A Proposed Analytic Framework for Determining the Impact of an Antimicrobial Resistance Intervention at the National Level

A Report from the NIMBioS Working Group on Modeling Antimicrobial Resistance Intervention

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Summary

The use of antimicrobials in human and veterinary medicine has brought tremendous benefits to both humans and animals; benefits that are increasingly threatened by the development of antimicrobial resistance (AMR). The Food and Drug Administration (FDA) is in the final year of implementing new risk mitigation measures to promote judicious use of medically-important antimicrobials in food animals. However, the relationship between antimicrobial use (AMU) and resistance is complex, and a suitable analytical framework to analyze this relationship, and evaluate the success of interventions, is not yet available. A systems science analysis, which clearly depicts the variables and associations among them, would greatly enable selection of mathematical or epidemiological options to evaluate the question at hand. Such an analysis would allow more efficient resource allocation, by identifying data that are most informative for evaluating surveillance and mitigation strategies.

This National Institute for Mathematical and Biological Synthesis (NIMBioS) AMR Working Group's report proposes a framework to fill the methodological gap for associating AMU in livestock (measured at the population level) and AMR in foodborne bacteria. The framework comprises specific analytical methods and sources of quantitative data to model selection pressure and effect. Results of such models can help evaluate how drug use practices (and interventions directed at changing them) impact AMR. Pharmacokinetic/dynamic modeling techniques are proposed for projecting selection pressure (i.e., enteric concentrations of antimicrobials) on bacteria that promotes AMR. Two methods are identified to model resistance: phenotypic methods, which model changes in AMR in the food supply (i.e., at farm, slaughter, and retail) based on antimicrobial susceptibility testing; and evolutionary genotypic analyses which determine the molecular details underlying changes in phenotypic AMR. Systems science analysis is used to integrate these methods to show how resistance in the food supply can be explained by drug use, and concurrent factors which influence how the whole system behaves. Overall, this modeling process is necessarily iterative in nature and will be continuously updated with new data to improve prediction accuracy and inform improvements for AMU and AMR surveillance systems.

The FDA's judicious use strategy will be fully implemented in 2017; the first data after this will be collected in 2017 and 2018, and likely will not be reported publicly until 2019. Meanwhile, the methods identified in this framework are sufficiently developed to be applied to existing data now. For example, the proposed phenotypic resistance methods can be applied to evaluate previous interventions, such as the 2005 withdrawal of fluoroquinolones from poultry water medications. The pharmacokinetic/dynamic modeling can be applied to identify AMU practices that impose the largest selective pressures. Each method in the framework would also benefit from further development.

Working group participants are pursuing research proposals to finish development, testing and validation of the proposed methods, as well as to more fully merge AMU, pharmacokinetic/dynamic, phenotypic, and evolutionary genetic models into an even more cohesive framework. The systems science analysis section could potentially be further refined through another NIMBioS Working Group.

1 Introduction

1.1 Drug Resistant Bacteria in the US Food Supply

The use of antimicrobial drugs in human and veterinary medicine has brought tremendous benefits to both humans and animals over the past century, benefits increasingly threatened by the development and progression of antimicrobial resistance (AMR). While AMR can be naturally-occurring, antimicrobial use (AMU) in animal agriculture, human medicine, and other settings is a major selection pressure for acquired AMR. Antimicrobial resistance and the resulting reduction in drug effectiveness threaten both public and animal health (Marshall and Levy, 2011; Oliver et al., 2011). It is thus vital that antimicrobials be used responsibly to preserve their effectiveness.

Globally and nationally, there is much attention to developing approaches to mitigate AMR. For example, the World Health Organization has created a Global Action Plan on Antimicrobial Resistance (http://apps.who.int/iris/bitstream/10665/193736/1/9789241509763_eng.pdf?ua=1). Several nations have developed national action plans (http://www.who.int/drugresistance/documents/situationanalysis/en/), including the United States; the White House recently released a National Action Plan for Combating Antibiotic Resistant Bacteria (https://www.whitehouse.gov/sites/default/files/docs/national_action_plan_for_combating_antibotic-resistant_bacteria.pdf; the NIMBioS working group is referenced under Sub-Objective 4.1.1).

Having considered scientific information and public policy, the FDA initiated a risk-mitigation strategy to promote judicious use of medically important antimicrobial drugs in food animals (FA). In April 2012, the FDA released its Guidance for Industry (GFI 209) which states: “Limit medically important antimicrobial drugs to uses in food-producing animals that are considered necessary for assuring animal health and that include veterinary oversight or consultation”. In December 2013, the FDA issued another guidance document, GFI 213, spelling out the process for achieving the objectives laid out in GFI 209. It included the process for pharmaceutical companies to withdraw growth-promotion claims from labels of products containing medically important antimicrobials. The FDA is giving the companies until December 2016 to make these changes. Also in December 2013, the FDA issued a proposed rule to amend its regulations relating to veterinary feed directive drugs to bring them into compliance with GFI 209. Hence, the FDA mitigation strategy has two principles: (1) phasing out growth-promotion use of these antimicrobials; and (2) phasing in veterinary oversight on the remaining therapeutic use of such drugs (FDA, 2012, 2013).

Determining the effectiveness of the FDA mitigation strategy is the subject of this report. Classic epidemiological methods commonly used to assess the effects of AMU on resistance must be improved to assess the impact of FDA guidances (and other potential system-wide interventions) because of the lack of standardized metrics (including data) on AMU (exposure) and the complexity of the measured outcome (patterns or prevalence of AMR).

Based on our work as the National Institute for Mathematical and Biological Synthesis (NIMBioS) AMR Working Group (NIMBioS WG (denoted ‘the Working Group’) (http://www.nimbios.org/workinggroups/WG_amr)), we have identified publicly available data and

modeling approaches which may be used. Data are measured at many levels (Figure 1). We recommend applying the systems approach – a widely, successfully used methodology that has supported decision-making and intervention evaluation in other realms of complex systems, such as engineering, public health and ecology, to the assessment of efficacy of mitigation of AMR. The methodology described in this report will allow much needed evaluation of intended risk reduction policies, providing useful feedback for informing both national and global efforts.

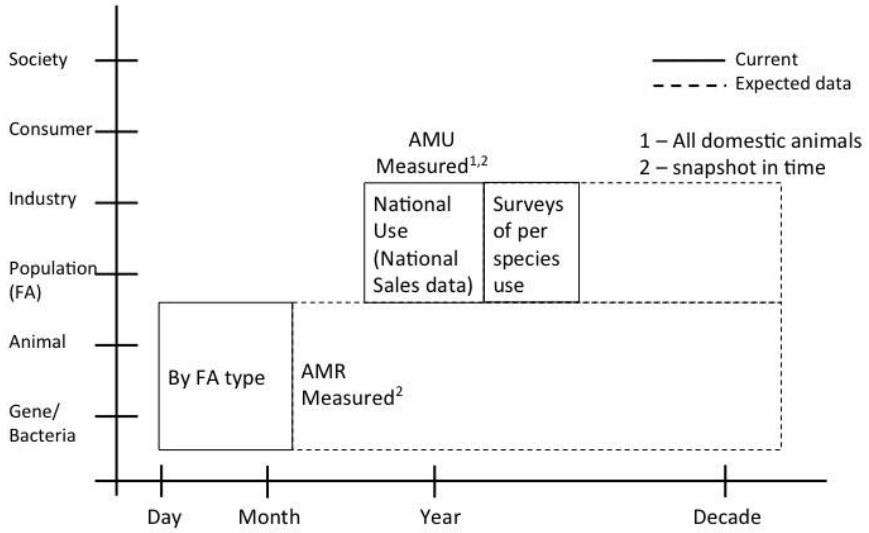

Figure 1. Temporal scale and hierarchical levels at which data are measured. Current measures (solid boxes) of the exposure - antimicrobial use (AMU) - are derived from yearly national sales data for food animal antimicrobials. Current indicators of antimicrobial resistance (AMR) are measured for the type of food animal (FA). Both measures provide a snapshot in time (point estimates) of the item measured. Initial models must make assumptions about the extent of time these point estimates cover until more temporal coverage data are obtained. Dashed boxes indicate the data expected to be available for model use.

1.2 The NIMBioS AMR Working Group

NIMBioS is a National Science Foundation (NSF) Synthesis Center supported through NSF's Biological Sciences Directorate via a Cooperative Agreement with the University of Tennessee. NIMBioS supports cutting-edge, cross-disciplinary research at the interface of mathematics and biology with a vision to:

1) address key biological questions by facilitating the assembly and productive collaboration of interdisciplinary teams; and
2) foster development of the critical and essential human capacity to deal with the complexities of the multi-scale systems that characterize modern biology.

The FDA's implementation of its risk-mitigation strategy creates a unique opportunity to evaluate the success of this imminent intervention. An interagency group (involving the FDA, USDA, and CDC) was formed to develop an approach to collect additional information to detect shifts in AMU and corresponding impacts on AMR due to the new FDA recommendations. These ongoing interagency activities require a suitable mathematical framework to identify and prioritize specific data and analytical approaches to analyze effects of the interventions on resistance. No suitable integrative model currently exists; development of such a framework requires integration of diverse fields and synthesis at multiple levels, and will benefit from development of new mathematical and computational approaches. A NIMBioS Working Group with scientists from the FDA, USDA, CDC, other government agencies, and academia was organized in 2014 to identify such an analytic framework and data needs (http://www.nimbios.org/workinggroups/WG_amr). The multi-disciplinary working group, which drafted this report, included veterinarians, epidemiologists, regulatory experts, mathematicians, microbiologists, and system engineers.

1.3 Objectives of this Project

1. Identify and develop an analytic methodology, applicable to the types of data that are or will be available, to analyze observed changes in AMU patterns and AMR (including conceptual/mathematical/epidemiologic/statistical models and parameter estimation). This is a vitally needed new integrative approach to assess this complex biological phenomenon.

2. Identify useful, quantitative variables based on the above framework allowing analysis of the relationship between observed changes in AMU practice in FA and AMR patterns in the food supply as the FDA implements its risk mitigation strategy. This is a unique opportunity to inform the approach to monitoring and assessing impacts of the interventions, allowing collaborating federal agencies to efficiently allocate limited resources by targeting the most valuable data.

This project is also represented as a one-year milestone under Sub-objective 4.1.1. of the US National Action Plan for Combating Antibiotic Resistant Bacteria (CARB)[1]:

> "A National Institute of Mathematical and Biological Synthesis (NIMBioS) working group will develop an analytic modeling framework for assessing the relationship between antibiotic use in livestock (measured at the population level) and the development of antibiotic resistance."

The underlying concept is that GFI #213 will impact resistance (in the food supply) by impacting AMU in food animals (Figure 2).

[1] https://www.whitehouse.gov/sites/default/files/docs/national_action_plan_for_combating_antibotic-resistant_bacteria.pdf

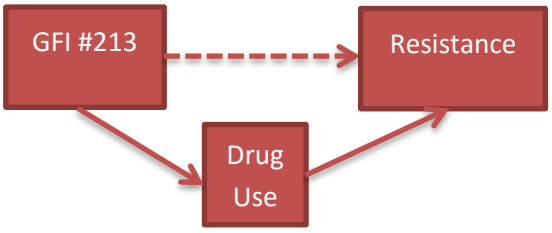

Figure 2. A simple depiction of an extremely complex underlying system of the anticipated relationship between Guidance for Industry 213, antimicrobial use in food animals, and antimicrobial resistance in the food supply.

The Working Group used the following basic process to develop the modeling framework:

1. Formalized the scientific question that the modeling framework will address
2. Established the boundaries of the system that need to be modeled to answer the question
3. Identified the analytic methods needed to model the system or subsystem(s) of interest
4. Evaluated the performance of the modeling framework (select aspects) using existing data
5. Identified additional data which were the most important for improving the performance of the modeling framework

To develop an analytic modeling framework we needed to further formalize the underlying scientific question under study. The Working Group's initial proposal used the following language:

> "…associating population-level changes in antimicrobial use in livestock with population-level changes in antimicrobial resistance."

The US National Action Plan used the following language:

> "…assessing the relationship between antibiotic use in livestock (measured at the population level) and the development of antibiotic resistance."

The Working Group discussed the need to demonstrate associations between use and resistance in order to gain support to conduct surveillance. This conflicts with having little real data to parameterize models to determine whether an association exists, resulting in circular discussions. Although these were challenging issues to communicate, the multidisciplinary team showed its strength by bringing together many viewpoints of technical experts in different fields. Ultimately the group determined that this was an epidemiologic question of association between an outcome factor (typically a disease outcome; in this case, AMR) and one or more explanatory factors (or risk factors; in this case specifically focused on AMU

in livestock). Further refining the concept of association to focus on explaining observed variation, the Working Group arrived at the following formal scientific question:

> ***What proportion of the observed variation in antimicrobial resistance within bacteria in the food supply can be explained by the observed variation in antimicrobial drug use in food-producing animals?***

Figure 3 illustrates the complexity of this question; there are several important points to note regarding this question. First, one must be careful to distinguish association from causality, the former being necessary but not sufficient for the latter.

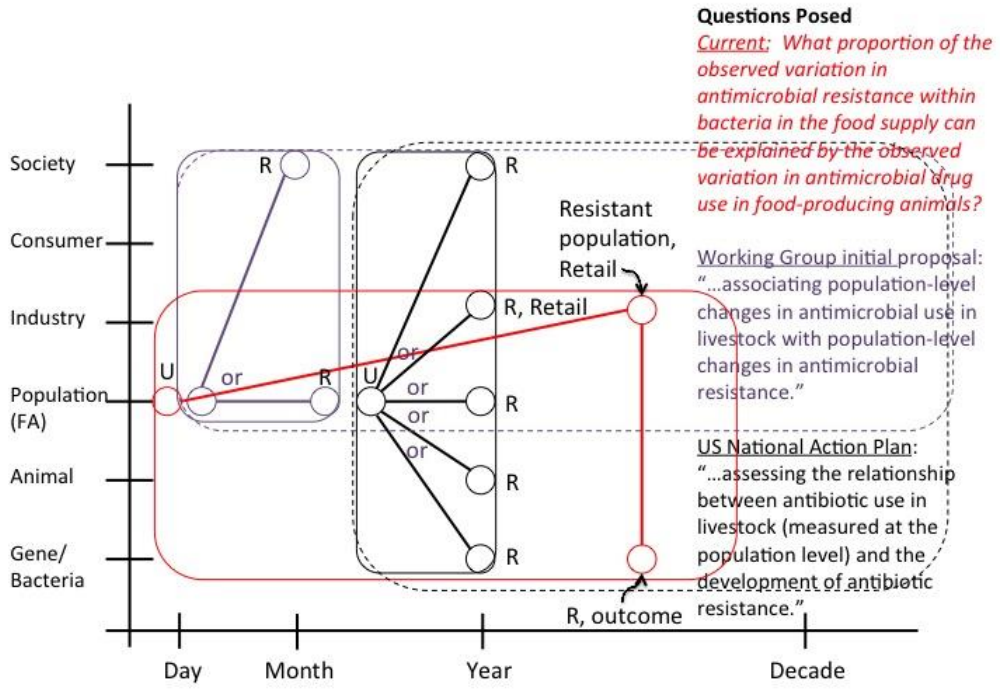

Figure 3. Temporal scale and hierarchical levels at which the scientific questions were posed. The figure indicates a) the hierarchical level(s) of Use (U) or Resistance (R) the questions pose by circles and b) the possible levels to which resistance is implied by the question. For example, the question posed in the US National Action Plan CARB points to the development of resistance, which can occur or be influenced at several levels. The initial working group posed question addresses resistance at the population level, which could be at the food animal (FA) or societal level, if measuring resistance in the human population. The currently posed question specifically examines resistance at the bacterial level in the food supply and whether any change in that resistance can be attributed to changes in antimicrobial use in the FA populations. Solid boxes capture individual questions (where the only temporal aspect is the implied use before resistance); dashed boxes indicate the temporal scope of answering the questions and the hierarchical level indicates the data required to answer the questions.

Second, as well as potentially missing subtle individual-level effects due to aggregation (and sampling delay), the population-level focus of this question raises concerns of the "ecological fallacy." This relates to the potential for an effect to be observed at the population level even though there is no effect at the individual level. We therefore take extra care to remember that while these methods are intended to detect national-level associations they do not allow us to fully understand underlying mechanisms.

Third, the outcome ("antimicrobial resistance in bacteria in domestically-produced meat and poultry") is actually a collection of several factors tested on each experimental unit (i.e., it is a multivariate outcome). For example, individual bacterial colonies (isolated from the food supply or any other place) are typically tested for susceptibility to a long (but nowhere near exhaustive) list of antimicrobial drugs, using one or more different techniques (e.g., broth microdilution assay +/- whole genome sequencing, etc.). This has bearing on the techniques appropriate for analyzing these data.

Fourth, the explanatory factor ("antimicrobial drug use in food animals") is neither the biological interaction of interest (exposure of bacteria to antimicrobials) nor does it have a consensus measure. Knowledge of the biology of AMR and animal-derived food supply system is thus critical to identify the most appropriate measures to characterize this explanatory factor for purposes of this project.

Several approaches to model select sections of the overall AMU/AMR problem space were discussed. All have advantages and disadvantages. While simplicity is desired and often requested, the working group concluded that no one model could adequately address all aspects of the AMU/AMR system at the same time. Rather a suite of models is necessary to provide estimates of uncertainty around the conclusions about associations between AMU and AMR and the effectiveness of policies which may affect either or both. These models can be used independently to answer questions geared towards a specific section of the system or in a combined manner to address questions geared towards the more complete system.

1.4 System Boundaries

A systems approach was needed to address the issue tasked to the Work Group. A systems approach aids in understanding complex systems[2] so that the subsystems making up the overall system can be designed, integrated and tested to effectively study the system's behavior. A systems approach has the advantages of helping to define the scope of the system (complex reality vs. what necessarily bounds the system according to the question posed), including key stakeholders' considerations while investigating solutions, making sure key data points and variables are considered, to link models created to evaluate select sections of the system, and to highlight the models that are proposed to answer the question vs. the models which could be used/validated based on currently available data.

[2] A **complex system** is any **system** featuring a large number of interacting components (agents, processes, etc.) whose aggregate behavior is nonlinear (not derivable from the summations of the activity of individual components) and typically exhibits hierarchical self-organization under selective pressures.

Most policymakers prefer simple systems, which AMU/AMR is not. How AMU in FA influences AMR in food products is not well understood. Many antimicrobials are used (for production purposes or therapeutically) in FA. Additional antimicrobial drugs will likely be developed, though likely few new antimicrobial classes in the near future. Antimicrobials are currently used both singly and in combinations, including in combinations with heavy metals.

There are numerous FA types and their production scenarios, e.g., cattle (dairy, beef, veal), chickens (broilers, table egg layers), turkeys, pigs, shellfish, finfish, sheep, goats, etc. The working group limited their scope to the major FA species (cattle, pigs, chickens, and turkeys). Also, numerous food products are derived from each FA, e.g., cattle products include beef, organ meat, veal, ground beef, and tartar. Processing factors at or before retail of these products must be included in a comprehensive approach to investigating an overall AMU/AMR association. The question posed includes these as a pathway between use and resistance, but current surveillance provides insufficient data to determine the scale of influence of the processing factors. Antimicrobial resistant bacteria occur naturally and more species and strains of these bacteria will continue to emerge. Hence, the framework for the study of AMU/AMR should be sufficiently generalizable for future inclusion of new bacteria and drugs. In addition, numerous genes confer AMR (e.g., dozens of tet genes confer resistance to tetracycline). Some genetic elements are chromosomal in nature and some are carried on transferable elements (plasmids, transposons, integrons). Figure 4 illustrates just part of the hierarchies (and aspects within them) involved in answering the AMU/AMR association question, looking only at FA.

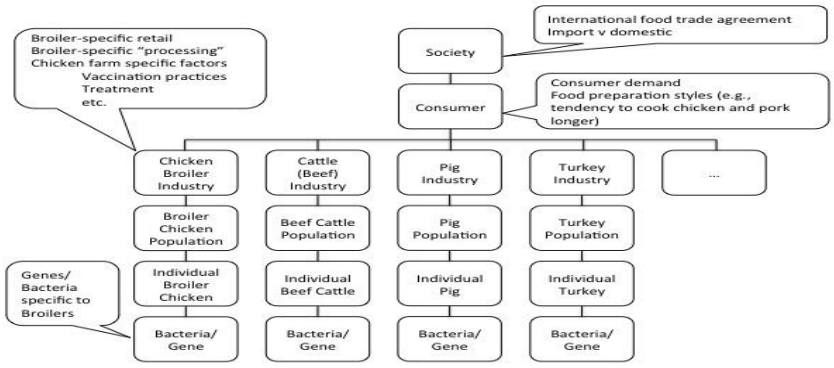

Figure 4. Hierarchical nature of the food animal (FA) industry and factors impacting antimicrobial resistance (AMR) with FA-specific factors. Pressures on AMR have many factors dependent on the specific FA and at different scales within the FA industry, consumer and society. For instance, using the lens of broiler chickens, there are resistance genes/bacteria specific to broilers, and commensals and pathogens of public health concern. Broiler-specific factors include vaccination practices, antimicrobial treatment etc. At the consumer level, demand for specific product types and food preparation styles play a role. At the societal level, factors like international trade agreements and whether a food item is imported or domestic play a role.

Given the scale and complexity of the AMR issue, even when restricted to foodborne bacteria and the selective pressures due to drug use in FA, it is important to clearly establish the scope and boundaries of this project (Figure 5).

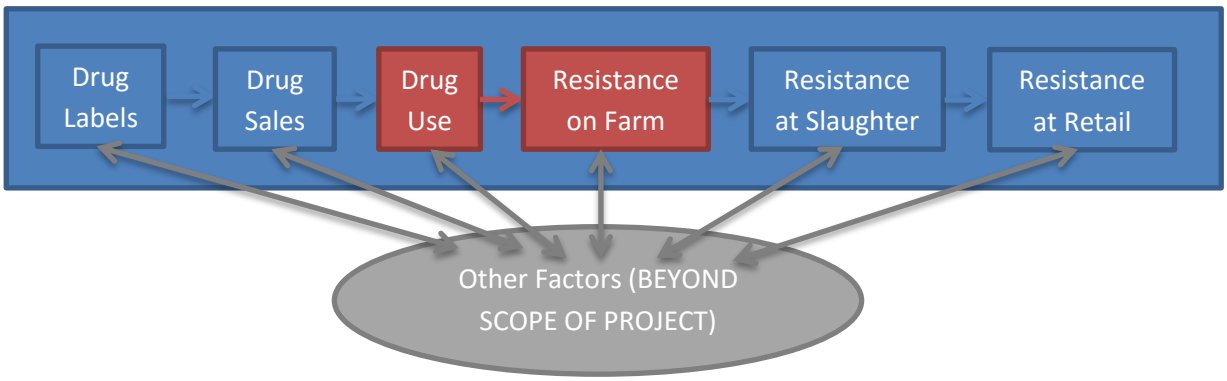

Figure 5. Summary of the scope of this project using the foodborne antimicrobial resistance risk pathway; red = primary focus, blue = in scope, grey = beyond the scope (e.g., disinfectant use in slaughter plant (may be linked with some resistances); non-antimicrobial changes in management practices; consumer demand).

First, population-level effects, particularly at the national scale, are the primary interest of this project. This is not to say that finer scales (e.g., animal, pathogen) of the system are beyond its scope—in fact they are likely critical to understanding the issue at the national level. However, we determined that this work would focus on addressing AMU and AMR at the national level. The relevance of international aspects (including movement of people, animals, food products, and drugs) is well-documented, but outside the scope of this particular project. The geospatial upper bound of our system will thus be the continental US. The lower bound will extend to the genetic/molecular level.

Second, the National Antimicrobial Resistance Monitoring System (NARMS) provides additional system boundaries which are both appropriate for this project (given similar focus on resistance in foodborne bacteria) and also practical in terms of data availability. These bounds include:

- bacteria (i.e., *Salmonella*, *Campylobacter*, *Escherichia coli*, and *Enterococcus*),
- animal species (i.e., those that the FDA considers the "major food species": cattle, swine, chickens, turkeys), and
- sampling site (e.g., animals at slaughter and retail meat).

Although NARMS also samples clinical isolates from humans with enteric infections (see CDC NARMS)[3], that part of the risk pathway was excluded from the scope of this project due to its distance from the primary biological point of interest (i.e., the lower gastrointestinal tract of food animals) and the expectation that observed effects at this site would be greatly "diluted" by other factors pertinent to

[3] http://www.cdc.gov/narms/reports/

human populations. Use of antimicrobials in human medicine is similarly beyond the scope of this project. Figure 6 shows an example of system boundaries for the food supply system.

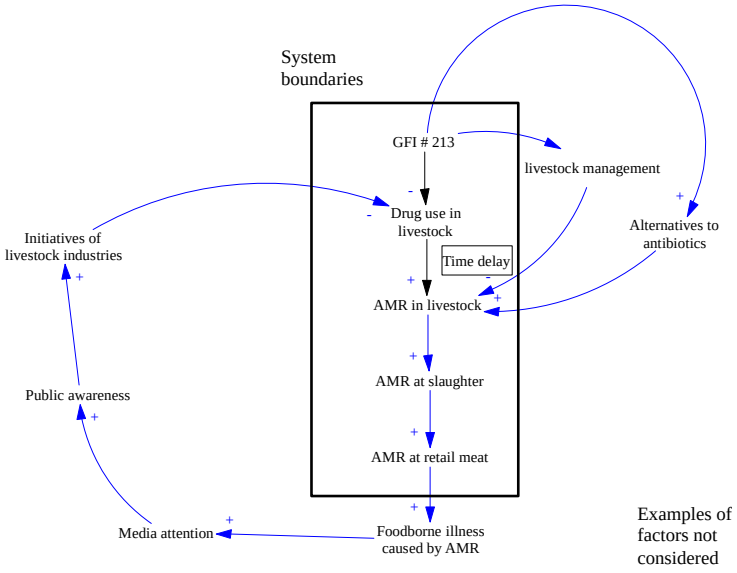

Figure 6. An example of boundary setting for food supply system (GFI= Guidance for Industry; AMR=antimicrobial resistance). The rectangle denotes the system boundaries. The factors outside the box are currently not considered.

Regarding the scope of drugs, the specific drugs NARMS evaluates within the abovementioned target bacteria are based mainly on human clinical relevance, and do not necessarily cover all drugs (or even drug classes) directly impacted by GFI #213. Furthermore, since we might anticipate (and be greatly interested in) changes in resistance to drugs that are not directly impacted by this (or any other) specific intervention, we are expanding our drugs of interest beyond the NARMS panels to all antimicrobial new animal drugs[4] approved by the FDA for use in FA. This excludes compounds with antimicrobial activity which are not considered "new animal drugs" (e.g., disinfectants) or otherwise not currently regulated by the FDA.

Given the complexity of the AMU/AMR system, and the numerous unknowns, for the first set of models we will not include environmental or climatological factors unless they are known selection pressures on resistance and there are data sufficient to not induce additional uncertainty.

[4] New animal drug: A **new animal drug** is defined, in part, as any drug intended for use in animals other than man, including any drug intended for use in animal feed, the composition of which is such that the drug is not generally recognized as safe and effective for the use under the conditions prescribed, recommended, or suggested in the labeling of the drug (21 U.S.C. § 321(v)).

As the boundary on the system under study becomes tighter, one might think that model results will become more precise. That may sometimes be true, but accuracy is more important than precision.

1.5 The Organization of This Report

Given the system scope and data availability, the following methods were identified as critical components of our analytic framework, those for analysis of:

> **Antimicrobial Drugs in Food Animals**: describes factors impacting AMU in FA; reviews indices describing use at the level of animals on farms; and presents methods for how exposure of enteric bacteria within the animals to the antimicrobials or their active metabolites can be estimated (extending the animal-level estimates to represent the actual antimicrobial exposure of foodborne pathogens)
>
> **Phenotypic Resistance in Bacteria in the Animal-Derived Food Supply**: identifies methods to model changes in antimicrobial susceptibility of bacteria isolated from various stages in the animal-derived food production system (e.g., farm, slaughter, retail)
>
> **Genotypic Resistance in Bacteria in the Animal-Derived Food Supply**: identifies methods to model changes in genotypic patterns associated with antimicrobial susceptibility of bacteria isolated from various stages in the animal-derived food production system (e.g., farm, slaughter, retail)
>
> **Systems:** identifies methods and considerations for system assumptions, system to be modeled, data requirements, model [result] integration, system boundaries, uncertainties associated with models/model levels/data availability, information needed, and the overall model framework

We discuss how they work together to create an analytic framework that addresses the question, ending with identification of specific next steps that are necessary to develop this framework more fully in order to assess the efficacy of interventions on AMU and AMR in FA.

2 Antimicrobial Drug Use in Food Animals

2.1 How the Population Structure of Food Animals Affects the Use of Antimicrobial Drugs

Population structure dynamics of FA include the rise or fall in numbers of farms and animals of individual species, production classes within species (e.g., breeder, finisher), and types of production to raise the animals (e.g., organic, conventional, intensive). Changes in animal demographics and population structure occur at varying spatial and time scales in response to consumer demand, market and other forces. These changes affect population-level need for and quantities of specific antimicrobials used via specific administration routes and treatment schedules. This is because the population structure determines the categories and numbers of animals experiencing specific health conditions which can necessitate AMU (e.g., animals of a given species, category and age in a given production type, location and season have a specific infectious disease incidence). The drug arsenal per label and extra-label use allowance, and AMU practices by veterinarians and producers, are also specific to animal species, category, age, and type of

production. Also, demographic changes lead to shifting age, weight and body composition of animals administered antimicrobials, influencing the pharmacokinetics (PK) of the drugs within the animals.

2.2 Factors Influencing the Need for Antimicrobial Drug Use in Food Animals

The need for AMU in FA depends on the incidence of animal health conditions necessitating AMU for disease prevention, control, therapy, or improving production efficacy. Disease detection, risk perception, responsiveness to provide antimicrobials by animal caregivers, and availability of alternatives to antimicrobials are also important. Factors influencing incidence of health conditions include animal husbandry, available technologies and biologicals (e.g., vaccines), animal production biosecurity, and epidemiology and evolution of animal pathogens. Factors influencing disease observation in FA and responsiveness to provide treatment include managerial practices for observing animal health, access to veterinary care, perception and judgement of the health risks which may necessitate AMU, and responsiveness to provide antimicrobials given need. Given a decision to treat, a choice can be made between an antimicrobial drug or its alternative upon availability of the latter.

2.3 Factors Influencing the Arsenal and Use Practices of Antimicrobial Drugs in Food Animals

The arsenal of antimicrobial drugs available for a given animal species, category, and age, in a given type of production, is determined by the per label and extra-label use allowances, i.e., by regulation and approval of antimicrobials for animals. For example, no antimicrobials should be used from the second day after hatching on farm during rearing of broiler chickens grown organically, but some can be used in broilers grown conventionally. Another example is that the FDA's Veterinary Feed Directive (21 CFR Section 558.6) regulates which antimicrobials can be administered in feed to address a health condition in a FA category. Given the arsenal, AMU practices are choices made by veterinarians or producers regarding antimicrobial classes and individual drugs, administration routes, and treatment schedules to address specific health conditions. Practicality of administration, production economics, and food safety regulations (e.g., the required drug withdrawal periods) affect the practices. Certain antimicrobials or administration routes are thus used more intensively in some species, categories or types of production. Beef cattle, for example, are more often treated with antimicrobials by injections than are broiler chickens due to practicality and production economics. Antimicrobial choices for broiler breeders can include drugs with a longer withdrawal period because of the birds' longer lifespan compared to drugs for grow-out broilers.

In addition to the arsenal available and practicality of administration to the animal species, category, age, and production type, in a case of disease occurrence and need for treatment, further factors influence the choice of antimicrobial drugs and treatment schedules by veterinarians or producers. These include: education, current professional information, and experience; knowledge of the health condition or the disease-causing pathogen occurrence within the farm or area; perceived efficacy of the antimicrobial or schedule given expectation or knowledge of the AMR epidemiology in the pathogen; apparent disease incidence (that can affect the decision to treat individual animals vs. administer the antimicrobial metaphylactically to the entire animal group); response to the drug manufacturer's behavior (e.g., price

insensitivity affecting the veterinarian's profit from drug sales); veterinarian-client-patient relationship and veterinarian-client expectations; and response to wider social pressures.

2.4 Indices of Antimicrobial Drug Use in Food Animals on Farms

Various indices describe AMU at animal-level on farms in both research settings and national surveillance programs (Berge et al., 2006; Callens et al., 2012; Carson et al., 2008; Chauvin et al., 2005; Dunlop et al., 1998; Grave et al., 2006; Jensen et al., 2004; Merle et al., 2014; Moon et al., 2011; Postma et al., 2015; Stevens et al., 2016; Timmerman et al., 2006; Vieira et al., 2011); see also reports of AMU monitoring systems listed below (e.g., CIPARS, 2012; DANMAP, 2014; MARAN, 2014; SVARM, 2014). Common indices are summarized in Table 1; different terms may refer to the same index, so we include the index definition. Other indices (not in Table 1) apply to specific antimicrobial uses, e.g., number of intra-mammary doses or the treated disease case rate in the population. Individual or a combination of the indices in Table 1 can describe AMU in a FA category for a farm, area, or at the national level when frequencies and amounts of different antimicrobials used are known. In the absence of such knowledge, some nations use an alternative approach to describe a drug's use in a FA category based on its main indication for the category. The drug's per label dosage and duration for this indication are used to derive Animal Defined Daily Dose (ADDD) and Animal Used Daily Dose (UDDD) indices (Table 1). These are used in combination with data on the total drug amounts used or sold, and characteristics of the population (number of animals and their weight), to approximate the intensity of the drug use, e.g., to estimate nADDD or Antimicrobial Drug Use Rate (ADUR) indices (Table 1). Knowledge of the production system can help judge how often during the animal production life-span this is applied.

Few national surveillance systems collect data on actual AMU or sales of antimicrobials intended for use in individual FA species. Those that do include systems in Denmark (DANMAP), Sweden (SVARM), Netherlands (MARAN), France (ANSES), and Canada (CIPARS). Species-specific data have been collected via different approaches; in the EU, approaches have included collecting data on farms, from veterinarians, and from pharmacies. Other countries collect only data on total sales of antimicrobials for veterinary use, e.g., Finland (EVIRA) and Ireland (HPRA). The European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) annually collates available AMU data for 26 countries of the EU and European Economic Area. Indices to approximate AMU based on the total sales data have been proposed, e.g., an index of Animal Level of Exposure to Antimicrobials (ALEA) for a FA species is estimated by dividing the body weight of animals treated (function of the ADDD and duration of treatment) by the animal mass that could potentially be treated by the antimicrobial drug, with the former approximated based on the sales data (ANSES). Research is underway to develop methods to stratify the total veterinary antimicrobial sales by animal species, utilizing supportive farm-level AMU data and different modeling approaches such as re-partitioning the sales data based on the animal body mass and known veterinary prescription patterns (Carmo et al., 2015). The US currently collects data on total antimicrobial drug sales for animals[5];

[5] FDA receives data for each antimicrobial product, but can only report summary data by class if there are 3 or more sponsors. Classes with fewer than 3 sponsors are grouped together in a category "not independently reported".

the data are reported by the FDA Center for Veterinary Medicine (FDA CVM 2015). Data on estimated antimicrobial drug sales for individual FA species will be collected by the FDA starting in 2016 and reported in 2017.

Table 1. Indices that have been used to describe AMU at the level of animals on farms.

Index	**Definition**
ADDD, Animal Defined Daily Dose	Per label dose for main drug indication in the animal category, amount active drug ingredient per day per kg animal weight[(a)] (Also called DDD_{vet})
PDDD, Animal Prescribed Daily Dose	Similar to ADDD but instead includes the dose prescribed; the ratio PDDD/ADDD reflects prescribing behavior
UDDD, Animal Used Daily Dose	Similar to ADDD but instead includes the dose actually used
UCD, Used Course Dose	Amount of active drug ingredient used in the treatment schedule per kg animal weight
TI_{xDDD}, Treatment Incidence per 1000 animal-head	Amount active drug ingredient used/(xDDD[(b)]*days at risk*kg animal weight)*1000. Relative usage can then be approximated as TI drug or drug class/TI all antimicrobials.
nADDD	Number of ADDDs in the animal population. Amount of active drug ingredient used in the animals/(ADDD*average animal weight)
ADUR, Antimicrobial Drug Use Rate	Number of xDDD per 1000 animal-days at risk (e.g., animal-days on the farm or in the production system)
Treatment frequency	Number of treatments in individual animals per 1000 animal-days at risk
AER, Antimicrobial Exposure Rate	Number of animals treated*days treated/total number of animals*total days (e.g., in the production cycle)
Level of antimicrobial exposure	Amount of active drug ingredient per 1000 animal-days at risk
Proportion exposed	Proportion of animals treated; proportion of farms that have used the antimicrobial

[a] The indices listed have been standardized as the active drug ingredient amount per kg animal meat, kg animal live weight, or Population Correction Unit (PCU). The PCU is defined as kg live or slaughtered animal

weight for the animal category, estimated as the number of live or slaughtered animals*standard or average animal weight at most likely time of the antimicrobial administration (European Medicines Agency ESVAC 2015). [b] xDDD – either ADDD or PDDD or UDDD

2.5 Relationships between Antimicrobial Drug Use in Food Animals and Antimicrobial Exposure of Enteric Bacteria within the Animals

In FA administered antimicrobials, enteric bacteria are exposed to concentrations of drugs or their antimicrobially active metabolites that are excreted or passed with the digesta to the intestine. Antimicrobials vary in their metabolism and excretion to the intestine, and in processes the drugs/active metabolites undergo in the intestine (e.g., degradation). Thus, the fraction of administered drug that is in the intestine in active form varies by drug, and relates non-linearly to the quantity at the animal level. This may explain the above-mentioned variability in associations of AMU with resistance in foodborne pathogens in studies that utilized animal-level AMU estimates. Relationships between AMU at the animal level and resulting antimicrobial exposure of the enteric bacteria within the animals can be determined using population PK-for-intestine models, which project the drug/active metabolite concentrations in the animals' intestines for different treatment schedules. Until recently, PK studies of antimicrobials focused on the drug concentrations in animals' central circulation to forecast effects against disease-causing pathogens, and in certain tissues to forecast the residues to ensure toxicological food safety. Those models and data will assist in developing PK-for-intestine models, but additional modeling and data are needed on processes antimicrobials undergo while transiting through the intestine. In FA populations, the antimicrobial's PK-for-intestine, and thus variation in intestinal antimicrobial concentrations, depend on the drug, its formulation, administration route, animal species and category, age and body composition, and disease pathophysiology (Brown et al., 1996; Gorden et al., 2016; Green and Duffull, 2004; Huang et al., 2015; Kissell et al., 2015; Lees and Shojaee Aliabadi, 2002; Rule et al., 1996; Sarwari and Mackowiak, 1996; Toutain et al., 2010; Volkova et al., 2016; Winter et al., 2010). Concurrent production practices may influence antimicrobial exposure (e.g., secondary exposure to drugs excreted to the environment (Call et al., 2013). PK-for-intestine models exist for cattle for the cephalosporin ceftiofur administered parenterally (Volkova et al., 2012) and chlortetracycline administered per os (Cazer et al., 2014). Foster et al. (2016) have reported experimental *in vivo* estimates of intestinal concentrations of ceftiofur and the fluoroquinolone enrofloxacin in cattle treated parenterally. Combinations of such modeling and experimental studies can fill the knowledge gap on relationships between animal-level AMU and antimicrobial exposure of enteric bacteria within animals.

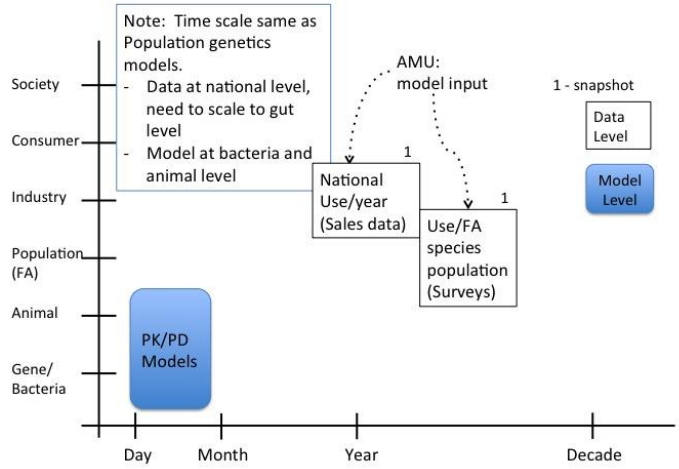

Figure 7. Pharmacokinetic/Pharmacodynamic (PK/PD) Models. Currently, antimicrobial use (AMU) is surveyed by the US National Animal Health Monitoring System at farm and food animal (FA) levels as point estimates. Pharmacokinetic (PK-for-intestine) models can be used at animal and population levels to extrapolate to the animals' intestines, to estimate antimicrobial exposure of enteric bacteria within treated animals due to AMU in the animals. Pharmacodynamic models can then be used to estimate effects from antimicrobial exposure on enteric bacteria and their AMR. Outputs that can be derived from PK/PD modeling are changes in AMR at the bacteria, individual animal, and animal population levels.

The effects on animal enteric bacteria from exposure to antimicrobial drugs or their active metabolites reaching the intestine can be studied using pharmacodynamics (PD) models. Until now, PD studies of antimicrobials have focused on the effects against the pathogens treated. This is often done using *in vitro* experiments with pure cultures of standardized densities of the pathogen isolates highly susceptible to the drug, with high bacterial population growth rates (Garcia, 2010); the predictions are validated *in vivo* through clinical efficacy of the treatments so designed. The approach is not applicable for PD modeling for animal enteric bacteria as these exist in the intestine in variable densities; are composed of sub-populations with variable antimicrobial susceptibilities; and are restricted in their growth due to nutrient and aeration conditions, and by other enteric microbiome components. Bacterial densities, population growth rates, and susceptibilities influence the PD of antimicrobials (Ahmad et al., 2015; Gehring and Riviere, 2013; Udekwu et al., 2009; Volkova et al., 2012). Further, feedback between antimicrobial exposure and susceptibility of the bacterial population is poorly understood (Hanberger, 1992; Jacobs et al., 1997; Levin et al., 2014; Livermore, 1987; Zeng and Lin, 2013). Bacterial susceptibility and drug/metabolite antimicrobial activity are also affected by intestinal pH or aeration conditions (Cid et al., 1994; DeMars et al., 2016; Schlessinger, 1988). Thus, capturing the effects of antimicrobials reaching the intestine on enteric bacteria will require new, significantly more complex PD models than those that have been used to predict the drugs' effects against the pathogens treated.

Accounting for relationships between AMU at the animal level and resulting antimicrobial exposure of the enteric bacteria within the animals (PK-for-intestine models), and the effects of that exposure on the bacteria (PD models for enteric bacteria), can provide the link for evaluating impact of AMU practices for different antimicrobials in FA on AMR in foodborne pathogens. Figure 7 depicts the levels at which data are required for operation of these models, where the data exist, where the models operate, and at what level the modeling results can answer the AMR question posed in the introduction.

2.6 Example – Exploratory work

We present an example of estimating population-level antimicrobial exposure of enteric bacteria in a FA category by combining estimates of AMU in the animals with the PK-for-intestine modeling for the drug. We also use the example to show the limitations (and differences in results) of the indices used to describe the AMU at the animal level that are standardized by animal body weight (BW) (listed in Table 1). The example is based on beef feedlot cattle and ceftiofur use for prevention or control of shipping fever. To approximate AMU at the animal level, we use data from a survey of beef feedlots with animal-head capacity >1000 conducted by NAHMS in 2011 in 12 US continental states (USDA APHIS NAHMS, 2011a,b,c). Such large feedlots housed 82% of the US beef cattle inventory in 2011, according to National Agricultural Statistics Service (NASS) data. Of those cattle, 95% lived in the 12 states surveyed. Hence, the cattle population surveyed included roughly 78% of the national inventory. Approximately 60% of cattle in this population were on feedlots with <8000 headage, and 40% on feedlots with ≥8000 headage. Roughly half of the cattle put on feed in 2011 had an initial BW below and the other half above 700 lb (318 kg); we assumed this was similar for feedlots with headage below and equal to or above 8000. For these 4 animal categories the survey reported the frequency of health conditions necessitating AMU, and frequency of specific drug choices by purpose. For illustrative purposes for the example, we consider a random sample of 1 million cattle from this population maintained on feed for a 6-month production cycle.

Estimated proportions of the cattle treated by an injectable antimicrobial drug for prevention or control of shipping fever were: 27% of animals placed on feed with a BW<700 lb on feedlots with headage <8000; 4% of animals at BW ≥700 lb on feedlots with headage <8000; 41% of animals at BW<700 lb on feedlots with headage ≥8000; and 5% of animals at BW≥700 lb on feedlots with headage ≥8000. The third generation cephalosporin ceftiofur was the drug of choice for the estimated 26% of the cattle treated on feedlots with headage <8000; and for 13% of the cattle treated on feedlots with headage ≥8000; we assumed this was similar for cattle with BW below and above 700 lb at placement. We assumed an animal received one complete treatment schedule of ceftiofur by injection for this purpose during the 6 months on feed, following per label daily dosage 2.2 mg/kg cattle BW, with a 5-day treatment schedule. A sustained-release ceftiofur formulation is also used for this purpose; we consider the 5-day treatment by a non sustained-release formulation for the purpose of the example.

We consider that a 650 lb (295 kg) BW steer and an 850 lb (386 kg) BW steer receive the 5-day ceftiofur treatment schedule. We consider an average or standard animal in the feedlot system weighed 800 lb (363 kg). We first describe the drug's use at the animal level using the indices in Table 1; we standardize

the indices by kg of animal live BW at the time of antimicrobial administration. The standard ADDD of ceftiofur for either animal was 2.2 mg/day/kg BW, and the standard Used Course Dose (UCD) over the 5-day schedule was 11 mg/kg BW. We next derive the antimicrobial exposure at the per-head level due to ceftiofur use, given the animal BW (Table 2).

Table 2. Use of ceftiofur for shipping fever control in US beef feedlot cattle by injection. Estimates of animal-level use indices standardized per kg of live animal body weight (BW), or based on total active ingredient quantities used, assuming the treatment schedule was once daily for 5 days in the dosage of 2.2 mg/day/kg animal BW.

Animal BW at treatment	**ADDD**[a]	**UCD**[b]	**Total active drug ingredient daily**	**Total active drug ingredient over the 5-day schedule**
Average animal, 800 lb (363 kg)	2.2 mg/day/kg BW	11 mg/kg BW	799 mg/day	3,993 mg
650 lb (295 kg)	2.2 mg/day/kg BW	11 mg/kg BW	649 mg/day	3,245 mg
850 lb (386 kg)	2.2 mg/day/kg BW	11 mg/kg BW	850 mg/day	4,246 mg

[a]Animal Defined Daily Dose. [b]Used Course Dose

The estimates presented in Table 2 show that AMU indices standardized per kg of BW (listed in Table 1) do not reflect differences in the antimicrobial exposure at the animal level arising from the differences in the animal BW at the time of drug administration.

Given the distribution of cattle with BW above and below 700 lb at placement in feedlots with below and equal to or above 8000 headage estimated in the survey, and assumptions listed above, we estimate ADUR, Antimicrobial Exposure Rate (AER), and proportion exposed for an entire random sample of 1 million cattle on feed in the population for 6 months vs. the categories put on feed at BW less than and greater than 700 lb across the feedlots of different sizes (Table 3). Taking a simplified assumption for illustration that at the time of ceftiofur treatment, all cattle placed on feed at BW<700 lb weighed 650 lb, and all cattle placed on feed at BW>700 lb weighed 850 lb, we can further estimate the index “level of antimicrobial exposure” that is based on the antimicrobial quantities per head (Table 3).

Table 3. Use of ceftiofur for shipping fever control in US beef feedlot cattle by injection. Estimates of population-level use indices standardized per kg of live animal body weight (BW), or based on total active ingredient quantities used. Based on the USDA APHIS NAHMS Beef Survey 2011 data, and assuming the treatment schedule was once daily for 5 days at 2.2 mg/day/kg animal BW.

Cattle placed on feed at BW	**Proportion of animals exposed**	**ADUR**[a]	**AER**[b]	**Level of antimicrobial exposure** (active drug ingredient per 1000 animal-days at risk)
Total random sample of 1 million cattle, at any BW	3.6%	0.99	0.0010	791 mg
Those at BW<700 lb	6.3%	1.74	0.0017	1128 mg
Those at BW>700 lb	0.9%	0.24	0.0002	206 mg

[a]Antimicrobial Drug Use Rate. [b]Antimicrobial Exposure Rate

We next consider exposure of enteric bacteria within treated cattle to antimicrobially active ceftiofur metabolites. Ceftiofur is rapidly metabolized in the animal body; a large fraction of the metabolites retains antimicrobial activity (Hornish and Kotarski, 2002; Ritter et al., 1996; Salmon et al., 1996). Based on experimental data and a published model of ceftiofur PK-for-intestine (Beconi-Barker et al., 1996; Volkova et al., 2012), we assume roughly 25-35% of the active ingredient administered daily to beef cattle can reach the animal intestine in the form of the drug's active metabolites. The metabolites likely undergo biotic degradation by enzymes produced by enteric bacteria (Gilbertson et al., 1990; Hornish and Kotarski, 2002). We assume degradation occurred for 6 hours before the metabolites reach the lower intestine, and use a published estimate of the degradation rate (Volkova et al., 2012). Under these assumptions, Table 4 presents the projected quantities of antimicrobially active ceftiofur metabolites to which enteric bacteria in the cattle lower intestine could be exposed on each of the 5 treatment days. These were 49-68 mg in a 650 lb steer, and 64-89 mg in an 850 lb steer. Further, we use a published allometric model to approximate the volume of cattle large intestine based on BW (Cazer et al., 2014). Given the metabolite quantities reaching the intestine, the degradation they undergo, and intestinal volume, projected concentrations of active ceftiofur metabolites in the large intestine can reach 1.7-2.4 μg/mL in either a 650 lb or 850 lb steer in the first hours post-injection (each of the 5 daily injections) (Table 4). These estimates compare well with experimental data (Foster et al., 2016). Thus, even though the metabolite quantities reaching the intestine may be higher in an animal with larger BW, because of also larger intestinal volume in the animal, the antimicrobial concentration to which enteric bacteria are exposed may be similar to that in a smaller animal treated under the same schedule. This is not captured by the estimates of AMU based on total quantities of the active drug ingredient administered (Table 2).

Table 4. Use of ceftiofur for shipping fever control in US beef feedlot cattle by injection. Estimates of animal-level quantities and concentrations of antimicrobially active drug metabolites to which bacteria of the lower intestine are exposed, assuming the treatment schedule was once daily for 5 days in the dosage of 2.2 mg/day/kg animal body weight (BW).

Animal body weight	**ADDD[a]**	**UCD[b]**	**Total amount of active drug ingredient administered daily**	**Total amount of active drug ingredient administered over the 5-day schedule**	**Projected quantity of antimicrobially active drug metabolites reaching the animal's lower intestine daily**	**Projected maximum concentration of antimicrobially active drug metabolites in the large intestine post-injection**
Average animal, 800 lb (363 kg)	2.2 mg/day/kg BW	11 mg/kg BW	799 mg/day	3993 mg	60-84 mg	1.7-2.4 µg/mL
650 lb (295 kg)	2.2 mg/day/kg BW	11 mg/kg BW	649 mg/day	3245 mg	49-68 mg	1.7-2.4 µg/mL
850 lb (386 kg)	2.2 mg/day/kg BW	11 mg/kg BW	850 mg/day	4246 mg	64-89 mg	1.7-2.4 µg/mL

[a]Animal Defined Daily Dose. [b]Used Course Dose

Table 5 gives examples of 3 hypothetical population-level indices of antimicrobial exposure of enteric bacteria within animals due to AMU in the FA category (population of interest), for the example of ceftiofur use for shipping fever control in beef cattle. The first index is the projected quantity of the drug/its active metabolites reaching the animal lower intestines, standardized per 1,000 animal-days. In the example, it is estimated by summing the quantities of active ceftiofur metabolites reaching the lower intestines during the 5-day schedule in all animals administered, standardized per 1000 total animal-days during the 6 month production cycle. Yet, as shown above, antimicrobial concentrations in the lower intestine may be similar in animals in which the antimicrobial quantities reaching the intestine are different (because of the allometric relationships between animal body size and intestinal volume).

A concept of an antimicrobial's No Observable Effect Level (NOEL) has been proposed (Carman et al., 2005; FDA-CVM, 2004, 2012; Perrin-Guyomard et al., 2001). We conjecture that the NOEL may be defined as the maximum antimicrobial concentration in animal intestines in the FA category at which there are

still no observable (measurable by currently available methods) effects on the enteric microbiome's structure, or, instead, on the microbiome's resistome. The NOEL may also be defined for a specific enteric bacterial species, as the maximum antimicrobial concentration in animal intestines in the FA category, at which there are still no observable effects on AMR in that bacteria species. Using antimicrobial concentrations in the lower intestines and NOEL, we derive 2 more hypothetical population-level indices of selective exposure on enteric bacteria within animals in the FA category:

(1) Duration of selective pressure: Total hours the antimicrobial's concentrations in the lower intestines of treated animals in the FA category exceeded the antimicrobial's NOEL, standardized by total animal-hours.
(2) Frequency of selective pressure: Total number of days the antimicrobial's concentrations in the lower intestines in treated animals in the FA category exceeded the antimicrobial's NOEL, standardized by total animal-days.

Table 5. Use of ceftiofur for shipping fever control in US beef feedlot cattle by injection. Estimates of hypothetical population-level indices reflecting the quantities and concentrations of the antimicrobially active drug metabolites to which bacteria of the lower intestines within cattle are exposed. Based on the USDA APHIS NAHMS Beef Survey 2011 data, and assuming the treatment schedule was once daily for 5 days in the dosage of 2.2 mg/day/kg animal body weight (BW).

Cattle placed on feed at BW	**Hypothetical index, quantity of antimicrobially active drug metabolites reaching animals' lower intestines, per 1000 animal-days at risk**	**Hypothetical index, duration in hours of selective pressure on enteric bacteria, per 100,000 animal-hours at risk**	**Hypothetical index, frequency of animal-days of selective pressure on enteric bacteria, per 1000 animal-days at risk**
Total random sample of 1 million cattle, at any BW. Assuming each treated animal was of average BW.	59-83 mg	74 hours	0.99
Those at BW<700 lb	85-118 mg	130 hours	1.74
Those at BW>700 lb	16-22 mg	18 hours	0.24

For the example, we define the $NOEL_{resistome}$ as the concentration of antimicrobially active ceftiofur metabolites in the lower intestines of beef cattle of feedlot age at which there are still no detectable changes in the enteric microbiome's resistome by current methods. To illustrate, we hypothetically assume the $NOEL_{resistome}$ to be 0.05 μg/mL. Given the above-described metabolite concentrations in the lower intestines projected by a published model of ceftiofur PK-for-intestine (Volkova et al., 2012) and experimental data (Foster et al., 2016), we assume the concentrations exceed the hypothetical

$NOEL_{resistome}$ for 18 hours each day of the 5-day schedule in either a 650 or 800-850 lb steer. With these assumptions, we derive:

(1) Duration of selective pressure: Total hours the concentrations of antimicrobially active ceftiofur metabolites in the lower intestines of all cattle that received the 5-day ceftiofur schedule for shipping fever control exceeded the $NOEL_{resistome}$ per 100,000 total animal-hours in the 6-month production cycle. The estimates are given in Table 5.
(2) Frequency of selective pressure: Total number of days in all cattle that received the 5-day ceftiofur schedule for shipping fever control when the concentrations of antimicrobially active ceftiofur metabolites in the lower intestines exceeded the $NOEL_{resistome}$ per 1,000 total animal-days in the 6-month production cycle. The estimates are given in Table 5.

This example demonstrates a need for further development of methods for informative estimation and assessment of AMU in FA at the population, animal, and within-animal levels, to determine selection pressure imposed. At animal level, standardizing AMU indices per kg of animal BW may mask AMU differences at head level in species experiencing extensive growth in BW during the production life-span. Development of the above methods will allow determining the antimicrobial concentrations to which enteric bacteria within animals are exposed, and associated impacts on AMR in foodborne pathogens (i.e., PK-PD models). This information can then be used to evaluate effects of changes in AMU (doses, regimens, routes of administration) on AMR. As discussed above, AMU changes can occur due to regulatory or other, e.g. industry-led or societal, initiatives. This type of model at a finite scale can thus infer selection pressure changes on foodborne pathogens arising due to changes in AMU practices in FA.

3 Phenotypic Antimicrobial Resistance in the Food Supply – Exploratory Work

3.1 Introduction

NARMS tests antimicrobial susceptibility of human clinical isolates of enteric pathogens and bacteria in retail meats and FA. The NARMS public-release isolate-level data contain susceptibility test results for each isolate (105,922 isolates in total) from samples collected at slaughter and in participating states, at the retail level (Table 6). NARMS reports minimum inhibitory concentration (MICs) for a panel of antimicrobial drugs tested for each isolate. The MIC is usually measured by a broth microdilution method: an isolate's growth is evaluated when exposed to successive twofold dilutions of antimicrobials. The test outcome, the MIC, is the lowest concentration with no visible growth after a specific time period, so the MIC for each bacteria-antimicrobial combination is a discrete variable. To aid interpretation of the susceptibility tests, MICs are often categorized into 3 classes (susceptible, intermediate, and resistant) using breakpoints.

Surveillance data collected before implementation of the FDA's interventions provide a baseline to compare changes in resistance outcomes. Changes in AMR levels after the policy implementation might be linked to the policy.

Table 6. Animal and bacterial species data available in the NARMS dataset

Stage	Animal species	Bacterial species and sampling years
Slaughter (non-cecal)	Chicken	*Salmonella* (1997-2013), *E. coli* (2000-2013), *Campylobacter* (mid-2001-2013)*, *Enterococcus* (2003-2011)
	Turkey	*Salmonella* (1997-2013)
	Cattle	
	Swine	
Slaughter (cecal)	Chicken	*Salmonella*, *E. coli*, *Campylobacter* and *Enterococcu*s (2013)
	Turkey	
	Cattle	
	Swine	
Retail	Chicken Parts	*Salmonella*, *E. coli*, *Campylobacter*, and *Enterococcu*s (2002-2013)
	Ground turkey	
	Ground beef	*Salmonella*, *E. coli*, *Enterococcu*s (2002-2013), *Campylobacter* (2002-2007)
	Pork chops	

*Isolation of *Campylobacter* from chicken at slaughter began in 1998, but nalidixic acid susceptibility and cephalothin resistance were used by the USDA as identification criteria for *Campylobacter jejuni/coli* until mid-2001, which likely resulted in underreporting of quinolone-resistant *Campylobacter* during this time period.

Trends in AMR phenotypic changes observed through NARMS are often analyzed and summarized using univariate approaches such as the percentage of non-susceptible isolates to drugs of interest in bacteria species for which resistance is of great concern. Clinically, this is an important and useful parameter to help guide therapy, but it is less informative for monitoring effects of mitigation strategies on resistance for several reasons, including (1) changes in MIC distribution that do not occur near the breakpoints may go undetected; (2) the background prevalence of resistance is high for some combinations of antimicrobials and bacteria species, making detection of changes due to mitigation strategies difficult, as stochastic variation may be large; and (3) for *Salmonella* specifically these percentages are highly influenced by serotype composition of the sample. Metrics identifying shifts in resistance should be refined; common ones such as percentage resistant based on breakpoints are not the most sensitive ones to evaluate resistance trends. The MIC values themselves may be a better choice than categorizing MIC as susceptible or non-susceptible. Also, NARMS data are multivariate. Each isolate has an MIC value for each antimicrobial drug in the panel. The number of antimicrobials included in a given panel varies by bacteria and year and ranges from 9-16 drugs. Covariance between MIC values is likely to be positive for some drug-bacteria combinations. Some drugs share biochemical mechanisms that confer resistance. Other drugs can be positively correlated if genetic linkage exists between their genes. Frequency of

multidrug resistance thus exceeds what would be expected by chance. Methods that expand beyond univariate approaches are needed to understand relationships among antimicrobials.

Resistance patterns have been analyzed using multivariate methods, e.g., principal component analysis or factor analysis (Wagner et al., 2003). These have been used to reduce the measured variables into fewer components that capture data variability. The main limitation of multivariate approaches that perform dimension reduction is the difficulty in interpreting the meaning of the created variables. Principal components are easier to interpret when clear separation of antimicrobial drugs exists among principal components, e.g., if each principal component represents a drug class (Poupard et al., 2002).

Graphical modeling can represent the multivariate relationships that exist within MIC data without performing dimension reduction. Markov networks, a form of undirected graphical models, can be used to represent variables in a set of data, where the variables are the vertices and the correlations between variables are represented by edges connecting the vertices (Taskar and Getoor, 2007). In applying graphical models to NARMS data, a vertex represents each drug resistance and edges joining drugs indicate drugs that are not conditionally independent. The most common types of graphical models are Bayesian networks and Markov networks.

Our exploratory work has two phases. The first focuses on baseline variation of individual drugs and the second on patterns of co-occurrence of resistance for multiple drugs.

The aim of the first stage was to study baseline AMR and how the information gained can be used to assess changes in policy. To do this, we studied historical NARMS data (2004-2012), looking at various microbe, host, and antimicrobial drug combinations. Having gained an understanding of the variation in these data, we wanted to know how much data should be collected to find a statistically significant difference in resistance, before and after policy change. Our objectives were to 1) evaluate the baseline trend and variations in the NARMS data, 2) examine the structure of the variation in resistance within/between years, and across geographic regions, and 3) using this baseline, estimate how much data must be collected to determine if the change in FDA policy had an effect.

The aim of the second phase was to develop methods to describe patterns in multidrug (> 1) resistance (MDR) and how these patterns change over time. Our objectives were to 1) estimate the Markov networks describing AMR relationships using the graphical least absolute shrinkage and selection operator (lasso), 2) apply log-linear models of contingency tables to infer more complex multi-drug relationships and 3) understand how these patterns have changed across time.

3.2 Materials and Methods

Phase 1:

To gain a basic understanding of AMR changes over time and across slaughter and retail in the food supply chain, we explored resistance data for each microbe/host/antimicrobial drug combination (Zawack et al., 2016). We examined many combinations of microbes, hosts and antimicrobials; we focus here on chicken

as the host and resistance of *Campylobacter jejuni* to tetracycline, *C. coli* to erythromycin, *Salmonella* Typhimurium to ampicillin, and *E. coli* to streptomycin. Chicken had the most consistent data across all time points and stages. Analyzed drugs were chosen for their significance in both human and veterinary medicine and extent of use in food production (FDA, 2003). Bacteria were chosen 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 then.

To assess AMR trends, we built models of resistance with AMR as a binary variable in logistic regression and as a censored continuous variable in linear regression, using $\log_2$ (MIC). Where isolates had resistance to a drug in excess of the highest tested concentration, the log2(MIC) was assigned $\log_2(MIC_{max}) + 1$ to indicate that this resistance was greater than those with MICs equal to the highest tested concentration. To assess sources of variation we used a linear mixed effects model. Coefficient significance was assessed by a likelihood ratio test.

We used logistic regression to model the log odds of resistance versus non-resistance as a function of slaughter/retail and year. To assess robustness as to choice of breakpoint, we ran regression at different breakpoints to evaluate similarity of the resulting coefficients, with *C. coli*-Erythromycin as an example.

To sidestep breakpoint choice, and add another view on AMR, we treated MIC as continuous and mean $\log_2$ MIC was modeled as a linear function of stage and year.

To study the sources of variation in resistance, $\log_2$ MIC was modeled as a function of a fixed intercept for state and a random intercept for year, in a linear fixed effects model.

To evaluate the effectiveness of the FDA policy change in line with our analyses, we propose a model with a constant base level of resistance around which yearly levels vary, a period of change, and then a new resistance level is established (Figure 8).

A hypothesis test of mean resistance level before and after policy change can assess change in resistance. Different variation within and between years is assumed so the test is carried out on mean yearly resistance. This test can be done with either percent resistance or MIC values. Power analysis was performed to benchmark the test's efficacy. Calculating power requires knowledge of standard deviation (SD), sample size, and magnitude of the effect to be detected. For the t-test of mean $\log_2$ MIC, SDs were chosen by calculating the empirical distribution for the SD of the mean $\log_2$ MIC per year. 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 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 drawn.

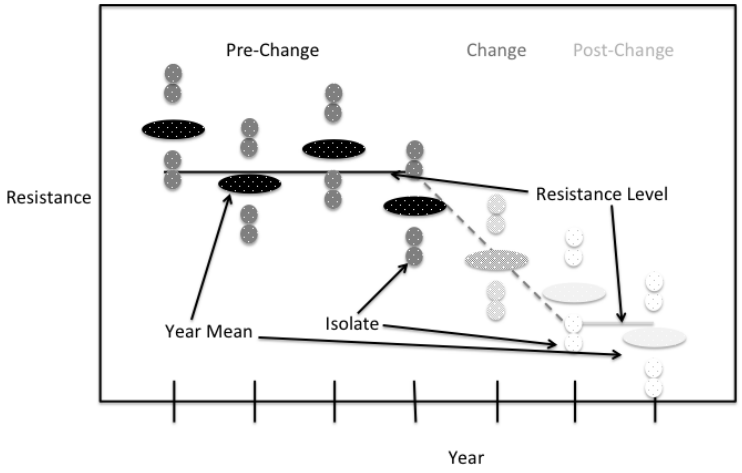

Figure 8. The model of antimicrobial resistance used in evaluating policy changes. It is assumed there is a constant base level of resistance around which the yearly levels vary, a period of change, and then a new resistance level is established.

Resistant/Susceptible counts are binomial quantities so their SDs are functions of proportions. Data are from a mix of conditions like geographical location, season, and production quality, so the proportions are over-dispersed with respect to binomial variation; the SD will also be a function of this over-dispersion parameter. Plotting the empirical distribution of each quantity yielded reasonable proportions and over-dispersion parameters. 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 to be detectable with such sample sizes. Power curves were plotted.

Role of isolate count was determined by calculating power as above, but replacing the single SD parameter with a combined SD for between and within year. The resulting power was plotted for isolate count between 1 and 2000 assuming $sd_{between}$ was 0.6, sd_{within} was 3, the pre-policy change sample size was 9 years, post-change sample size was 5, and effect size was a 1 fold change in MIC. In assessing power, 0.8 was chosen as the desirable threshold.

Phase 2:

To study collateral resistance patterns we took as our data set the resistance measurements for all drugs against *E. coli* from chickens at both slaughter and retail.

Collateral resistance is the positive or negative impact of one drug resistance trait on other resistances. When a bacteria population is exposed to an antimicrobial, resistant bacteria are selected for and prevalence of resistance to the antimicrobial in the population increases. Other resistances correlated with the selected resistance will also change frequency in the population. We developed Markov networks to describe these collateral resistances and evaluate changes in resistance patterns (Love et al., 2016). Presence or absence of specific edges at various times can provide information about resistance

relationships between specific drugs. Edges consistently present during the study period represent stable resistance relationships, and are expected to be found amongst drugs that share common mechanisms for resistance, e.g., stable relationships are expected amongst drugs in the β-lactam class since β-lactamase enzymes are effective, albeit to varying degrees, in all drugs in the class. Edges that were absent during early time periods and became consistently present in later time periods may represent emergent patterns of drug resistance in the microbial population. The NARMS data were stratified by year; MIC values were $\log_2$ transformed. A Spearman rank correlation matrix of the transformed MIC values was calculated, and the associated penalized precision matrix was estimated using the graphical least absolute shrinkage and selection operator (GLASSO method) (Friedman et al., 2008). This step removed trivial partial correlations, making the resultant Markov networks sparser.

Modeling collateral resistance as graphical models provides a number of advantages. First, graphical models provide excellent visualization of the systems they represent. Another advantage is that a wide variety of parameters have been described to summarize the structure of networks. These parameters include but are not limited to: density, modularity, vertex degree, and vertex centrality. These parameters describe different structural characteristics and can be tracked over time to summarize structural trends. Algorithms also exist to find particularly dense subregions of networks. These correlate with sets of potentially important phenotypic resistance traits in Markov networks of resistance.

Building on the graphical models inferred using GLASSO we examined the AMR data with a log-linear model, providing a different means of inferring pairwise dependencies and of inferring multi-way dependencies. Log_2 MIC for each antimicrobial test was converted to a binary susceptible/resistant value and collected in a contingency table. A log-linear model was then built for the joint density of the cells in this table, using forward inclusion, manually enforcing the hierarchy principle.

Once we had a strong understanding of the MDR patterns in single years, we examined trends in the patterns over time. For the GLASSO models we manually inspected changes in the graphical model parameters; application of more objective comparison methods, including multivariate time series, is planned for future work. For the log-linear approach year was included as a random effect.

3.3 Preliminary Results

Slaughter and retail had similar trends. It was important to consider both AMR proportion and MIC: each captured different AMR changes over time. Most variation was within, not between, years; little additional variation was explained by accounting for geographic location (state) in retail data where this information is available. We estimated at current rates of data collection, a one-fold change in MIC should be detectable in 5 years and a 6% decrease in percent resistance could be detected in 6 years following establishment of the new resistance rate. As most variation is within years, the number of isolates tested each year is important in determining this power. The current level of 200 samples per year provides a high degree of power; the standard power threshold of 0.8 could be achieved with 100 samples per year.

Figure 9 shows an example of a Markov network for MIC data for 2004. During the 9 years of the study, 33 unique edges of the 119 possible were identified, and 19 were present in 6 years or more. The remaining unique edges that were identified tended to be weak and only sporadically present. Graphical density describes the proportion of all edges that are present and represents overall graph interconnectivity ($m = m_{obs}/{}_kC_2$). The density of AMR Markov networks ranged from 16.2% to 24.8% and did not appear to change over time (Spearman's rho -0.14, p = 0.71). Modularity in these networks describes how frequently resistance to similar drugs were connected (Gomez et al., 2009; Newman, 2004). Assortativity was always positive in these networks and did not change significantly over time (Spearman's rho = -0.48, p = 0.18). Two subgraphs of high density appeared frequently. The first contained the β-lactam antimicrobials (penicillins and cephalosporins) and in all years was complete, i.e., all possible edges were present ($m = {}_5C_2 = 10$). The other dense subgraph contained sulfonamides, tetracycline and most of the aminoglycosides. This subgraph had a density of 50% or more in six of the nine years in the study.

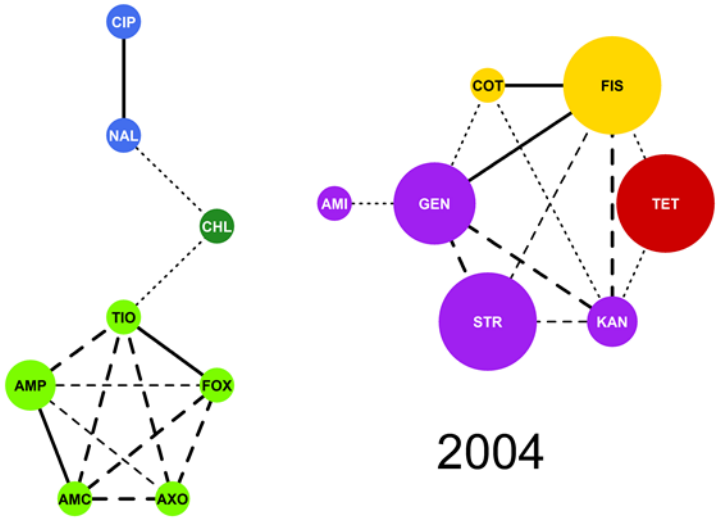

Figure 9. Markov network of drug resistance relationships in *Escherichia coli* isolated from chicken collected in 2004 and tested by the FDA and USDA. The following 15 drugs from the NARMS panel for *E.coli* were included in the graph: ampicillin (AMP), amoxicillin and clavulanic acid (AMC), ceftriaxone (AXO), cefoxitime (FOX), ceftiofur (TIO), amikacin (AMI), gentamicin (GEN), kanamycin (KAN), streptomycin (STR), nalidixic acid (NAL), ciprofloxacin (CIP), sulfisoxazole (FIS), trimethoprim and sulfamethoxazole (COT), chloramphenicol (CHL), and tetracycline (TET). Vertex color indicates antimicrobial drug class as follows: β-lactams (light green), quinolones (blue), aminoglycosides (purple), sulfonamides (yellow), amphenicols (dark green), and tetracyclines (red) . Vertex size is proportional to the percentage of non-susceptible isolates. The presence of an edge between two drugs indicates that the two drugs are correlated, when all other variables are controlled. Line weight reflects the strength of the partial correlation defining the edge.

3.4 Discussion

The exploratory analysis done here shows that over the past decade percent resistance and mean $\log_2$ MIC at both slaughter and retail have fluctuated up and down in a bacteria-drug specific manner. Logistic and linear models confirm this; both year and stage were significant in likelihood ratio tests. Logistic and linear models revealed the same overall trends, but each provides a distinctly useful lens on AMR. Logistic regression provides a straightforward characterization of situations where a known epidemiologic cutoff or clinical breakpoint between resistance and susceptibility exists. Logistic regression, however, is highly sensitive to choice of breakpoint and difficult to interpret when no clear breakpoint exists, as for *C. coli*-erythromycin, among others (Jaspers et al., 2014). In this case linear regression provides a more straightforward characterization of resistance patterns. Also, because linear regression explains the mean resistance level, it can in general provide a more holistic lens on AMR.

Our analysis showed that by performing a hypothesis test comparing level of resistance before and after a change, one can detect a change in resistance level as small as 1 $\log_2$ MIC in 5 years or a 6% change in resistance in only 6 years. Moreover, the current 200 isolates per year level of data collection provides sufficient power and could drop to 100 samples per year without significant reduction in power. It might be interesting to test change in slope of the trend line before and after a policy change, but our analysis showed that resistance patterns do not often have clear trends, so we focused on a comparison test of resistance levels. Although 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. Thus, although it may be difficult to predict the outcome of the current change in FDA policy, it will not be difficult to assess changes in AMR. An implicit assumption of the proposed approach is that changes in AMR levels after policy implementation are due largely to the policy. Combined analysis of AMU and resistance would provide more evidence (EFSA, 2006); and one method has been proposed earlier in the document. However, in the US, AMU data are limited to sales of active compounds aggregated by drug class intended for use in all FA since 2009, and data for some antimicrobial classes cannot be independently reported (FDA, 2013).

Overall, the Markov networks of AMR showed a relatively stable collateral resistance structure in this population of *E. coli*. Many of the edges found were consistently present in most or all years during the study. The density did not change significantly over time and had less than a 10% range. There may be evidence of change in modularity over time. The correlation between modularity and time was substantial, but was not found to be statistically significant at $\alpha = 0.05$. If more years had been included in the study, the trend may have been found to be significant. Decreasing modularity over time would indicate that collateral resistance involving drugs of different classes was becoming more frequent, which could in turn lead to less predictable evolution of MDR strains of bacteria in response to monodrug therapy.

With AMR an increasing threat worldwide, studies such as these are crucial to understand its complex dynamics among hosts, microbes, and drugs. Such studies can pave the way for future mitigation strategies and policy changes to decrease its threat.

4 Evolutionary Genetic Perspective on Antimicrobial Dynamics

The following discussion provides methodological and strategic suggestions on how to evaluate changes in resistance at the genetic level (i.e., do new regulations regarding agricultural AMU result in a change in AMR?). The Appendix (Section 7) provides suggestions for deriving more detailed explanations regarding what might underlie those genetic changes.

4.1 Introduction

Antimicrobial resistance is often divided into intrinsic and acquired. Intrinsic resistance may be referred to as insensitivity, as it occurs in a group of bacteria (a species, genus, or even a larger group) that have never been susceptible to the drug, because of some structural or functional trait common to all members of that group. For example, Enterobacteriaceae are considered insensitive to erythromycin. Acquired resistance, about which we are concerned here, is a trait associated with only some strains of a particular species. Acquired resistance is due to a genetic change in the bacterial genome, which can be due to a mutation (endogenous resistance) or horizontal acquisition of foreign genetic information (exogenous resistance). Either way, acquired resistance is due to the genetic complement of that strain, i.e., its DNA sequence information. Exogenous resistance involves uptake by bacteria of free pieces of DNA (or plasmids) through transformation, transfer of DNA by bacteriophages via transduction or cell-to-cell transfer via conjugation. Exogenous resistance can involve entire genes, groups of genes, or fragments of genes that are incorporated in the recipient genome via homologous recombination. Endogenous resistance involves mutations that occur spontaneously in antimicrobial target genes, that are fixed through the process of natural selection, and which confer resistance to those antimicrobials.

Decades of research subsequent to the development and widespread use of antimicrobials has gradually identified the genes, and mutations within genes, responsible for conferring resistance to commonly used antimicrobials, for the vast majority of important pathogens. With knowledge of the genes and mutations within antimicrobial target genes, that are responsible for conferring resistance, combined with the advent of the polymerase chain reaction (PCR), molecular diagnostics became a valuable tool for characterizing the resistance profile of bacteria isolates. For many antibiotics, no single gene, but multiple genes, are responsible for resistance, so multiplex PCR was the germane approach for molecular diagnosis of resistance, followed by microarray analysis which enabled screening of many molecular targets simultaneously. Today we are in another revolution in molecular technology, with the advent of Next Generation Sequencing (NGS) technologies and their progressively decreasing cost, which has made whole genome sequencing (WGS) of bacteria isolates a rapid, cost-effective way to obtain all of the DNA sequence information for a bacterial strain and thus all relevant genetic information associated with its resistance genotype. WGS accompanied by the library of accumulated information on antibiotic relevant genes and target mutations now enables the prediction of an isolate's resistance profile from its genome sequence information (Bradley et al., 2015; Gordon et al., 2014; McDermott et al., 2016; Walker et al., 2015; Zhao et al., 2015). This has led to development of software tools that can do this rapidly, in a user-friendly fashion, on laptop computers (Bradley et al., 2015).

4.2 The Data

4.2.1 Bacteria isolates

The key to understanding the genetics underlying the effectiveness of new regulations and policies on AMU and AMR is access to collections of bacterial strains, and ultimately, if further details on origins of resistance are desired, possibly also microbiome samples. In terms of the bacterial strains, ideally this would involve collections of different key foodborne pathogens – from a) archived samples over a time period dating back several years, and from different states (the control group; the pre-regulation sample set), and b) contemporary samples collected over the next few years (the experimental group; the post-regulation sample set) that would serve as a direct comparison to the archived samples. Samples involving several key pathogenic species andcommensals, including non-typhoidal *Salmonella*, *E. coli*, and possibly *Enterococcus* and *Campylobacter*, derived from farms (or slaughterhouse), for both swine and beef cattle (ideally poultry as well, if the metadata are available) are desirable. Further understanding of the flow or path of resistance through the food-processing pipeline could be gained by analysis of slaughterhouse and retail data, but for basic understanding of changes in resistance, these additional sources of data are not essential. To have the experimental group as meaningful a comparison as possible, to the control group, the post-regulation picture should involve sampling isolates of the same pathogen species from farms of beef cattle and swine, comprising the same states (ideally the same farms) included in the control. That is, the post-regulation sample should mirror as much as feasible, the control sample. An ideal set of farm samples, comprising both the pre and post regulation period, would cover a period of several years - 2 or 3 years pre-regulation, and 3-5 years post-regulation. Having multiple years pre-regulation will allow evaluation of stochastic fluctuations in genotypes and resistance profiles, and having multiple years post regulation will provide an opportunity to evaluate changes that might not be immediately evident.

The ideal sampling regime proposed above may not be readily attainable; if isolates from replicate farms are unavailable, within states, both pre and post regulation, the most germane plan would be to drop replicate farms within a given state, while keeping one farm from each of more than one state and have those same states (ideally same farms) represented both pre and post sampling. In terms of hosts, if samples were not available for swine and beef cattle, evaluating just beef cattle would still provide valuable data.

4.2.2 Genetic Data

Genome sequences of bacterial isolates derived using NGS technology represent the current best source of genetic data to evaluate changes in AMR. Acquiring the genome sequence of an isolate is a more rapid and cheaper approach than for example, multiplex PCR of relevant resistance genes, and having the complete genome sequence has other benefits like the ability to determine changes in clonal composition over time. To attain an effective picture of changes in resistance pre and post new regulations, and the genetics underlying it, many genome sequences (at least several hundred – e.g., 500-800) are needed from each bacteria species, from both hosts, from each farm, at every time point. This is a great deal of data, but due to the genetic diversity of the pathogens and because genetic changes over this relatively

short time may be quite subtle, it is the only effective way to get a reasonable approximation of actual changes in genotypes and in the prevalence of resistance substitutions. Costs associated with NGS have dropped sharply; with the relatively small size of these bacterial genomes many strains can be indexed and run on a single run. Sequencing entire bacterial genomes has now become much less expensive than doing 7 gene PCR based multi-locus sequencing typing (MLST), with the advantage of having all of the genome sequence and not just fragments of 7 genes. The entire genome sequence can provide a much higher resolution genotype than just MLST (Stanhope - and others - have found that a single MLST clonal group is often comprised of multiple distinct genetic lineages when examined at the genome level) but more importantly, for the present problem, it provides the ability to examine resistance substitutions in all the relevant resistance conferring loci and to employ powerful new tools for examining the genetics of resistance that are based on the analysis of genome sequences (described below – section 4.3.2.1).

4.3 Genetic Analysis

4.3.1 General Comments

Genetically, AMR is ultimately due to a) a subset of bacteria strains within a species carrying particular resistance conferring loci (i.e., not all strains of the same species carry the same set of genes, and some strains may not carry a resistance conferring locus), or b) key substitutions in some strains, in potentially resistance conferring loci (generally the target locus of a particular antibiotic), that are carried by most strains of that species (in this case the vast majority of strains carry the gene in question, but some strains have nucleotide changes (substitutions) in that gene, that confer resistance to particular antibiotics). Thus, if one can evaluate changes in the frequency of these genes or substitutions within genes, over time, within bacteria populations, one can determine changes in AMR. This is one level of understanding of the problem – the first, most basic, and necessary level of understanding – with additional levels of genetic detail about why such changes might have transpired, and their origins, requiring further investigation. For example, changes in resistance could be because of a) changes in the clonal composition (represented lineages) over time – that is, clones carrying resistance being more or less represented in the population at different time points and the origins and/or spread of resistance loci could be through such clonal dissemination or b) via lateral gene transfer (LGT) from other bacteria within the environment – either conspecifics, or other species represented in the host microbiome.

Genetic analysis of AMR change in the context of the present problem has 2 phases, with different levels of understanding. The first phase is examining changes in the presence/absence of resistance loci and in resistance conferring substitutions in target loci over time. This is essential; if no additional level of understanding about why or how this might have occurred is needed, it might be considered adequate. That is, one could address the basic questions of whether there were changes in resistance and in the genetic basis underlying that resistance, by doing only this. However, knowing something about the roles of clonal dissemination, LGT and the microbiome in explaining those observed genetic changes requires more analyses (second phase) (see Appendix (Section 7)). Similarly, the present issue, like all biological questions, can be considered at both proximate and ultimate levels of causation. The proximate cause for changes in AMR is genetic change in key resistance conferring loci; this can be influenced by, or have as

separate components, the above mentioned factors (clonal dissemination, etc.). The ultimate causation is changes in selection pressure, i.e., changes in antibiotic usage practices.

4.3.2 Changes in Frequency of Resistance Loci and Resistance Substitutions

4.3.2.1 Genome-based Prediction of Resistance

Some resistance loci, such as those carried on plasmids, are more or less represented in a population of bacteria isolates and thus lead to more or less resistance. Other loci, like antibiotic target loci, occur in nearly all bacteria isolates of a certain species, but may have different key substitutions within that gene, leading to more or less resistance. Sequencing bacteria genomes would recover both such pieces of genetic information. One can now use genome sequence data to provide accurate genotypic assays of susceptibility or resistance to relevant antibiotics and in general bypass phenotypic drug-susceptibility testing. This has been shown to be effective for a number of pathogens including *Mycobacterium tuberculosis* (Bradley et al., 2015; Walker et al. 2015), *Staphylococcus aureus* (Gordon et al., 2014), *Salmonella* (McDermott et al., 2016), and *Campylobacter* (Zhao et al., 2015).

4.3.2.2 Correlating Resistance Gene Substitutions with MICs

Despite the power and utility of the genome sequencing approach for the present problem, classical MIC testing of these genome sequenced isolates is also needed; such data are a fundamental component in an evaluation of resistance change. This is because some resistance mechanisms, such as efflux pumps, may make it difficult to specifically correlate genotype with resistance phenotypes. MIC data have a range of values and the genome sequencing approaches and their associated software (e.g., Bradley et al., 2015) tend to generate a result that predicts an isolate to be resistant or susceptible based on currently recognized clinical breakpoints. Changes in resistance may be evident from a reduced proportion of the bacteria population with resistance carrying alleles – i.e., a greater proportion of the population below the clinically recognized breakpoint – but may also be evident in overall lower MIC values. Reduction in frequency of isolates with MIC of 64 mg/L to 32mg/L for an antibiotic like streptomycin represents a change but not below the resistant/susceptible breakpoint for organisms like *E. coli* and *Salmonella*. So, MIC data for at least a subset of these genome sequenced isolates are a valuable source of information to augment the genome derived picture of resistance change. Having the genome sequence means we can enumerate all the substitutions of resistance loci, whether they are currently recognized as key to resistance or not.

Although most of the principal resistance conferring substitutions are known for most resistance genes, for many organisms and antibiotics, MICs are more subtly altered by secondary substitutions in the same genes (e.g. Stanhope et al., 2008). If we obtain MIC data for a subset of strains for which we also have genome sequence data, we can correlate the substitutions with MIC values and acquire a detailed picture of genetic change in resistance genes and their relationship with conferring slightly lower or higher MICs. The approximate cost of doing a MIC test for a panel of about 20 antibiotics, using an automated 96 well Sensititre apparatus (AHDC Cornell), is $25 per isolate. This is an additional significant cost, when one is considering thousands of isolates, but we believe that acquiring both MIC and genome sequence data for

a subset of the total genome sequenced set, for each time point, increases the potential predictive value of the genome sequence data, particularly in identifying minor substitutions which could be linked to subtle modifications of MIC.

A useful method to make such correlations of sequence data with MIC values involves correspondence discriminant analysis (CDA), a multivariate statistical method that can be used on contingency tables. Classical discriminant analysis is limited to quantitative variables; CDA can be used on frequency tables. Thus, CDA can incorporate codon or amino acid frequencies from sequence data. One may also test for sites (genetic codons) within the resistance conferring loci that are under positive selection – Darwinian selection at the molecular level. Positive selection is the fixation of advantageous mutations driven by natural selection, and is a fundamental process behind adaptive changes in genes and genomes. Powerful methods can assess positive selection pressure using both phylogenetic and population genetic approaches (e.g., Cornejo et al., 2013; Yang and Nielsen, 2000). Stanhope et al. (2008) have performed such analyses with over 1000 pbp (penicillin binding protein; targets of beta-lactam antibiotics) sequences from *Streptococcus pneumoniae* isolates and correlated the positively selected sites, and other mutations in these genes not identified as being under positive selection, with amoxicillin MIC values, using CDA. As well as this analysis of selection pressure in pbp sequences, CDA has e.g., also been used in molecular biology to separate bacterial proteins according to their subcellular location (Perriére et al., 1996; Perriére and Thioulouse, 2003), and in showing that genetic expression profiles of cortical regions can predict to what functional cortical network ring these regions belong (Cioli et al., 2014). Stanhope et al.'s CDA of pbp sequences and amoxicillin resistance revealed that not only known resistance substitutions, but also many previously unrecognized substitutions that were under positive selection, were important in discriminating different amoxicillin MICs. They found evidence of positive selection in all three of these pbp sequences with many positively selected sites strongly correlated with discriminating amoxicillin MICs, sometimes discriminating low from intermediate MICs, and for other sites, intermediate from fully resistant. Many of the positively selected sites could be directly associated with functional inferences based on the crystal structures of these proteins.

Similar analysis could be used with resistance loci from some of the foodborne species proposed here for genome sequencing. This will work better for some species and loci better than others, due to levels of sequence divergence typical of some species and genes. Phylogenetic based methods of positive selection, although having a very low Type I error, require a moderate level of sequence divergence (at least a few percentage points). Whether one wants to perform positive selection analysis or not, it would be of significant benefit to simply correlate all substitutions in resistance conferring loci sampled at different time points with the MICs obtained for those same time points. The MICs indicate what temporal changes have occurred; CDA analysis of substitutions and MICs provide more specific molecular information on what substitutions in these genes may have been associated with change in MICs.

4.4 Summary

The genetics of AMR is complex; it has several layers of understanding, but with the development of NGS technology concomitant with convenient new approaches to analyze these genome sequence data for

resistance substitutions, the principal genetic goals of the present program can be relatively simply attained: is there any change in resistance after onset of new regulations and what are the key genetic changes which can be linked to any changes in resistance? Additional levels of understanding like the role of clonal dissemination versus LGT and the role of the microbiome would require more detailed analysis and sampling. Although such additional analyses would provide a more satisfactory picture of the overall biological details behind development and spread (or decline) of AMR, they are not absolutely necessary for addressing the principal goal. For a brief explanation of how such additional analyses could be used to add value to the present problem, please consult the Appendix (Section 7).

5 Integration of Methods and Remaining Uncertainty

Some of the methods proposed in previous sections can provide some evidence on the efficacy of interventions in the absence of data on exact AMU in FA. For example, changes in phenotypic and genotypic AMR before and after the intervention can be evaluated using the methods described in sections 3 and 4. However, these types of "before and after" studies have weaknesses, as changes are assumed to be linked to the policy when no other data on AMU or possible confounders are available. Assessing the relationship between AMU and AMR before and after implementation of the guidelines would provide stronger evidence.

Several methods have been previously used to integrate data on AMU and AMR. Time series have been used to analyze the relationship between AMU and AMR surveillance data and evaluate the efficacy of antimicrobial stewardship interventions at the population level. Time series analyses are statistical methods for data series with non-independent observations, e.g., in variables measured repeatedly over time. To quantify the effect of AMU on resistance, transfer function models in which AMU is an input variable and AMR the output series have been built (Willmann et al., 2013). Population genetics and biology frameworks have been used to quantify the relationship between the frequency of AMR and AMU (e.g., Austin et al., 1997 and 1999). Population genetics models track changes in the frequency of genes (or DNA haplotype data). Longitudinal data on the frequency of AMR have been fitted to simple haploid models of selection to estimate changes in fitness of sensitive and resistant genotypes during periods of presence or absence of AMU (Johnsen et al., 2011). Antimicrobial use effects on AMR are mediated by direct effects at the individual level and indirect population level effects by changes in colonization and transmission (Lipsitch and Samore, 2002). Population biology models address explicitly the transmission dynamics of resistance. Population genetics and biology frameworks have been integrated to address the effects of AMU on AMR and relate to observed population level changes in AMR (Austin et al., 1999).

Overall, the analytic frameworks described above require longitudinal data on AMU and AMR, but available data on AMU and AMR are limited and highly uncertain (see section on model uncertainty). Figures 10 and 11 depict the levels at which data are required for model operation for the network and time-series models, where data exist for the model type, where the model operates, and at what level the model results answer the AMR question posed in the introduction. Overall, available AMU (sales) and AMR data are insufficient for the modeling approaches used in the past to address AMU and AMR relations.

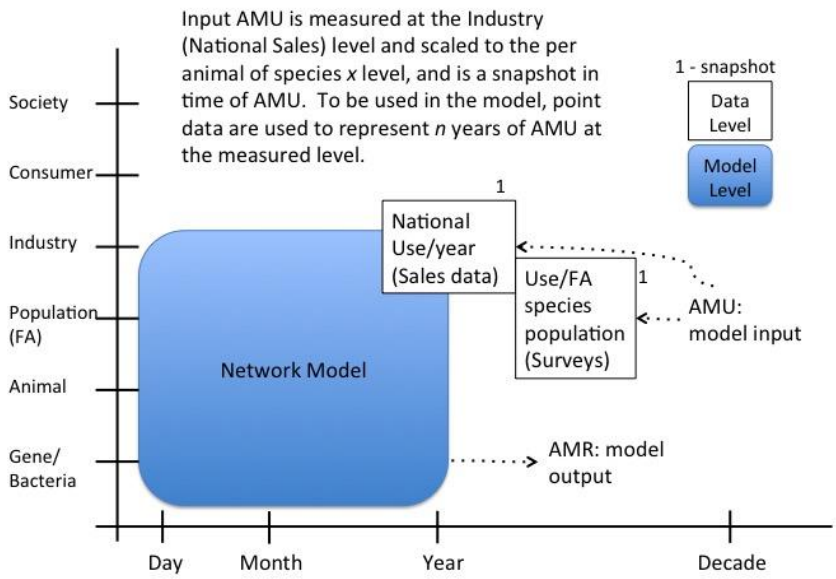

Figure 10. Network model. A network model can be used at the gene/bacteria scale. Input antimicrobial use (AMU) is measured at the Industry (National Sales) level and scaled to the per animal species level, and represents a point estimate for AMU. Assumptions must be made as to the length of time that the point value indicates until such time as multi-year AMU and antimicrobial resistance (AMR) profiles are developed. Output AMR is provided at the gene/bacteria scale. (FA=food animal)

Few frameworks suitable for modeling systems having much uncertainty and allowing integration of diverse data types like expert knowledge or accumulated data exist. Probabilistic graphical models use a graph-based representation to represent complex distributions. In section 3, we propose the use of Markov graphical models to characterize multidrug resistance outcomes. Another type of probabilistic graphical model is the Bayesian network (BN). In the following section, we discuss this emerging modeling technique that integrates multiple sources of complex data and provides a practical and explicit mathematical formalism. The dynamic Bayesian Network (DBN) is a particular form of the BN that allows representation of the evolution of stochastic processes through time.

The BN is a directed acyclic graph encapsulating a joint distribution over all variables and their probabilistic dependencies. Recently, DBN has been applied to model complex dynamical systems tainted with uncertainty (Baudrit et al., 2010; Izadi et al., 2014). Complexity is dealt with through a hierarchical structure incorporating available data expressed as different characteristics (e.g., numerical, categorical, etc.), at multiple scales (e.g., animal, bacteria, genes, etc.), and from diverse sources (e.g., expert opinions, databases, equations, etc.); the uncertainty pertaining to the system is accounted for by quantifying probabilistic dependencies between variables (Baudrit et al., 2010). Prior probabilities of variables and parameters can be continuously updated using new data (Smid et al., 2011). The DBN would thus be an

intuitive and effective tool to synthesize and integrate the multiple facets of AMU and AMR monitoring systems and assess the associations between the two as the FDA implements its risk mitigation strategy.

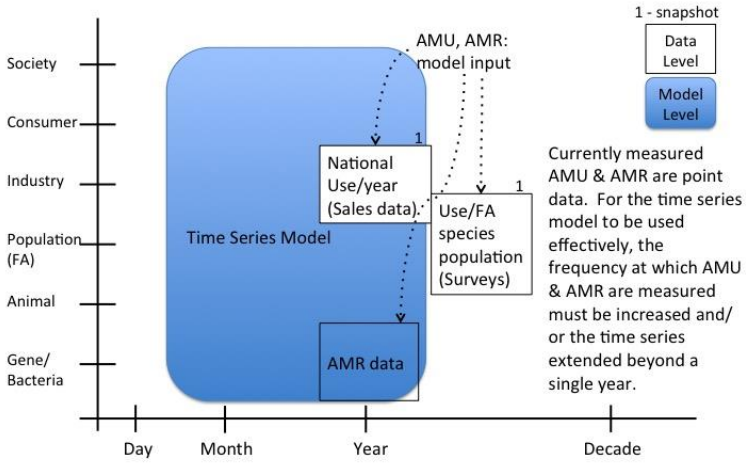

Figure 11. Time-series model. Time-series models can be used at multiple levels in the hierarchy, depending on the level at which the question is posed and available data reside. As discussed elsewhere in this report, currently measured antimicrobial use (AMU) and antimicrobial resistance (AMR) are point estimates. For the time series model to be used effectively, the frequency at which AMU and AMR are measured must be increased and/or the time series extended beyond a single year. (FA=food animal)

Classic epidemiological methods commonly used to assess the effects of AMU on resistance are of limited value to assess FDA guidelines because of few data on AMU (exposure) and the complexity of the measured outcome (patterns/prevalence of AMR). Also, "before and after" studies, per se (i.e., comparing AMU and resistance before and after guideline implementation), have weaknesses, e.g., other influential factors may also change simultaneously with the policy change. Our strategy is thus first to establish baseline levels of AMR at different points of the food chain (e.g., slaughter, retail) using historical data and Bayesian methods. We will then use change-point analysis to see if resistance is decreasing, or increasing, at the same rate before and after policy change. To understand the relationship between resistance at the current point (e.g., retail) and preceding point (slaughter) and ultimately on the farm, all of these stages of the food chain can be included in the Bayesian framework.

To model the AMR system we will break it into 3 conceptual modules. The first examines dynamics of AMR over time, especially in response to two simultaneous interventions: phase out growth-promotion claims and phase in veterinary oversight. The second looks at dynamics across slaughter and retail. The third examines MDR. Each module will be constructed from a single basic building block (Figure 12), expressing the true, hidden prevalence of resistance as a function of a known specificity and sensitivity and observed prevalence with measurement error. This module provides a more accurate estimate of prevalence by accounting for errors in the acquired resistance data and making it possible to study effects

of different types and rates of error on the ability to correctly infer AMR prevalence. This module can be thought of as inferring the prevalence based on a single time point (e.g., month or year) of collected data. Data from different locations can be aggregated to infer AMR prevalence on regional or national scales.

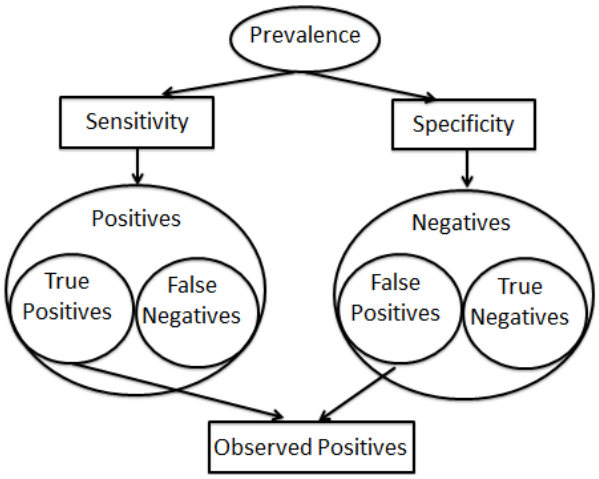

Figure 12. Diagram of the basic building block showing the relationships among prevalence, sensitivity, specificity, positives, negatives, and observations. Circles are inferred values, squares are known values.

To investigate changes in AMR over time, multiple instances of this basic building block can be connected in series (Figure 13) to model a data set that is being continually updated over time. Comparing the results of a model of this type constructed before a change in policy to one constructed after the change provides a natural framework for evaluating the effect of the policy, e.g., for each major *Salmonella* serotype. Through simulation studies one may determine which measurements are most critical to making high quality inferences and how many of these measurements are necessary.

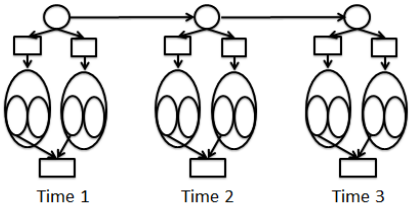

Figure 13. Diagram of 3 basic building blocks connected in series to jointly model antimicrobial resistance over 3 time periods.

The AMR dynamics across slaughter and retail can be modeled by combining 2 basic building blocks to simultaneously estimate AMR prevalence in both settings. This sub-module can be connected in series into an Object-Oriented Bayesian Network (OOBN) (Philippe and Lionel, 2006) describing stochastic

changes of AMR in 2 discrete time scales: across slaughter and retail stages and over calendar sampling time (Figure 14). Again, simulation studies can be carried out to optimize the data gathering process.

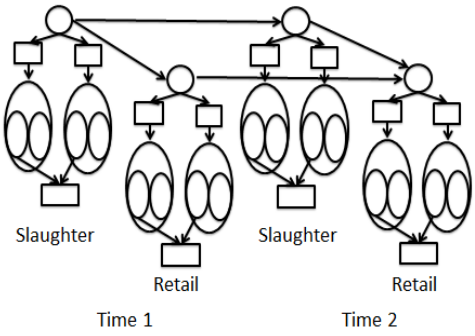

Figure 14. Diagram of 2 basic building blocks connected to form a sub-module modeling antimicrobial resistance (AMR) across slaughter and retail which is in turn connected in series to a second copy of the same sub-module to simultaneously model AMR across time.

Although insights can be gained from examining each antimicrobial in isolation, a true systems approach requires modeling the effects of all antimicrobials jointly, to uncover interactions between them. Statistical power may also be gained through such a meta-analysis involving multiple drugs. Analysis of the dynamic changes of patterns of MDR is needed to foresee complex responses to FDA interventions and resistance emergence because of MDR caused by genes linked in transferable resistance elements.

Despite the care with which the modeling framework was developed, uncertainty will remain in the results. This is true for any data analysis, modeling or simulation effort. What is important is that the uncertainty be quantified or bound and communicated to stakeholders so that the meaning of results is understood. This understanding is required for science based decision-making.

Optimizing strategies to control AMR must occur through understanding of the system as a whole, including all its complexities. This requires merging disciplines ranging from molecular biology to applied math to social sciences to gain a better understanding of complex systems like AMR dynamics and influential factors in the food supply chain.

Traditionally, one first compartmentalizes the whole system into convenient subsystems or parts of subsystems. Each part is described, and one tries to understand the system as a whole by aggregating knowledge of its parts. However, this approach ignores interdependence and instead simplifies what are actually quite complex, multidirectional relationships into convenient, unidirectional, relationships. Importantly, this approach ignores that in complex systems the dynamics of the whole is a non-linear (not a summation) function of the parts.

The structure of the conceptual model to address the complexity of AMR in the food supply system encloses, at its core, the food industry sectors interconnected with food safety and public health

authorities, industry self-monitoring agencies, and media outlets. A food supply chain consists of producers, processing facilities, retail outlets and consumers. The model allows time dependent assessment of food safety standards, while locating potential hot spots of AMR risk in specified food sectors.

Another quantitative perspective on AMR is the economic impact it has on business and governance costs for implementing tests and controls, e.g., in response to regulatory interventions. Key interventions imposed on the industry and the correlation with economic costs act, in turn, as feedback loops to modeling pathogen emergence and spread in the food supply system. In optimization, a complex system often lacks centralized control, i.e., actors may have different objectives. The overall optimization of AMU policies has therefore a dual and competing role that aims to minimize risk of AMR and, at the same time, observes the allocated economic costs for preventing potential spread of AMR.

Because media/consumer feedback may be an important but neglected influence in models addressing AMR, the media should be included as a modulatory element affecting all constituencies. The media is the most widespread and connected component of the systems epidemiology scheme in our model. The strength of the "media feedback" may influence whether industry and government take reactive vs. preventive actions on AMR. A weak/short-lived media feedback that does not translate into sustained consumer activism and/or government actions may favor short-term, limited reactive actions, but does not translate into longer-term, far-reaching changes in legislation/regulation. Yet, such intricate connectivity demands input from social sciences, connective media, networks, and behavioral studies. For now, we assume that the behavioral component accounting for human attitudes and motivation in each food industry sector, among policy makers, and consumers, is accounted for in the conceptual model through self-regulatory feedback loops internalized in response to such media-related influences.

5.1 Sources of Model Uncertainty

Uncertainty can arise from numerous sources, including data (e.g., data quality issues), processes conducted on the data (e.g., data extraction/cleaning), use of proxy data, data aging, model selection, data analytics and simulation uncertainty, etc. (Figure 15). Communicating uncertainty to stakeholders, including decision makers, can also add uncertainty, or provide an opportunity to clarify what the results (including the uncertainty) mean, and enable the decision-maker to effectively act upon the results.

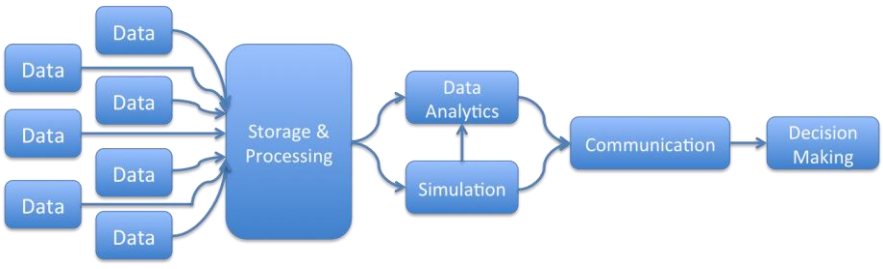

Figure 15. Data-to-decision pipeline and potential sources of uncertainty

Uncertainty in data includes potential observation bias, collection errors, and data quality issues such as missing, incorrect or incomplete data. Processes performed on the data (data aggregation, scaling, etc.) and use of proxy data introduce uncertainty. For instance, AMU is available as a presumed subset of national sales data. National sales data are a proxy for national use data; the actual amount of antimicrobials used is uncertain (we assume a certain percentage of sales is used). Also, AMR data are available at group/species level; to correctly compare AMU and AMR, one must make further divisions of the national "sales to use" data to determine the amount of antimicrobials used in an animal species/group. As the exact numbers of animals in a group vary at least daily and estimates are available at a gross level, uncertainty occurs in the amount of antimicrobials used per animal group. Uncertainty also occurs when the data are a snapshot in time (measured once) of a naturally occurring factor or outcome.

Uncertainty related to modeling choices and data availability is illustrated in Figures 10 and 11 above. For models, simulations or analysis, stochasticity inherent in the problem space (e.g., different feeding or treatment practices) adds uncertainty. Furthermore, it is important to understand how uncertainty propagates through the "pipeline" (data and modeling effort).

Confidence bounds on quantitative results have long been used to communicate the scale of uncertainty. Uncertainty can also be effectively communicated using graphic visualization approaches and by other means.

5.2 Uncertainty Reduction

The amount of uncertainty in assessing the association between population-level changes in AMU and AMR may appear enormous given the multiple and varied sources of uncertainty described above. It is no doubt a significant issue. Recognizing this is one matter; what can be done about it is another. Suggestions for the reduction of uncertainty include:

- Move the observation of data closer to the need for the data. For example, measure on farm use of antimicrobials. This may not be practical; however, there are other means such as veterinary disclosure or farm self reporting, which carry their own biases.
- Increase the frequency at which data are collected. For example, measure the use of antimicrobials (yearly, seasonally, etc.).
- Increase the location(s) at which data are collected. Since we have seen that geography had little influence on the association of AMU and AMR, location data may not be helpful in reducing that uncertainty, but they can help reduce the uncertainty in determining the use per animal since the animals in different geographic locations can be determined.
- Report data collection processes with the data, including any data cleaning processes that may have been conducted.
- Identify model uncertainty and error propagation.

This is not an exhaustive list of actions that can be taken to reduce uncertainty; however, they are necessary first steps to enable the effective modeling and subsequent communication of the association between population-level use of and resistance to antimicrobials.

Note that there will always remain some uncertainty in the results, regardless of the steps taken, because the factors associated with AMR are not static and, at least at this point, are not all known and thus cannot be considered in this or any AMR modeling framework.

6 Conclusions/Future Directions

Antimicrobial resistance is a growing threat with serious implications for human and veterinary medicine, the food supply, animal agriculture and the economy. The FDA has thus proposed tightening restrictions on AMU in FA. This will have consequences for food production and pricing. It is thus essential to understand historical baseline resistance trends and ensure it will be possible to assess the effects of the policy change on future levels of resistance.

Limitations of the proposed modeling process relate to gaps in knowledge and data, especially at the farm, animal product processing, and consumer levels, on the AMU and AMR dynamics of interest. Until such data are available, this can be overcome by using slaughter and retail data: the former reflect "collective" AMR (i.e., both farm and potential contamination and changes during transport and processing), and the latter tell us what consumers are receiving. Also, an inaccurate network structure specification may occur, and hinder model learning/inference, affecting the validity of predicted dynamics. This can be overcome by incorporating more measurements in monitoring systems and expert knowledge, to reduce model uncertainty and obtain more reliable predictions. This systems approach could be further refined through another NIMBioS Working Group.

By exploring systematically several metrics of AMU and AMR, including MDR patterns, we will better understand what changes can be expected due to the FDA guidance, and provide a methodology to maximize the information generated from analysis of costly longitudinal surveillance data on AMR. The proposed modeling framework will present an explicit overview of AMR dynamics and improve our understanding of the complex ecological and evolutionary processes at play. This quantitative analytical framework will allow one to determine the impact of the FDA's policy changes on AMR, and not only identify a prioritized list of important quantitative variables, but also the optimal sampling frequency and sample size for AMU and AMR data, which is critical for improving the existing national monitoring systems.

7 Appendix

7.1 Clonal Dissemination

Bacteria clones are difficult to define precisely as bacteria are not entirely asexual, and recombination results in diversification of the ancestral genotype, to produce a group of highly similar but nonetheless increasingly diverse genotypes (a clonal complex). The rate at which clonal diversification occurs depends

on mutation rate, but significantly, also on level of recombination, which varies among bacteria species. MultiLocus Sequence Typing (MLST) has long been the chosen method for genotyping clones and clonal complexes, but is becoming outmoded due to the advent of NGS sequencing, the lower NGS sequencing costs, and importantly, the increased resolution that WGS can provide in demarcating "sub-clones" of putative clinical importance. Phylogenetically, sub-clones, clones, and clonal complexes are all monophyletic – each has descended from the same common ancestor – with clones descending from within clonal complexes, and sub-clones from within clones. Approaches utilizing genome sequence data for phylogenetic reconstruction can provide additional resolution beyond that afforded by MLST and identify any clones or sub-clones, which drop in or out of a sample set during the temporal period involved herein. This in turn is of importance to the present problem, as certain lineages may bear certain resistance conferring mutations in chromosomally carried loci, or may preferentially carry extra-chromosomal elements, such as plasmids with resistance loci. If genome sequence data are used for resistance/susceptibility evaluation, the sequence data will already be available and afford the additional purpose of this genome-wide phylogenetic reconstruction. Originally, this was accomplished by assembling sequence reads into contigs, annotating the open reading frames, identifying orthologous open reading frames across all genomes, aligning the orthologous coding regions, and reconstructing a phylogenetic tree from these multiple alignments. This is very labor intensive and requires considerable bioinformatics processing of the sequence data.

An alternative approach has recently been adopted: sequence reads are directly mapped to a single reference sequence, single nucleotide polymorphisms (SNPs) are extracted, and a phylogenetic tree is inferred using maximum likelihood methods from the aligned SNP positions. Most recently, however, Bertels et al. (2014) found that both the exclusion of non-polymorphic positions and alignment to a single reference genome introduce errors in genome wide phylogeny reconstruction. They developed a new method that combines alignments from sequence read mappings to multiple reference sequences, eliminating biases in the resulting phylogenetic reconstructions. They implement this method as a web server, fully automating phylogenetic reconstruction from raw sequence reads of bacterial genomes. They refer to their program and webserver as REALPHY (Reference sequence Alignment-based Phylogeny builder; a Java version of the program is also available for download); this method can be used to examine changes in lineage composition over the temporal period examined here. Reconstructing phylogenies using REALPHY from a mixture of genome sequence data obtained from successive, or at least a mixture of, different time points represented here, should prove most informative: knowing whether lineages are more or less represented over time cannot be evaluated effectively without knowing the relationships of isolates involving different years. This could involve many hundreds of bacterial genome sequences in a phylogenetic reconstruction. We have run REALPHY on a lab workstation (30 CPU; 512 GB memory) employing 11 reference genomes, involving a data set of approximately 300 genomes (2 Mb each), which took several days; another set of approximately 800 genomes (2 Mb each), 12 reference genomes, on the same workstation took just over one week.

7.2 Lateral Gene Transfer

Lateral gene transfer (LGT) is movement of genetic material between organisms via a process other than

vertical inheritance (transmission of DNA from parent to offspring). In bacteria this occurs by 3 principal mechanisms: conjugation, transformation, and transduction. All are involved in transfer of antibiotic resistance loci. Detecting LGT is fairly simple when donor and recipient are different species. If an antibiotic resistance locus is vertically inherited, phylogenetic reconstructions of this gene acquired from multiple isolates of a particular species should conform to the monophyly of the species. That is, sequences of this gene from multiple isolates of species X, included in alignments of the same gene from other species of bacteria (obtained via The Basic Local Alignment Search Tool (BLAST) searches with the query gene from species X), will result in monophyly of the antibiotic resistance locus from species X if there is no LGT involving those isolates. If there is LGT of this locus involving one or more of the isolates of species X then for these isolates this gene will fall outside the monophyly of the species, and instead will fall within the clade of outgroup taxa, probably closely related to one of these outgroup taxa.

Detecting intraspecific LGT is more difficult. The analytical premise is similar, but here one derives a control phylogeny for the isolates in question (e.g., using REALPHY with the antibiotic resistant locus in question excised) and compares the phylogeny of the antibiotic resistant locus to that control phylogeny. If the phylogeny of the antibiotic locus provides evidence for strongly supported conflicting nodes compared to the control phylogeny, this is evidence for intraspecific LGT of that antibiotic locus. If, however, the antibiotic phylogeny and the control phylogeny are incongruent, but such incongruence does not include strongly-supported conflicting nodes, the evidence for LGT of the antibiotic locus is inconclusive. This approach is described in an analysis of the relative role of LGT vs. clonal dissemination in the spread of amoxicillin resistance in *Streptococcus pneumoniae* (Stanhope et al., 2007); for this species and antibiotic, clonal dissemination was a more important factor than LGT. Technically, intraspecific LGT detection is more difficult than interspecific LGT detection because the phylogenetic signal afforded by a single antibiotic locus is generally insufficient to reconstruct robust intraspecific phylogenies to reliably compare to the control, and any sample of isolates used in an intraspecific phylogeny is a minute subsample of the total possible set, so many donor lineages will not be represented in the analysis. Generally, therefore, an approach such as described in Stanhope et al. (2007) will yield a conservative minimum estimate of the levels of intraspecific LGT.

7.3 Metagenomics

Metagenomics is the study of genetic material in environmental samples. It includes 2 approaches: (1) 16S metagenomics to identify species diversity in different environments and (2) environmental shotgun sequencing to identify genome contents favored by an environment. In the present case of examining the role of the environment in AMR of foodborne pathogens and how this might change in the context of new regulations, it would involve environmental shotgun sequencing that focused on recovering AMR loci. Testing for AMR loci in metagenomics samples has been performed in different situations, including the resistome in manure, soil and wastewater from dairy and beef production systems (Noyes et al., 2016b). The methodology reported in this paper is state of the art; the technical aspects of this study can be used as a framework on which to base an analogous study for the present situation. However, in order to assess the impact of the new regulations on environmental AMR, archived metagenomics samples are required (sizable in number and from several locations and years); our present understanding of available samples

suggests that this is unlikely. If they were somehow made available, we recommend evaluating AMR loci from metagenomics samples pre and post the new regulations using Noyes et al. (2016b)'s procedures. This would entail identifying the resistance loci, with standard bioinformatics techniques (e.g., BLAST), using a curated list of known resistance loci from foodborne pathogens as queries and assessing changes in frequency of these loci and specific alleles of these loci in the post regulation samples. In the present context of assessing effects of the new regulations on metagenomic resistance loci, the key is to have pre and post regulation metagenomic samples; this is a difficult, and unfortunately, unlikely sample set to come by.

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