: Hypothetical contingency tables representing the counts for various susceptible/resistant (S/R) combinations of three drugs X, Y and Z. Table 1a is for when drug Z is susceptible and Table 1b is for when drug Z is resistant.

a.)

		Y	
		S	R
X	S	190	100
	R	10	100

b.)

		Y	
		S	R
X	S	15	0
	R	185	4

susceptibility pattern among the drugs. For example, if there are three drugs (X, Y and Z), the most general model is given by equation 1

$$\log \mu_{ijk} = \lambda + \lambda_i^X + \lambda_j^Y + \lambda_k^Z + \lambda_{ij}^{XY} + \lambda_{ik}^{XZ} + \lambda_{jk}^{YZ} + \lambda_{ijk}^{XYZ} \qquad (1)$$

where μ is the expected cell count, and the indices, i, j, k, take values 1 and 2 for susceptible and resistant, respectively. In order for the model to be identifiable, all parameters with a 1 subscript are set to zero. The parameter λ is then the log expected cell count of cell 111. The last coefficient in the model is the difference in the log odds-ratio for the two partial tables for X and Y, one when Z is resistant and one when Z is susceptible. This coefficient is zero only if there is no 3-way interaction.

Inference concerning coefficients in log-linear models is typically done by fitting the model via maximum likelihood, and then testing hypotheses about coefficients using likelihood-ratio or Wald statistics. The null distributions of these test statistics are approximately chi-squared provided most of the counts in the contingency table are not too small. For example, a rule of thumb that is often used

when testing for association in a 2 by 2 table is that all the estimated expected counts should be at least 5 (Cochran, 1954). Unfortunately, this assumption is often violated in our data. One example of this is a four-way interaction among amoxicillin-clavulanic acid, ceftriaxone, cefoxitin, and ceftiofur for which the contingency table is shown in Table 2. In these cases the interactions will not be reflected in the log-linear model fit. Table 3 shows this for the simpler case of drugs X, Y and Z, where the interaction term is not significant. This is due to the failure of the chi-squared approximation to the null distribution of the Wald statistic caused by the low counts (and some zeros) in the contingency table.

Exact conditional testing

The most common way to overcome violations of the large expected counts assumption necessary for asymptotic inference concerning coefficients in log-linear models is to carry out an exact conditional test. The simplest and most famous example of an exact test for associations in a contingency table is Fisher's exact test for two-way associations between binary variables (Fisher, 1935; Irwin, 1935). Consider a general 2-way table like that of Table 4. If the row and column sums are fixed, the entire table can be determined from a single known cell value. Assuming multinomial sampling, if there is no association between the two variables, the count in the (1,1)-cell has a hypergeometric distribution given in equation 2.

$$p(n_{11}) = \frac{\binom{n_{.1}}{n_{11}}\binom{n_{.2}}{n_{1.}-n_{11}}}{\sum_{u=l}^{h}\binom{n_{.1}}{u}\binom{n_{.2}}{n_{1.}-u}} \qquad (2)$$

Table 2: Contingency table for the four-way interaction among amoxicillin-clavulanic acid, ceftriaxone, cefoxitin, and ceftiofur. S signifies susceptible and R signifies resistant.

Ceftiofur=S

Amoxicillin-Clavulanic Acid								
S					R			
		Cefoxitin				Cefoxitin		
		S	R			S	R	
Ceftriaxone	S	2437	3		Ceftriaxone	S	5	13
	R	3	0			R	2	24

Ceftiofur=R

Amoxicillin-Clavulanic Acid								
S					R			
		Cefoxitin				Cefoxitin		
		S	R			S	R	
Ceftriaxone	S	0	0		Ceftriaxone	S	0	0
	R	3	0			R	1	200

Table 3: Results of fitting a log-linear model to the three-way contingency table for hypothetical drugs X, Y and Z.

	Coefficient	Standard Error	p-value
Intercept	5.247	0.0726	<.000001
X	-2.944	0.324	<.000001
Y	-0.642	0.124	<.000001
Z	-2.539	0.268	<.000001
X:Y	2.944	0.354	<.000001
X:Z	5.457	0.421	<.000001
Y:Z	-24.37	4,225	1.0
X:Y:Z	18.23	4,225	1.0

Table 4: Notation for a generic contingency table. S signifies susceptible and R signifies resistant.

		Y		
		S	R	
X	S	n_{11}	n_{12}	$n_{1.}$
	R	n_{21}	n_{22}	$n_{2.}$
		$n_{.1}$	$n_{.2}$	$n_{..}$

where $l = \max(0, n_{.1} + n_{1.} - n_{..})$ and $h = \min(n_{.1}, n_{1.})$. The p-value for Fisher's exact test is usually defined as the cumulative probability of all tables as or less probable than the one we observed. The logic of this test can be extended to test for three way interactions as long as we fix the additional margin so that the entire table can be determined from a single cell count value. The null distribution for this test, a special case of Zelen's test for no interaction in a 2x2xK table (Zelen, 1971), is given in equation 3.

$$p(n_{111}) = \frac{\binom{n_{.11}}{n_{111}}\binom{n_{.21}}{n_{1.1}-n_{111}}\binom{n_{.12}}{n_{11.}-n_{111}}\binom{n_{.22}}{n_{1.2}-n_{11.}+n_{111}}}{\sum_{u=l}^{h}\binom{n_{.11}}{u}\binom{n_{.21}}{n_{1.1}-u}\binom{n_{.12}}{n_{11.}-u}\binom{n_{.22}}{n_{1.2}-n_{11.}+u}} \qquad (3)$$

where $l = \max(0, n_{.11}+n_{1.1}-n_{..1}, n_{.11}+n_{11.}-n_{.1.}, n_{11.}+n_{1.1}-n_{1..})$ and $h = \min(n_{.11}, n_{1.1}, n_{11.}, n_{.11}+n_{1.1}+n_{11.}-n_{..1}-n_{.1.}-n_{1..}+n_{...})$. These bounds arise from applying the bounds in Fisher's exact test to the 2x2 "slices" that contain the 111 and 112 cells.

While exact conditional tests can be used even if some of the counts are small, in extreme situations conditioning on the table margins can remove the information about the association of interest. For example, there are only 5 tables with the same marginal totals as the 2x2x2 table given in Table 1. In this instance the 111 cell can take values 190 to 194, and the observed value, 190, has the highest null probability equal to 0.983. Clearly this "exact" test fails to reject the hypothesis of no interaction.

A Bayesian approach

To address the problems that result from conditioning on all of the table margins we propose a Bayesian test conditioned only on the total number of observations. Conditional on the total number of observations the vector of cell counts

in the contingency table is a multinomial variable with cell probabilities π_{ijk}. The conjugate prior for the multinomial distribution is the Dirichlet distribution (Kotz et al., 2000, pp. 485). This implies that the posterior distribution of the vector of cell probabilities also has a Dirichlet distribution. For example, in the 3-way table case, with prior concentration parameters α_{ijk}, the posterior distribution is given by equation 4.

$$p(\pi|\alpha, n_{ijk}) = \frac{\Gamma(\sum_{IJK}(\alpha_{ijk}+n_{ijk}))}{\prod_{IJK}\Gamma(\alpha_{ijk}+n_{ijk})}\prod_{IJK}\pi_{ijk}^{\alpha_{ijk}+n_{ijk}-1} \qquad (4)$$

That is, the posterior concentration parameters are the sum of the prior values and their corresponding cell counts. In our analyses, we use an uninformative Dirichlet prior with concentration parameters all set to 1, or equivalently a uniform distribution over the K-dimensional simplex, where K is the number of drugs under consideration.

The posterior distribution of any function of the cell probabilities can be estimated based on 1 million draws from this Dirichlet distribution. For example, to test for interaction in a 3-way table we can estimate the posterior distribution of the interaction coefficient

$$\lambda_{ijk}^{XYZ} = log\left(\frac{\pi_{111}\pi_{221}}{\pi_{211}\pi_{121}}\right) - log\left(\frac{\pi_{112}\pi_{222}}{\pi_{212}\pi_{122}}\right) \qquad (5)$$

and determine if 0 is a plausible value. The posterior distribution for the three-way interaction in table 1 is given in Figure 1 and shows that the Bayesian procedure is able to detect this interaction because the entire posterior distribution of the interaction coefficient lies to one side of 0. In what follows we define the significance of a coefficient as 1 minus the coverage of the largest posterior credible interval that does

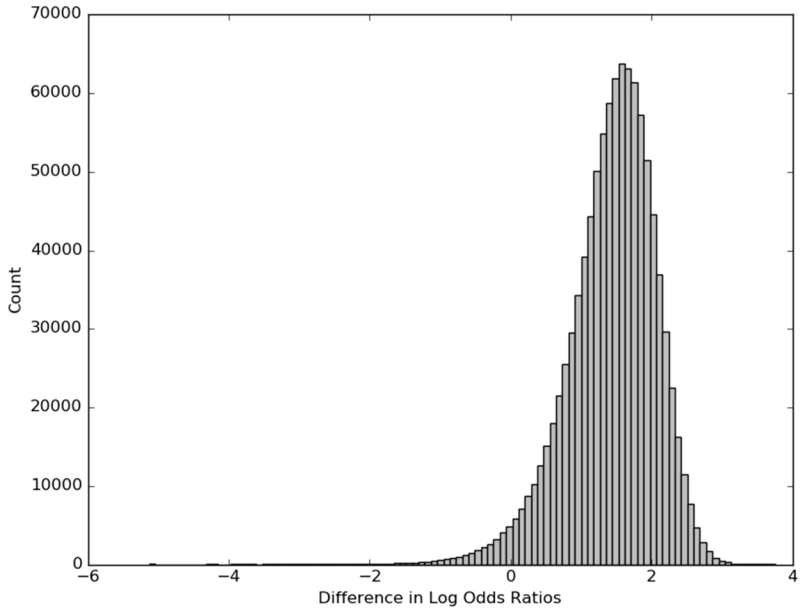


Figure 1: Posterior distribution of the interaction coefficient of hypothetical drugs X, Y and Z.

not contain 0. We choose .01 as our significance threshold to account for multiple testing, but many of the probabilities are substantially lower.

Results

We first tested all possible pairs of drugs using the Dirichlet-multinomial model described above. At the .01 significance threshold, there was an association between almost every pair of drugs. Lowering the threshold to .00001 provides a more informative picture of the pairwise associations and results in the graph in Figure 2. This graph suggests that dependences between the drugs can be decomposed into two dense sub-networks with a few ancillary drugs, a finding consistent with that of Love et al., (2016). The first subnetwork consists of the β-lactams ampicillin, ceftriaxone,

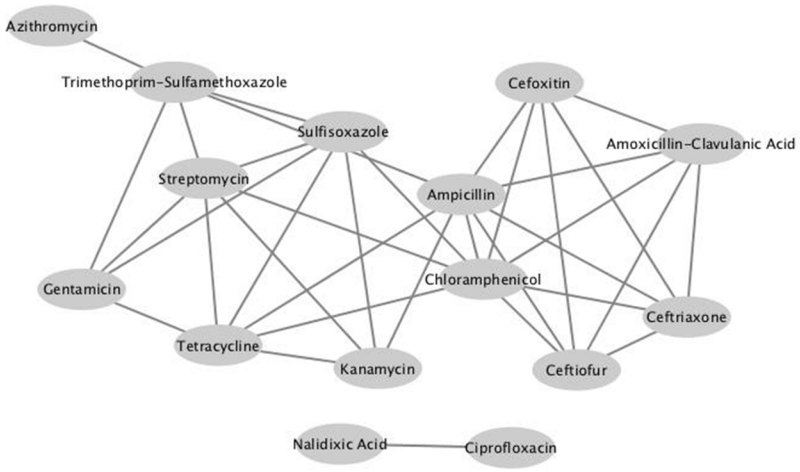


Figure 2: Pairwise associations between antibiotics. This network can be decomposed into 2 subnetworks, the one of the left composed of β-lactams the other composed of a mixture of drug classes.

cefoxitin, amoxicillin-clavulanic acid, and ceftiofur, as well as the phenicol chloramphenicol. The second subnetwork bridges from chloramphenicol and ampicillin to the aminoglycosides streptomycin, gentamicin, and kanamycin, tetracycline, and the sulfonamides sulfisoxazole and trimethoprim-sulfamethoxazole. Additionally, the macrolid azithromycin is attached to trimethoprim-sulfamethoxazole alone and the fluoroquinolones nalidixic acid and ciprofloxacin are connected to one another. The dense structure of each of the subnetworks suggests they might contain multi-drug interactions. These subnetworks are small enough that it is feasible to test all possible interactions within each of them.

The first sub-network is characterized by a significant β-lactam only four-way interaction among amoxicillin-clavulanic acid, ceftriaxone, cefoxitin and ceftiofur with posterior probability 0.00583. This interaction results from a hierarchy where the

resistance status of amoxicillin-clavulanic acid, ceftriaxone, and cefoxitin are generally identical and susceptibility to any of these three implies susceptibility to ceftiofur. The near equivalence among amoxicillin-clavulanic acid, ceftriaxone, and cefoxitin is shown in Table 5 with almost all isolates being susceptible to all three antibiotics or resistant to all three antibiotics. The hierarchical relationship among amoxicillin-clavulanic acid, ceftriaxone, and ceftiofur can be seen in Table 6 where isolates susceptible to amoxicillin-clavulanic acid or ceftriaxone are almost always susceptible to ceftiofur.

The second subnetwork is characterized by 2 four-way interactions and 10 three-way interactions that are not contained in a four-way interaction as shown in Table 7. All of these interactions include at least 2 different antibiotic classes. Both four way interactions involve 4 different drug classes, the aminoglycoside gentamicin, tetracycline, the β-lactam ampicillin, and a sulfonamide either trimethoprim-sulfamethoxazole or sulfisoxazole. Many of the three factor interactions are also among 3 different drug classes for example the interaction among the aminoglycoside streptomycin, the sulfonamide sulfisoxazole and tetracycline. The contingency tables underlying these interactions, like that for the streptomycin:sulfisoxazole:tetracycline interaction shown in Table 8, tend to have more nuanced count patterns than the all or nothing hierarches of the β-lactam interaction.

Discussion

Three approaches to interaction testing

The results of applying the three different approaches to inferring higher order interactions reveal the strengths and weaknesses of these different methods as well as some unique features of antibiotic co-resistance relationships.

The inability of approximate chi-squared tests to detect obvious relationships like that among amoxicillin-clavulanic acid, ceftriaxone, cefoxitin and ceftiofur highlights the sensitivity of such approximations to zero (and small) cell counts. The failure of exact testing to detect the interaction is due to the severe restriction on the sample space imposed by conditioning on table margins in extremely sparse contingency tables. In such cases, the margins of a contingency table can contain some of, and possibly all, the information about associations. The Bayesian approach by contrast side steps both problems and leads to robust inferences based on exact marginal posterior distributions. This approach has clear advantages in terms of making the inference possible in this setting. The method does require sampling from the posterior distribution for each interaction that is tested. However, this is straightforward in this setting because the posteriors are all Dirichlet distributions that are easy to simulate from.

The small cell counts and restrictions on sample space that plague the approximate and exact conditional methods have a common source fundamental to antimicrobial resistance. They arise from structured relationships between antibiotics like the hierarchy among the β-lactams. These relationships make some resistance combinations extremely rare and create cells and sometimes even marginal counts that are near zero.

Table 5: Contingency table showing the equivalency among amoxicillin-clavulanic acid, ceftriaxone, and cefoxitin. Almost all isolates are either susceptible to all three drugs or resistant to all three. S signifies susceptible and R signifies resistant.

Amoxicillin-Clavulanic Acid								
S					R			
		Cefoxitin					Cefoxitin	
		S	R				S	R
Ceftriaxone	S	2437	3		Ceftriaxone	S	5	13
	R	6	0			R	3	224

Table 6: Contingency table showing the hierarchy among amoxicillin-clavulanic acid, ceftriaxone, and ceftiofur. Almost all isolates susceptible to either amoxicillin-clavulanic acid or ceftriaxone are also susceptible to ceftiofur. S signifies susceptible and R signifies resistant.

Amoxicillin-Clavulanic Acid								
S					R			
		Ceftiofur					Ceftiofur	
		S	R				S	R
Ceftriaxone	S	2440	0		Ceftriaxone	S	18	0
	R	3	3			R	26	201

Table 7: Significance of interactions underlying a multi-drug resistance subnetwork composed of several antibiotic classes. A) Four-way interactions. B) Three-way interactions not part of a four-way interaction.

A)

Interaction	Significance Level
Gentamicin:Sulfisoxazole:Tetracycline:Ampicillin	0.000070
Trimethoprim-Sulfamethoxazole:Gentamicin:Tetracycline:Ampicillin	0.00444

B)

Interaction	Significance Level
Gentamicin:Streptomycin:Tetracycline	<.000001
Streptomycin:Sulfisoxazole:Tetracycline	<.000001
Gentamicin:Streptomycin:Ampicillin	<.000001
Gentamicin:Sulfisoxazole:Kanamycin	0.0000340
Trimethoprim-Sulfamethoxazole:Streptomycin:Tetracycline	0.0000560
Streptomycin:Sulfisoxazole:Chloramphenicol	0.00155
Tetracycline:Kanamycin:Ampicillin	0.00183
Gentamicin:Tetracycline:Ampicillin	0.00528
Streptomycin:Sulfisoxazole:Kanamycin	0.00703
Sulfisoxazole:Tetracycline:Kanamycin	0.00799

Table 8: Contingency table for the three-way interaction among streptomycin, sulfisoxazole, and tetracycline. All cell counts in this table are large indicating a non-hierarchical relationship between these 3 drugs. S signifies susceptible and R signifies resistant.

Streptomycin								
S					R			
		Tetracycline				Tetracycline		
		S	R			S	R	
Sulfisoxazole	S	883	292		Sulfisoxazole	S	138	133
	R	131	208			R	357	549

The structure of multi-drug resistance

Application of the Bayesian test reveals that patterns of multidrug resistance can be broken down into two sub-networks with two distinct sets of features. One subnetwork consists of a single antibiotic class and has an interaction structure that results from a hierarchical relationship. The other contains a number of antibiotic classes. The interactions in this subnetwork are all between antibiotic classes and are characterized by dense contingency tables with more subtle patterns to their counts.

The β-lactam sub-network

All of the drugs in the first network are β-lactams with the exception of chloramphenicol which primarily serves to connect this subnetwork with the second subnetwork. β-lactams are antibiotics that work by inhibiting the synthesis of

peptidoglycan, which is necessary for, among other things, constructing the bacterial cell wall (Brunton, 2011, pp. 1480). This subnetwork is underpinned by a single β-lactam only interaction resulting from a hierarchy in which amoxicillin-clavulanic acid, ceftriaxone, cefoxitin, have nearly identical resistance patters and susceptibility to any one of these three implies susceptibility to ceftiofur. This hierarchy is in agreement with the known pharmacology of these drugs. The first tier of antibiotics is composed of drugs that are not affected by the presence of β-lactamases, enzymes focused on metabolizing β-lactams. Amoxicillin-clavulanic acid explicitly contains the β-lactamase inhibitor clavulanic acid and ceftriaxone as well as cefoxitin are known to be inherently resistant to β-lactamases (Brunton, 2011, pp. 1480). The second tier, ceftiofur, has the least amount of resistance of the β-lactams in this dataset.

The other sub-network

The second sub-network is made up of aminoglycosides which inhibit protein synthesis by binding to the 30S ribosomal subunit, tetracycline which also functions by binding to the ribosome but binds specifically to the A site, phenicols which inhibit ribosomal peptydal transferase activity, sulphafonamids which inhibit folate synthesis and β-lactams (Brunton, 2011). The fact that the interactions in this subnetwork link such disparate antibiotic functions make it unlikely that they result from a single pharmacological mechanism like the β-lactam subnetwork. Also, while multi-drug resistance can result from pumps that move a number of antibiotics out of the bacterial cytoplasm (Brunton, 2011, 1480) the large difference in the structures of the linked antibiotics make this unlikely.

Two mechanisms that could explain the interactions in this second network are linked genes and linked selection pressures. Integrons could very easily link together genes for resistance to different antibiotic classes. In fact, some of the inferred linkages such as that between sulfisoxazole and streptomycin and are known to be present in integrons (Fluit, 2004). The dense nature of the contingency tables for these interactions would naturally result from a large variety of resistance cassettes with overlapping patterns of genetic linkage. These patterns could also result from many overlapping hierarchies of antibiotic administration as an ensemble of clinicians respond to developing patterns of multidrug resistance.

Comparison to previous work

In this paper, we take a log-linear model approach to explore interactions among the resistance or susceptibility of various antibiotics. Love et al. (2016) applied the graphical lasso to continuous minimum inhibitory concentration values from a similar dataset to create networks of dependencies between drugs. This study similarly found that the dependencies could largely be divided into two sub-networks, one for the β-lactams and another for the other drugs. The main difference between the two resulting networks is that the ones presented here are much denser. This may be the result of differences in the effective significance level of the two tests. While the hypothesis testing approach employed here uses an explicit 1% significance level the graphical lasso relies on a tunable parameter to determine how sparse or dense the resulting network should be.

Conclusion

Antibiotic resistance is a serious health threat that has captured the attention of politicians, regulators and clinicians. Even more threating than the development of resistance to a single antibiotic is the development of simultaneous resistance to multiple drugs as it increases the likelihood of an infection that cannot be treated with any known antibiotic. To understand these multidrug resistances, we must understand the various interactions that characterize them. By employing a Bayesian hypothesis testing approach, we have explored not only the pairwise associations but also the interactions involving three or more drugs. This analysis has revealed that multidrug resistance is characterized by a broad range of interaction patterns. One example involves a clear-cut and biologically meaningful hierarchical relationship in a single drug family. This relationship creates an extreme sparsity pattern that cannot be analyzed using standard asymptotic chi-squared tests or even exact conditional tests, but which can be detected using a Bayesian approach described in this paper. In other cases the interaction pattern is more complex, with overlapping sets of antibiotics from a variety of drug classes. These results validate concerns over the complex etiology of multidrug resistance. They also suggest the need for detailed population genetic studies to tease apart their molecular underpinnings so that the extent of multidrug resistance can be controlled.

REFERENCES

Print References

Agresti, A., 2002. Catagorical Data Analysis, 2nd edition. John Wiley & Sons, Hoboken, New Jersey.

Brunton, L. L., Chabner, B.A., Knollmann, B. C., 2011. Goodman and Gilman's the pharmacological basis of therapeutics, 12th edition. McGraw Hill Medical, New York, New York.

Cochran, W. G., 1954. Some methods of strengthening the common chi-square tests. Biom. 10, 417-451.

European Commission Directorate-General for Health and Consumers., 2011, Action plan against the rising threats from antimicrobial resistance.

Fisher, R. A., 1935. The logic of inductive inference. J. of the R. Stat. Soc. Ser. A. 98, 39–54.

Fluit, A. C., Schmitz, F. J., 2004. Resistance integrons and super-integrons. Clin. Microbiol. Infect. 10, 272–288.

Irwin J. O., 1935. Tests of significance for differences between percentages based on small numbers. Metron. 12, 83–94.

Kotz, S., Balakrishnan, N., Johnson, N. L., 2000. Continuous Multivariate Distributions. Volume 1: Models and Applications. John Wiley & Sons, New York.

Love, W. J., Zawack, K. A., Booth, J. G., Grohn, Y. T., Lanzas, C., 2016. Markov Networks of Collateral Resistance: National Antimicrobial Resistance Monitoring System Surveillance Results from *Escherichia coli* Isolates, 2004-2012. PLoS. Comput. Biol. 12(11), e1005160. doi:10.1371/journal.pcbi.1005160.

Ludwig, A., Berthiaume, P., Boerlin, P., Gow, S., Léger, D., Lewis F.I., 2013. Identifying associations in *Escherichia coli* antimicrobial resistance patterns using additive Bayesian networks. Prev. Vet. Med. 110, 64-75. http://dx.doi.org/10.1016/j.prevetmed.2013.02.005.

Perez, F., Granger, B. E., Hunter, J. D., 2011. Python: An Ecosystem for Scientific Computing. Comput. in Sci. Eng. 13, 2, 13-21. 10.1109/MCSE.2010.119.

Seabold, S. Perktold, J., 2010. Statsmodels: Econometric and statistical modeling with python. Proc. of the 9th Python in Sci. Conf.

Van Rossum, G., 1995. Python tutorial. Technical Report CS-R9526, Centrum voor Wiskunde en Informatica (CWI).

White House, 2015, National action plan for combating antibiotic-resistant bacteria.

Zelen, M., 1971. The Analysis of Several 2x2 Contingency Tables. Biom. 58, 129–137.

Web References

CDC. https://www.cdc.gov/drugresistance. 11-25-2016

European Commision Directorate-Generals on Health and Food Safety. http://ec.europa.eu/dgs/health_food-safety/amr/index_en.htm, 11-25-2016

[Dataset] National Antimicrobial Monitoring system. http://www.fda.gov/AnimalVeterinary/SafetyHealth/AntimicrobialResistance/NationalAntimicrobialResistanceMonitoringSystem/ucm416741.htm. 11-25-2016

CHAPTER 4

ANALYSIS OF VARIATION IN MULTIDRUG INTERACTIONS INFERED BASED ON THE NATIONAL ANTIMICRIBIAL MONITORING SYSTEM

Introduction

Antimicrobial resistance develops through evolution. Resistance traits enter a population through mutation and gene transfer and then grow or shrink in proportion based on the fitness of the organisms that possess them. As such antimicrobial resistance is affected by the myriad variables that influence microbial fitness. This includes factors related to antimicrobials such as the amount of antimicrobials present, but also factors unrelated to antimicrobials like the available carbon source. This makes it difficult to make sense of long term trends in resistance as latent variables inflate the variation in measured resistance. The problem can be compounded in surveillance programs that cover large geographic areas and long periods of time. In the United States such surveillance is carried out by the National Antimicrobial Monitoring System (NARMS). Analysis of resistance to single antibiotics in the NARMS data set has shown significant amounts of over dispersion (Zawack, 2016). More concerning to public health than resistance to a single antimicrobial is simultaneous resistance to multiple antimicrobials. This paper examines the variation in the NARMS dataset, focusing on multidrug resistance variation in the animal production surveillance components.

There are many factors that can potentially influence antimicrobial resistance. Beginning on the farm resistance may be influenced by the particular husbandry

practices. Cow-Calf farms have been shown to have different levels of resistance than feed lots (Carson, 2008) and differences in resistance patterns have been detected among dairy, beef, and veal farms (Catry, 2016). Also, organic production processes have been shown to have lower resistance levels than conventional approaches (Miranda, 2008). Husbandry practices may also interact with animal age as some authors have found increases in resistance with age for cattle (Carson, 2008) while others have found decreases (Gow, 2008). Moreover, because there is evidence that animal microbiomes change over time (Holman, 2015), resistance may also be affected by the particular microbial make up of a given environment. In light of this, resistance would then be affected by any factor that influences the microbe content of an environment, such as temperature, moisture, and nutrient sources. At slaughter such a dependency on microbe content indicates that resistance may be influenced by overall hygiene (Lerma, 2014a). Additionally, specific hygiene practices can also influence resistance as analyses of genetic relatedness of bacteria in different locations in a slaughterhouse indicate that many of the bacteria throughout the slaughter facility, including those on products headed to retailers, come directly from the live animals (Lerma, 2014b). Disinfectant usage may also affect resistance. These effects may be indirect, by affecting general microbial ecology, or more direct, through linkages between antimicrobial resistance and disinfectant resistance (Lerma, 2015). These factors are likely to play similar roles in retail facilities.

NARMS has three components, two covering animal production, and a third covering human medicine (NARMS, 2016). The first animal production component monitors animal slaughter facilities and the second monitors retail meat. The

slaughter facility surveillance obtains its samples from the food safety monitoring of slaughter houses carried out by the United States Department of Agriculture's Food Safety Inspection Service (FSIS). Food safety, not antimicrobial resistance is the mandate of FSIS, so its sampling procedures are targeted not at resistance surveillance, but at food safety and change as these food safety needs change. The slaughter component of NARMS began in 1997. At that time most isolates came from random inspections that sampled each facility almost every year. A small proportion of isolates came from follow-up inspections of facilities that failed initial inspection. There is no data available on which isolates were obtained from which regime. Beginning in 2006 FSIS adopted a risk based approach to focus on facilities with higher rates of salmonella contamination. FSIS data contains information on year, host animal, bacteria, antibiotic, minimum inhibitory concentration as a measure of resistance and that the isolate came from FSIS sampling. There is no data about how the animals were raised or about the hygiene processes in the slaughter house. The collection of retail samples is carried out through FoodNet, a collaboration of federal, state and local food safety officials. In contrast to the slaughter program the retail program is focused solely on antimicrobial resistance surveillance. Retail surveillance began in 2001 at Connecticut, Georgia, Maryland, Minnesota, New Mexico, Oregon, Tennessee, California, Colorado, and New York. It has since expanded to include Louisiana, Missouri, Pennsylvania, Washington, Iowa, Kansas, South Dakota, and Texas. Each Foodnet site selects samples by dividing the zip codes within fifty miles into 4 quadrants and randomly assigning the quadrants across the months. Each month a list of grocery stores is randomly selected from the assigned quadrant and a

total of 40 meat products are sampled from these stores. Retail data contains information on state, year, host animal, bacteria, antibiotic, minimum inhibitory concentration and that the isolate came from retail sampling. There is no data about which slaughter facility the meat came from or about the hygiene processes at the store.

Antibiotic resistance is problematic because it weakens our arsenal to cure infections. Multidrug resistance is even more significant because it represents a compound weakening of this arsenal. One way to quantify multidrug resistance is to categorize each isolate as susceptible or resistant to each drug and then organize these isolates into a multiway contingency table. This approach has been used in the previous chapter to investigate the structure of multidrug resistance. In addition to many others, associations were found among gentamicin, sulfisoxazole, tetracycline and ampicillin.

Here we assess the variability of multidrug resistance in the NARMS dataset. We do this by examining contingency tables categorizing gentamicin, sulfisoxazole, tetracycline and ampicillin resistance of *Escherichia coli* isolates sampled from chicken at slaughter and retail. We also examine effects of this variability on the inference of associations between drug resistances to uncover potential improvements to the NARMS system.

Materials and Methods

The data

For the purposes of this study the publicly available data sheets for the slaughter and retail surveillance were downloaded from the NARMS website. In order

to account for variability due to host and bacteria type we focus here on a single host and bacterial species. Chicken was chosen as the host and *E. coli* as the bacteria because this maximized sample size. We focus on the drugs gentamicin, sulfisoxazole, tetracycline and ampicillin, because susceptibility and resistance to these drugs were previously observed to be associated with one another (Zawack, 2016; Love 2016). These choices produced a dataset covering the years 2004 to 2012. Susceptibility status was determined using the NARMS guidelines. In order to appear in the analysis a sample had to be tested for each of these drugs. The processed data then consisted of a table with isolates as the rows and antimicrobial resistance test results as well as the year and production stage of the isolate in the columns. These data were then in turn organized into contingency tables giving the count of isolates with each resistance pattern for a given setting of year and stage. All data processing and analyses were carried out in Python (Van Rossum, 1995), using the Scipy package (Perez, 2011) for statistical analyses.

Analysis of variation in contingency table cell counts

The most general type of variability in the data is the variability between contingency tables. A higher than expected level of variability could result from systematic trends over time, or excess variation (overdispersion) due to unaccounted for factors like hygiene practices. To test whether the differences in contingency table counts between years was in accord with random multinomial variation with no time trend we compared the cell frequencies from individual years with the aggregate frequency over all years. Specifically we calculated the Pearson goodness-of-fit statistic (Agresti, 2002, pp.22)

$$\sum_{year \in Years} \sum_{cell \in Cells} \frac{n_{year}\left(\hat{\pi}_{year,cell} - \hat{\pi}_{cell}\right)^2}{\hat{\pi}_{cell}} \qquad (1)$$

where $\hat{\pi}$ is an estimated cell proportion, and compared it to a chi-squared distribution with $(years - 1)$ x $(cells - 1)$ degrees of freedom. We did this for slaughter data alone, retail data alone, and the full data set. Large values of the Pearson statistic provide evidence of lack-of-fit (relative to no trend) or overdispersion due to unaccounted for factors.

Overdispersion with respect to univariate measures

It is difficult to distinguish between overdispersion due to unaccounted for factors and a time trend by looking solely at contingency tables because of their multivariate nature. A 2x2x2 table for instance has 8 cells. The (log) odds-ratio is the natural metric for measuring association in 2x2 tables. Higher-order associations (interactions involving three or more drugs) are quantified by contrasts between log odds-ratios in partial 2x2 tables. For example, the most general association model for a 2x2x2 table is given by:

$$\log \mu_{ijk} = \lambda + \lambda_i^X + \lambda_j^Y + \lambda_k^Z + \lambda_{ij}^{XY} + \lambda_{ik}^{XZ} + \lambda_{jk}^{YZ} + \lambda_{ijk}^{XYZ} \qquad (2)$$

where X, Y, and Z denote three different drugs, subscripts i, j, and k indicate the susceptible/resistant combination for the three drugs (1=susceptible, 2=resistant), the $\mu's$ are the expected cell counts and the $\lambda's$ are model coefficients constrained to be zero whenever i, j, or k equal 1 for identifiability. In this case the parameter λ_{222}^{XYZ} is a contrast between log odds-ratios in two partial tables, and quantifies the level of interaction between the three drugs. If λ_{222}^{XYZ}=0, the log odds-ratios for drugs X and Y is the same whether drug Z is resistant or susceptible. On the other hand, if λ_{ijk}^{XYZ}=0.5,

the X-Y odds-ratio is $e^{0.5}$=1.65 times higher when Z is resistant than when it is susceptible.

For a univariate statistic we can measure overdispersion by taking the ratio of observed variance over the years in the data set to expected variance assuming multinomial variation and no time trend, by calculating the test statistic

$$\sum_{year \in Years} \frac{n_{year}\left(f(\hat{\boldsymbol{\pi}}_{year}) - f(\hat{\boldsymbol{\pi}})\right)^2}{\Delta f(\hat{\boldsymbol{\pi}})' \Sigma(\hat{\boldsymbol{\pi}}) \Delta f(\hat{\boldsymbol{\pi}})} \qquad (3)$$

where n is the total number of observations, $\hat{\boldsymbol{\pi}}$ the vector of estimated contingency table cell frequencies, $f()$ is a function of the cell proportions, Δ indicates the gradient, and Σ is a covariance matrix. Under the null hypothesis this test statistic has a chi-squared distribution with Years-1 degrees of freedom. In the special case in which $f()$ is the coefficient for the K-drug association in a 2^K table, the expected variance assuming multinomial variation and no time trend can be approximated using the delta method as

$$\frac{\sum_{cell \in Cells} \frac{1}{\hat{\boldsymbol{\pi}}_{cell}}}{n} \qquad (4)$$

where $\hat{\boldsymbol{\pi}}_{cell}$ denotes an estimated cell frequency in the marginal table obtained by collapsing over years and n is the total sample size. As with the general Pearson statistic, large values relative to this null distribution are an indication of overdispersion.

Asymptotic power analysis

Under multinomial sampling, the ratio of an estimated coefficient to its standard deviation approximately follows a standard normal distribution asymptotically in the null case. In the non-null case the distribution of this ratio

approximately follows a non-central standard normal distribution with a non-centrality parameter given by

$$\Delta = \frac{\lambda}{s(\lambda)} \qquad (5)$$

where λ is the coefficient, and $s(\lambda)$ is the standard error of the estimated coefficient. Equivalently, the square of this statistic approximately follows a non-central chi-squared distribution with non-centrality parameter Δ^2.

One of the primary problems with overdispersion is that it decreases the power to reject a null hypothesis that is in fact false. This is due to the fact that overdispersion inflates the variance of an estimated coefficient by a factor ϕ >1. In this case, one might consider adjusting the non-centrality parameter to

$$\Delta = \frac{\lambda}{s(\lambda)\sqrt{\phi}} \qquad (6)$$

Power simulation

The analysis above assumes the ratio of an estimated coefficient to its standard deviation follows a (possibly non-central) standard normal distribution. This may be essentially true when the expected counts in all cells are sufficiently large, but will hold less exactly for tables with small cell counts resulting from large interaction coefficients, or high levels of overdispersion. Figure 1 shows qq-plots comparing the theoretical normal distribution for a given sample size and log-linear model with that resulting from a process similar to the one generating the NARMS data. Specifically, cell frequencies are drawn from a Direchlet distribution with parameters chosen to produce expected counts satisfying the given model with a specified level of overdispersion and then cell counts are drawn from a multinomial with the just drawn

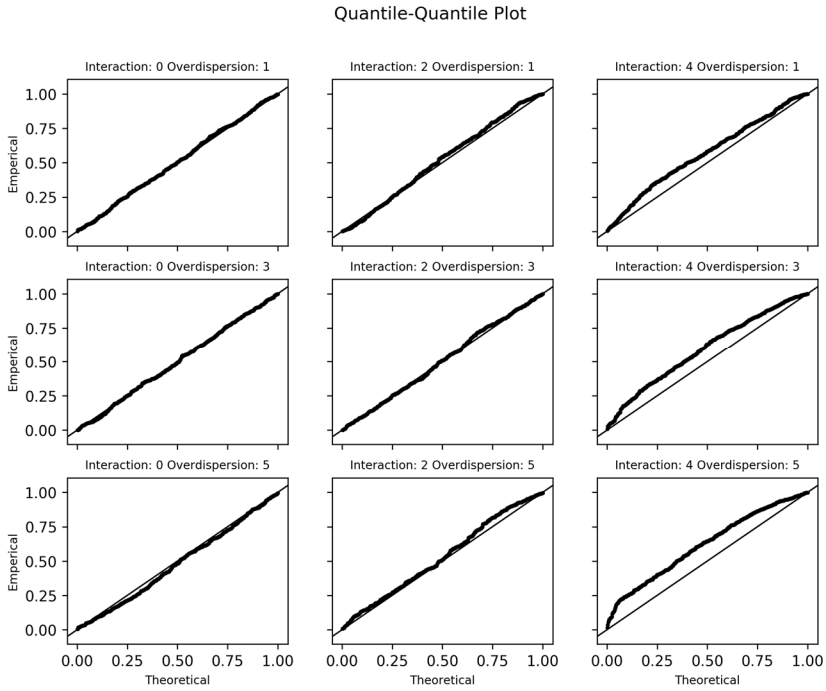


Figure 1: Quantile-quantile plots comparing the theoretical normal distribution for the highest order interaction term in a log linear model of a contingency table to a Dirichlet-multinomial distribution that more accurately models the process generating the underlying contingency table. The null model in the upper left corner has a total count of 1000 and form $\log \mu_{ijk} = -1.836 - .4055 + .04886 - .8598 + .4055 + .4055 + .4055$ which corresponds to a contingency table for three variables with resistance probabilities .4, .5, and .6 where all two-way odds ratios are 1.5.

cell frequencies and the chosen sample size. The plots begin with a null model having a total count of 1000 and form $\log \mu_{ijk} = -1.836 - .4055 + .04886 - .8598 + .4055 + .4055 + .4055$ which corresponds to a contingency table for three variables with resistance probabilities .4, .5, and .6 where all two-way odds ratios are 1.5. As both the interaction coefficient and the amount of overdispersion increase the qq-plots leave the unit slope diagonal to a larger degree indicating that the distributions are becoming more different.

To account for possible inaccuracy of the normal/chisquared approximation, we recalculated the power via the Dirichlet-multinomial simulation. The critical value for a test of the null model (at, say, significance level alpha) was determined as the empirical (1-alpha)-quantile of the test-statistic. The power to detect the alternatives to the null was estimated by the proportion of test-statistics simulated under the alternative that exceeded the null critical value.

Results

Analysis of multivariate overdispersion

For each of the 7 years at slaughter, retail and the combined data there are 5 multiway tables to consider, the four-way table for gentamicin, sulfisoxazole, tetracycline and ampicillin and the 4 subsidiary three-way tables. With the exception of 2007 when there was no testing for sulfisoxazole, sample sizes range from 600 to 2200 a year for slaughter, 300 to 400 at retail, and 300 to 2600 over all (Table 1). At slaughter and over all the probabilities of observing as much or more variability than seen in the data by chance alone is less than .00001 (Table 2). The p-values for retail

Table 1. Sample sizes by year and production stage.

Year	Slaughter	Retail	Total
2004	1695	400	2095
2005	2232	393	2625
2006	1357	418	1775
2007	0	299	299
2008	986	306	1292
2009	876	314	1190
2010	941	357	1298
2011	613	341	954
2012	990	386	1376

Table 2. P-values for the test of overdispersion in year to year variation of cell counts in multiway antimicrobial resistance interaction contingency tables.

Interaction	Slaughter	Retail	Total
Gentamicin:Sulfisoxazole:Tetracycline	<.000001	.0000691	<.000001
Gentamicin:Sulfisoxazole: Ampicillin	<.000001	0.000611	<.000001
Gentamicin:Tetracycline:Ampicillin	<.000001	.0000223	<.000001
Sulfisoxazole:Tetracycline:Ampicillin	<.000001	.0000307	<.000001
Gentamicin:Sulfisoxazole:Tetracycline: Ampicillin	<.000001	0.000318	<.000001

are slightly higher ranging from .0000975 for Gentamicin:Sulfisoxazole:Tetracycline to 0.00166 for the four-way table.

Overdispersion of the interaction parameters

Consistent with the multinomial analysis the interaction parameters also exhibit a greater than expected amount of variability (Table 2, Table 3, Table 4). The three-way interaction coefficient among gentamicin, sulfisoxazole, and tetracycline has no trend at any stage (Figure 2). It is generally positive with a mean of 0.859 at slaughter, 0.970 at retail, and .883 in the full dataset, but it does have a negative estimate in 2008 at retail. The overdispersion factor for this interaction is 3.05 at slaughter, 3.11 at retail and 4.83 in the combined data. The three-way interaction among gentamicin, sulfisoxazole and ampicillin is generally positive with no trend at any stage, but 2005 has a negative estimate at all three sages (Figure 3). The mean interaction parameter is 1.03 at slaughter, 2.19 at retail, and 1.34 over all. The overdispersion factors are 3.15 at slaughter, 4.14 at retail and 4.65 in the combined data. The three-factor interaction for gentamicin, tetracycline, and ampicillin has one negative estimate in 2004 at retail, otherwise it is positive with no trend (Figure 4). The interaction parameter means are .46 at slaughter, .75 at retail, and .53 overall. The overdispersion factors are 2.01 at slaughter, 3.04 at retail, and 2.65 combined. The three factor sulfisoxazole, tetracycline, ampicillin interaction is fairly close to 0 with no trend over time (Figure 5). Its mean is .32 at slaughter, .16 at retail, and .30 overall. The overdispersion factors are 3.00 at slaughter, 1.93 at retail, and 2.43 overall. The four-factor interaction among gentamicin, sulfisoxazole, tetracycline, and

ampicillin has an increasing trend from -2.5 to 2.5 at slaughter and overall (Figure 6), but it must

Table 3. Overdispersion factor for the highest order interaction parameter in the log-liner model of the multidrug contingency table.

Interaction	Slaughter	Retail	Total
Gentamicin:Sulfisoxazole:Tetracycline:	3.05	3.11	4.83
Gentamicin:Sulfisoxazole:Ampicillin	3.15	4.14	4.65
Gentamicin:Tetracycline:Ampicillin	2.01	3.04	2.65
Sulfisoxazole:Tetracycline:Ampicillin	3.00	1.93	2.43
Gentamicin:Sulfisoxazole:Tetracycline: Ampicillin	5.87	3.09	5.77

Table 4. Overdispersion test p-values for the highest order interaction parameter in the log-liner model of the multidrug contingency table.

Interaction	Slaughter	Retail	Total
Gentamicin:Sulfisoxazole:Tetracycline	<.000001	<.000001	<.000001
Gentamicin:Sulfisoxazole: Ampicillin	<.000001	<.000001	<.000001
Gentamicin:Tetracycline:Ampicillin	<.000001	<.000001	<.000001
Sulfisoxazole:Tetracycline:Ampicillin	<.000001	<.000001	<.000001
Gentamicin:Sulfisoxazole:Tetracycline: Ampicillin	<.000001	<.000001	<.000001

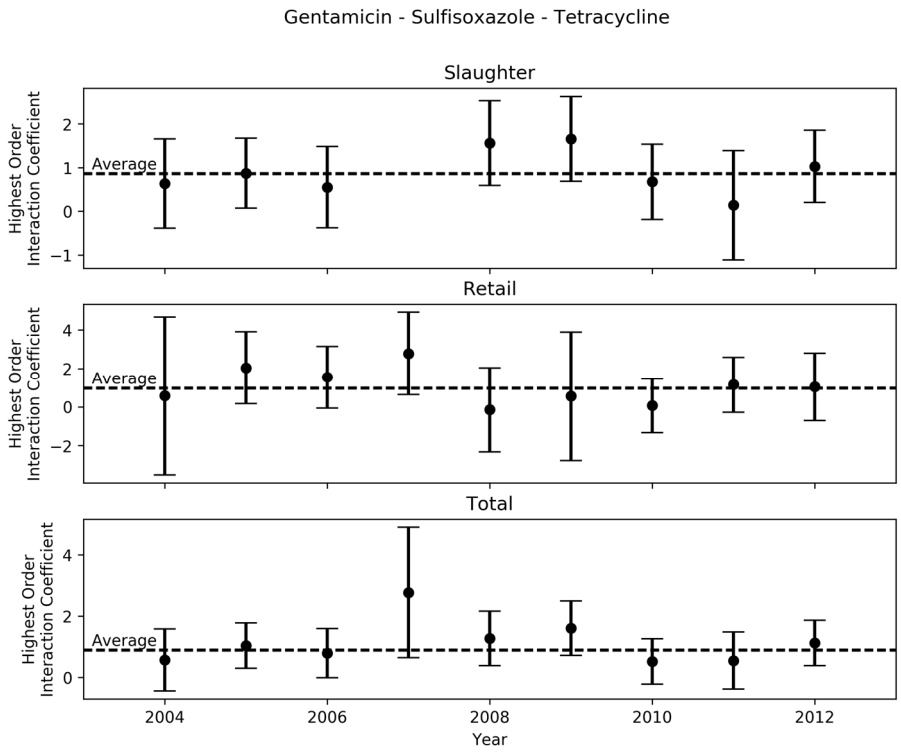


Figure 2: Plot of the highest order interaction parameter in the log-liner model of the gentamicin, sulfisoxazole, and tetracycline multidrug contingency table by year and stage.

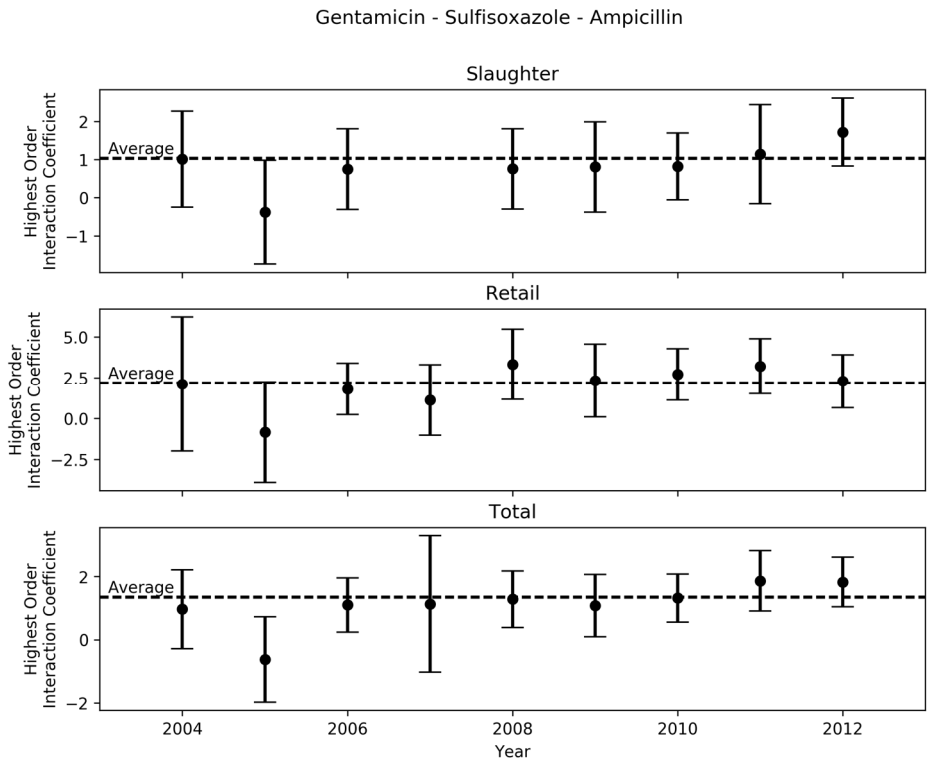


Figure 3: Plot of the highest order interaction parameter in the log-liner model of the gentamicin, sulfisoxazole, and ampicillin multidrug contingency table by year and stage.

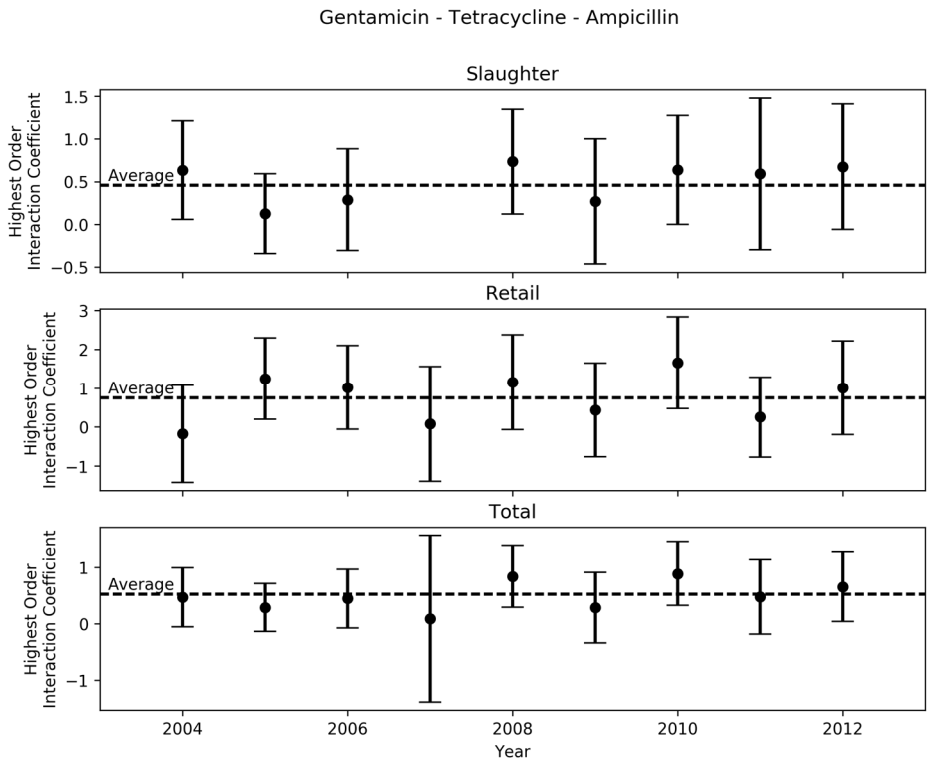


Figure 4: Plot of the highest order interaction parameter in the log-liner model of the gentamicin, tetracycline, and ampicillin multidrug contingency table by year and stage.

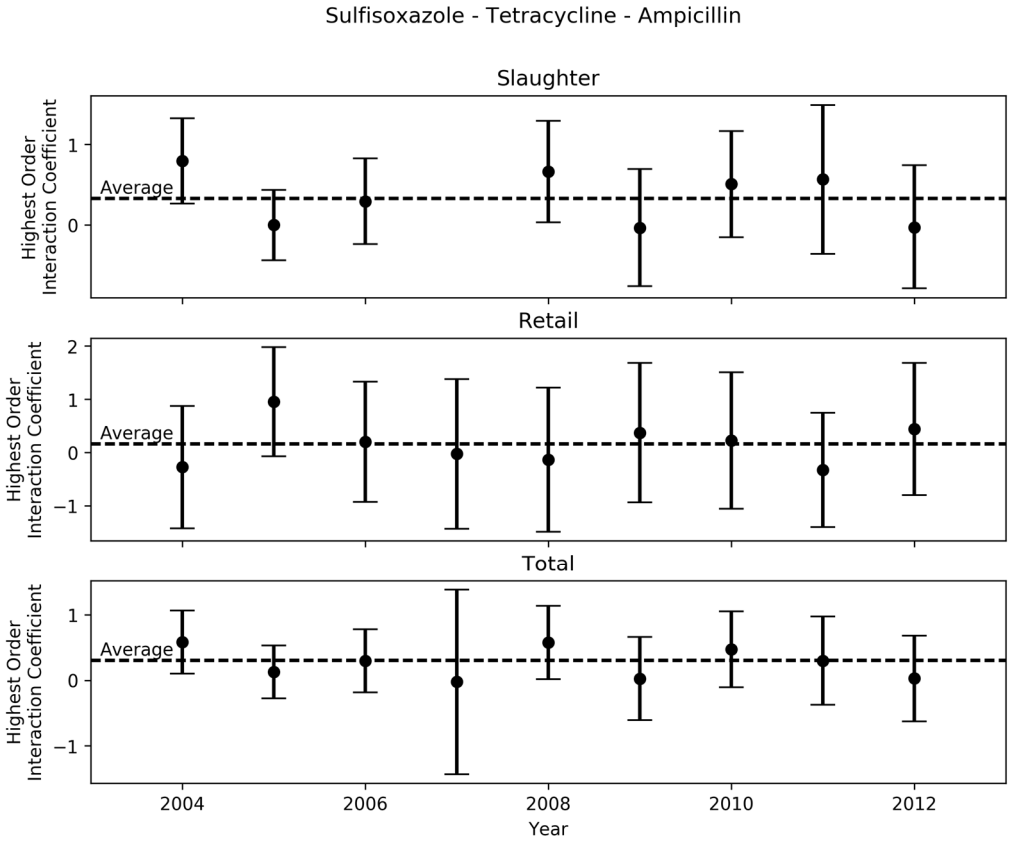


Figure 5: Plot of the highest order interaction parameter in the log-liner model of the sulfisoxazole, tetracycline, and ampicillin multidrug contingency table by year and stage.

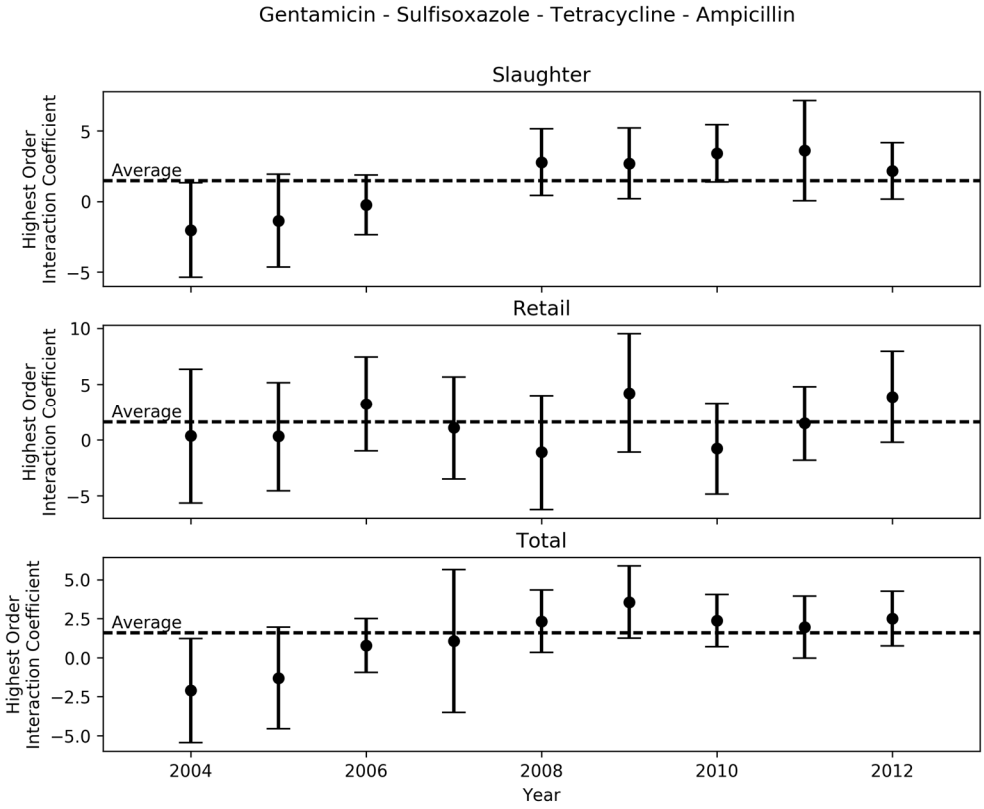


Figure 6: Plot of the highest order interaction parameter in the log-liner model of the gentamicin, sulfisoxazole, tetracycline, and ampicillin multidrug contingency table by year and stage.

be kept in mind that such a trend could just be the product of overdispersion. At retail it has a mean of 1.60 with no trend and an over dispersion factor of 3.09.

Power analysis

In our data interaction parameters ranged in magnitude from 0 to 4, overdispersion parameters ranged from 2 to 6 and sample sized ranged from 300 to 10,000 depending on how many years were pooled. We consider .8 to be a minimum desirable power.

For the null model with form $\log \mu_{ijk} = -1.836 - .4055 + .04886 - .8598 + .4055 + .4055 + .4055$ and an overdispersion of 3 a sample size of 2,500 would be required to detect an interaction parameter of 1 and a sample of 500 would be required to detect an interaction parameter of 2 (Figure 7). For sample sizes of 100 and 500 the powers determined from the simulation are slightly lower than theoretical values based on the normal assumption. For a sample of 100 the theoretical power is about .01 lower than the theoretical value at an interaction parameter of 2.

When the interaction parameter is fixed and the overdispersion is instead allowed to vary a sample size of 1000 would be sufficient to detect an interaction parameter of 1 at an overdispersion of 1.75, and a sample size of 2,500 could detect such an interaction at an overdispersion of 4.5 (Figure 8). Interestingly, at an interaction parameter of 1 there is little difference between the powers calculated from the simulated and theoretical distributions. For detecting an interaction parameter of 2 a sample size of 500 would be sufficient up to an overdispersion of 3 and a sample

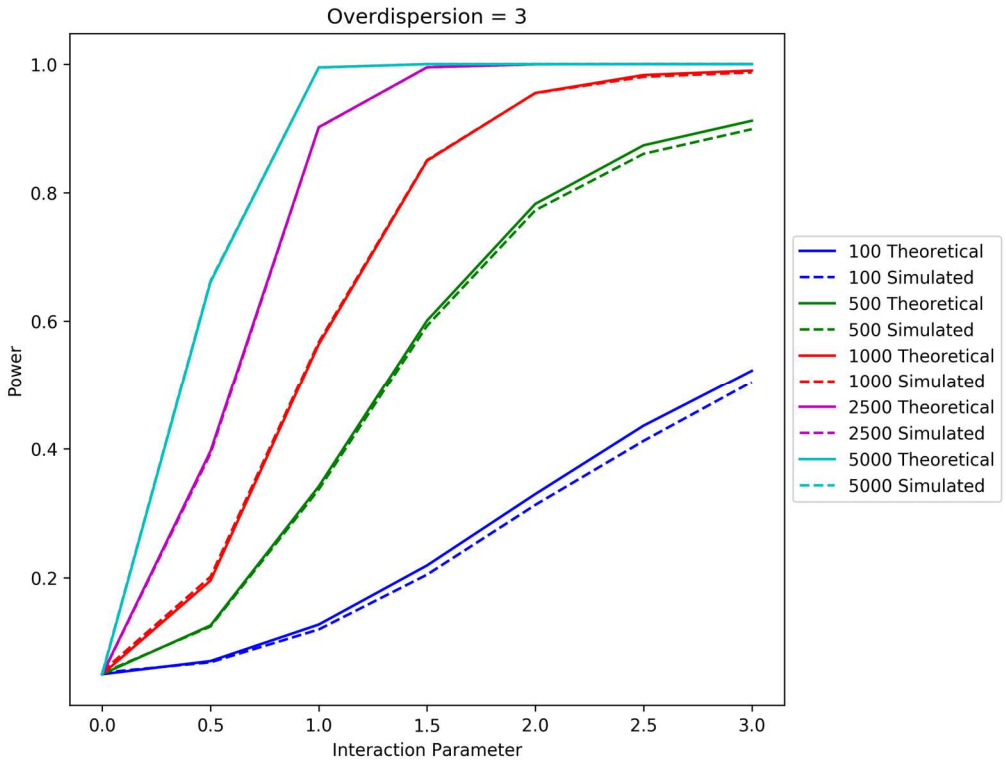


Figure 7: Power curves for detecting the highest order interaction in a log-linear model of a contingency table under various settings of the interaction parameter and sample size at a fixed overdispersion of 3 when the null model has form $\log \mu_{ijk} = -1.836 - .4055 + .04886 - .8598 + .4055 + .4055 + .4055$. Solid lines are calculated using the assumption of asymptotic normality, and dashed lines are calculated using simulations from a Dirichlet-multinomial distribution.

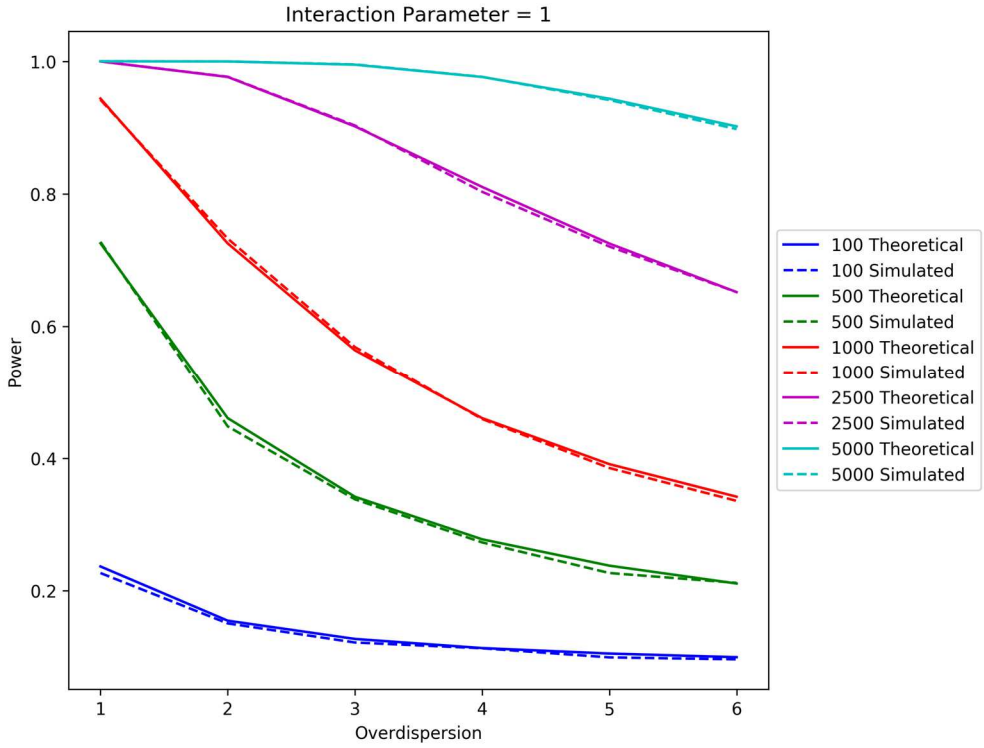


Figure 8: Power curves for detecting the highest order interaction in a log-linear model of a contingency table under various settings of the overdispersion and sample size at a fixed interaction parameter of 1 when the null model has form $\log \mu_{ijk} = -1.836 - .4055 + .04886 - .8598 + .4055 + .4055 + .4055$. Solid lines are calculated using the assumption of asymptotic normality, and dashed lines are calculated using simulations from a Dirichlet-multinomial distribution.

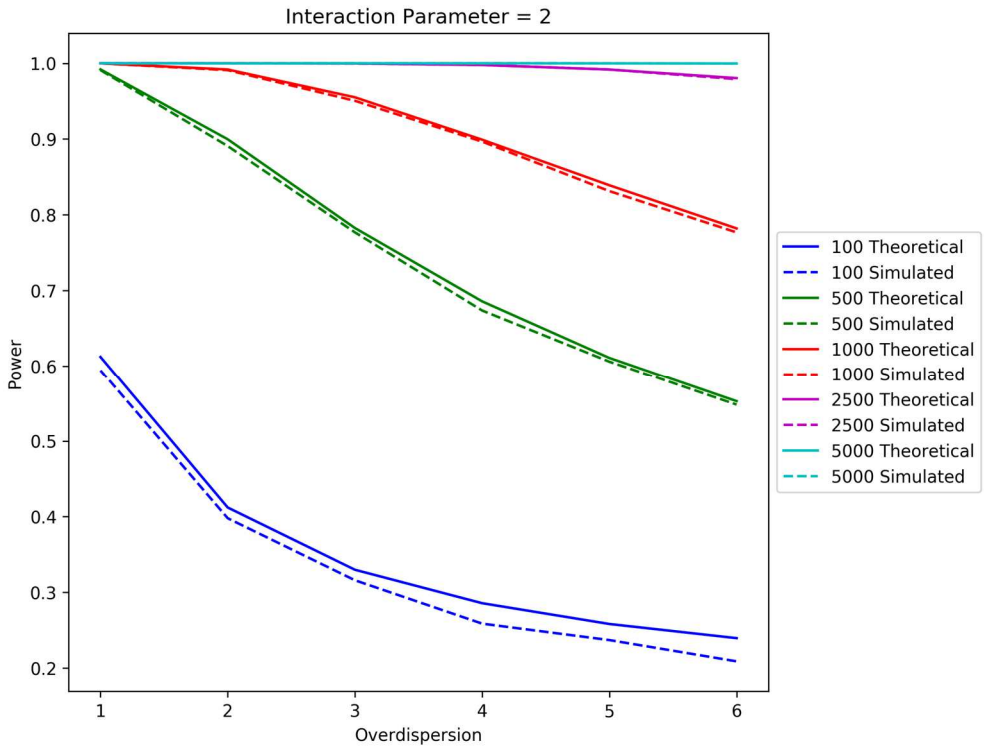


Figure 9: Power curves for detecting the highest order interaction in a log-linear model of a contingency table under various settings of the overdispersion and sample size at a fixed interaction parameter of 2 when the null model has form $\log \mu_{ijk} = -1.836 - .4055 + .04886 - .8598 + .4055 + .4055 + .4055$. Solid lines are calculated using the assumption of asymptotic normality, and dashed lines are calculated using simulations from a Dirichlet-multinomial distribution.

size of 1000 would be sufficient up to an overdispersion of 6 (Figure 9). At this level of the interaction parameter there begins to be a discrepancy between theoretical and simulated powers. At a sample size of 100, the power calculated from the theoretical distribution is almost .025 higher than that calculated through simulation.

Conclusion

Year on year contingency table analysis of multidrug resistance to gentamicin, sulfisoxazole, tetracycline and ampicillin by *E. coli* isolated from chicken at slaughter and retail in the years 2004 to 2012 by NARMS suggests overdispersion with respect to multinomial variation. The overdispersion is apparent both at the multivariate (whole table) level, and on the level of individual interactions. In some cases, like the interaction among gentamicin, sulfisoxazole, and tetracycline at retail, this overdispersion comes from hyper-variability around a consistent mean even sometimes switching signs in different years. In other cases, like the interaction among gentamicin, sulfisoxazole, tetracycline, and ampicillin at slaughter and overall, there seems to be an increasing trend in the interaction parameter, although this may simply be the result of overdispersion.

This overdispersion is problematic for surveillance systems like NARMS because it increases the sample sizes needed to detect interactions. In fact, the sample sizes needed to achieve a power greater than .8 for detecting a highest order interaction parameter of 1 at an overdispersion of 3 exceed 2,500 and thus, at current rates of NARMS data collection would take several years to achieve. This power can be accurately approximated with the asymptotic normal assumption at high sample sizes, low overdispersions and small interaction parameters. The approximation is not

strictly valid, however, for low sample sizes, high overdispersions and large interaction parameters where an improved power estimate can be obtained using a Dirichlet-multinomial simulation approach.

Much of the overdispersion observed in this study is likely do to uncontrolled latent variables, like antimicrobial use, husbandry and hygiene practices. Interestingly though, the slaughter data which has a less consistent sampling design than the retail program in some cases had lower levels of overdispersion. This indicates that while more careful sampling design could help to improve power in surveillance systems like NARMS, much of the variability is likely to be endemic to the enterprise of antimicrobial resistance surveillance.

REFERENCES

Print References

Agresti, A., 2002. Catagorical Data Analysis, 2nd edition. John Wiley & Sons, Hoboken, New Jersey.

Carson, C. A., Reid-Smith, R., Irwin, R. J., Martin, W. S., McEwen S. A., 2008. Antimicrobial resistance in generic fecal *Escherichia coli* from 29 beef farms in Ontario. Can. J. Vet. Res. 72 (2), 119-128.

Catry, B., Dewulf, J., Maes, D., Pardon, B., Callens, B., Vanrobaeys, M., de Kruif, A., Haesebrouck, F., 2016. Effect of Antimicrobial Consumption and Production Type on Antibacterial Resistance in the Bovine Respiratory and Digestive Tract. PLoS ONE. 11(1), e0146488. doi:10.1371/journal.pone.0146488.

Gow, S. P., Waldner, C. L., Rajić, A., McFall, M. E., Reid-Smith, R., 2008. Prevalence of antimicrobial resistance in fecal generic *Escherichia coli* isolated in western Canadian cow-calf herds. Part I — Beef calves. Canadian J. Vet. Res. 72 (2), 82-90.

Holman D. B., Timsit E., Alexander T.W., 2015. The nasopharyngeal microbiota of feedlot cattle. Scientific Reports. 5, 15557. doi:10.1038/srep15557.

Lerma, L. L., Benomar N., Knapp, C.W., Galeote, D. C., Gálvez, A., Abriouel, H., 2014a Diversity, Distribution and Quantification of Antibiotic Resistance Genes in Goat and Lamb Slaughterhouse Surfaces and Meat Products. PLoS ONE. 9 (12), e114252. doi:10.1371/journal.pone.0114252.

Lerma, L. L., Benomar, N., Muñoz, M. C. C., Gálvez, A., Abriouel, H., 2014b. Antibiotic Multiresistance Analysis of Mesophilic and Psychrotrophic *Pseudomonas*

spp. Isolated from Goat and Lamb Slaughterhouse Surfaces throughout the Meat Production Process. App. Env. Microb. 80 (21), 6792-6806. doi:10.1128/AEM.01998-14.

Lerma, L. L., Nabil, N., Muñoz, M. C. C., Gálvez, A., Abriouel, H., 2015. Correlation between antibiotic and biocide resistance in mesophilic and psychrotrophic *Pseudomonas* spp. isolated from slaughterhouse surfaces throughout meat chain production, Food Microb. 51, 33-44. http://dx.doi.org/10.1016/j.fm.2015.04.010.

Miranda, J. M., Vazquez, B. I., Fente, C.A., Calo-Mata, P., Cepeda, A., Franco, C.M., 2008. Comparison of antimicrobial resistance in *Escherichia coli*, *Staphylococcus aureus*, and *Listeria monocytogenes* strains isolated from organic and conventional poultry meat. J. Food. Prot. 71, 2537–2542.

Perez, F., Granger, B. E., Hunter, J. D., 2011. Python: An Ecosystem for Scientific Computing. Comput. in Sci. Eng. 13, 2, 13-21. 10.1109/MCSE.2010.119.

The National Antimicrobial Resistance Monitoring System., 2016a. Methods.

Van Rossum, G., 1995. Python tutorial. Technical Report CS-R9526, Centrum voor Wiskunde en Informatica (CWI).

Zawack, K., Li, M., Booth, J.G., Love, W., Lanzas, C., Gröhn, Y.T., 2016. Monitoring Antimicrobial Resistance in the Food Supply Chain and Its Implications for FDA Policy Initiatives. Antimicrob. Agents. Chemother. 60 (9), 5302-11. doi: 10.1128/AAC.00688-16.

Web References

[Dataset] National Antimicrobial Monitoring system. http://www.fda.gov/AnimalVeterinary/SafetyHealth/AntimicrobialResistance/NationalAntimicrobialResistanceMonitoringSystem/ucm416741.htm. 11-25-2016

CHAPTER 5

CONCLUSION

Summary

Surveillance is a key component of controlling antimicrobial resistance. In the United States this surveillance is carried out by the National Antimicrobial Resistance Monitoring System (NARMS). In this system, the United States Department of Agriculture Food Safety Inspection Service collects isolates from slaughter facilities, the Food and Drug Administration collects Isolates from Grocery Stores, and the Center for Disease Control and Prevention collect isolates from human medicine.

Examination of resistance to single drugs reveals that resistance patterns depend very much on the antibiotic, microbe, host and stage under consideration. Resistance of *Eschereschia coli* isolated from chicken to streptomycin has decreased steadily over time at both slaughter and retail. By contrast resistance of *Salmonella* Typhimurium isolated from chickens to ampicillin has increased slightly at retail, but decreased at slaughter. This dependence on context occurs both when then magnitude of resistance is measured as a minimum inhibitory concentration and for binary resistance/susceptibility determined using a threshold. What does change, is the trend in resistance. This may at first seem problematic, but makes sense when we consider that modifying the choice of resistance cutoff effectively askes a different question about resistance, a new question with a new answer. Another consistent aspect of the data is higher than expected variability. The standard deviation for counts of resistant isolates exceeds expected binomial standard deviation and the amount by which it exceeds the expected value is independent of samples size. The variation in MICs is

greater within a given year than between years. This greater than expected variability makes it difficult to detect changes in resistance. In particular, it would take up to 6 years of data collection at the current rate to detect even a 6% change in resistance.

As serious a threat as single drug resistance poses to our ability to control infections multidrug resistance compounds this effect. Fortunately, the NARMS data provides a trove of information about this problem. By constructing contingency tables of resistance counts and modeling them with log-linear models we can detect not just pairwise associations but also higher order interactions among antibiotic resistances. A prime example of this is the four-way interaction among the β-lactams amoxicillin-clavulanic acid, ceftriaxone, cefoxitin and ceftiofur. Many of these interactions, such as this four way β-lactam interaction have such extreme odds ratios they cannot be tested for using asymptotic or exact conditional methods, but instead require a Bayesian approach. Inference of these interactions sheds important light on the structure of multidrug resistance. This structure can help inform clinical practices on which antibiotics to prescribe. They also can shed light into the mechanisms by which resistance develops as in the case of the hierarchal ordering of β-lactam resistance that creates its extreme four-way interaction.

Examination of the multidrug interactions over time shows they exhibit greater than expected variability. This is not surprising given that there is over dispersion in the single drug resistances. The overdispersion makes it more difficult to detect these associations as it would take a sample size of 2,500 to detect an interaction parameter of 1 at the levels of overdispersion observed in the NARMS data.

The overdispersion observed in both the single drug and multidrug data make it difficult to estimate with high precision quantities of interest such as interaction parameters or changes in resistance. The existence of this overdispersion indicates the existence of unmeasured covariates. The list of potential covariates is extensive, including antimicrobial use, climate, animal husbandry practices, hygiene practices, and available carbon sources. In fact, because antimicrobial resistance is a product of natural selection any factor that effects the fitness of microbes carrying resistance traits is a relevant covariate. The observation that most variability in single drug resistance is within year not between years and that the more consistent sampling design of the retail program than the slaughter program did not uniformly decrease overdispersion suggests that there are unlikely to be any silver bullet covariates that by themselves produce a dramatic decrease in overdispersion. Undoubtedly increasing the number of measured covariates would help decrease overdispersion in the NARMS data. There are a number of variables, like measuring the state in which slaughter samples were collected, that could be collected at little to no extra cost, and are ripe for the picking. Nonetheless overdispersion is likely to be a fundamental part of antimicrobial resistance surveillance, especially in a country as large as the United States.

Future Directions

All the work up to this point has been phenotypic, focusing simply on resistance with no data on the underlying genotypes. Arguably the most important addition that could be made to the current work is adding in this genotypic information. On the single drug level whole genome sequence data would allow us to

identify which genes are most important in leading to the increases in resistance. It would help in determining if changes in resistance in different regions are the result of the same strains or different strains. On the multi-drug level whole genome sequencing would provide an important actionable way of following up on the potential mechanisms identified in the log linear models.